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Food and Drug Administration
CDER, Office of Regulatory Policy
10903 New Hampshire Avenue,
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Silver Spring MD 20993-0002

APR 0 3 2018

Attention: Beverly Friedman

The attached application for patent term extension of U.S. Patent No. 7,718,175 was filed on January 11, 2018, under 35 U.S.C. § 156. Please note that a patent term extension application for U.S. Patent No. 7,179,464 and 8,101,185 for BLA 761070 for the human biological product FASENRA™ (benralizumab) were filed concurrently, pursuant to the provisions of 37 C.F.R. § 1.785.

The assistance of your Office is requested in confirming that the product identified in the application, FASENRA™ (benralizumab), has been subject to a regulatory review period within the meaning of 35 U.S.C. § 156(g) before its first commercial marketing or use and that the application for patent term extension was filed within the sixty-day period beginning on the date the product was approved. Since a determination has not been made whether the patent in question claims a product which has been subject to the Federal Food, Drug and Cosmetic Act, or a method of manufacturing or use of such a product, this communication is NOT to be considered as notice which may be made in the future pursuant to 35 U.S.C. § 156(d)(2)(A).

Our review of the application to date indicates that the subject patent would be eligible for extension of the patent term under 35 U.S.C. § 156.

Inquiries regarding this communication should be directed to Ali Salimi at (571) 272-0909 (telephone) or (571) 273-0909 (facsimile) or by e-mail at ali.salimi@uspto.gov.

Ali Salimi
Senior Legal Advisor
Office of Patent Legal Administration
Office of the Deputy Commissioner
for Patent Examination Policy

cc: Carl A. Morales
Dechert LLP
1095 Avenue of the Americas
New York, NY 10036-6797

POWER OF ATTORNEY
FOR PATENT TERM EXTENSION APPLICATIONS AND REQUESTS
UNDER 35 U.S.C. § 156

Kyowa Hakko Kirin Co., Ltd., a Japanese Corporation

hereby appoints **Carl A. Morales**, Registration No. 57,415, and the **Dechert LLP** practitioners associated with **Customer Number 37509** as its attorneys and agents with full power of substitution and revocation

to prosecute applications and requests for patent term extension under 35 U.S.C. § 156 for **U.S. Patent Nos. 7,179,464; 7,718,175; and 8,101,185** based on the regulatory review of the pharmaceutical product, **FASENRA™ (benralizumab)**, as approved by the U.S. Food and Drug Administration under **Biologics License Application (BLA) No. 761070**, on which Kyowa Hakko Kirin Co., Ltd. has been authorized to rely by the BLA holder, AstraZeneca AB, and to transact all business in the United States Patent and Trademark Office connected with respect to such applications and requests for patent term extension;

provided that if any one of said attorneys or agents ceases to be affiliated with the law firm of Dechert LLP as partner, employee, or of counsel, such attorney or agent's appointment as attorney and all powers derived therefrom shall terminate on the date such attorney or agent ceases being so affiliated; and

revokes all previous powers of attorney with respect to such applications and requests for patent term extension.

Please direct all correspondence regarding patent term extension for the above-identified patents to:

☒ Customer No. 37509
Dechert LLP
c/o Carl A. Morales
1095 Avenue of the Americas
New York, NY 10036-6797
Tel: 212-698-3500
Fax: 212-698-3599

The undersigned, whose title is supplied below, is authorized to act on behalf of Kyowa Hakko Kirin Co., Ltd.

Signed: Kenya Shiota Date: March 20, 2018

Print Name: Kenya Shiota

Print Title: Executive Officer and Director, Legal and Intellectual Property Department, KYOWA HAKKO KIRIN CO., LTD.

PTO/SB/96 (07-09)
Approved for use through 07/31/2012. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

STATEMENT UNDER 37 CFR 3.73(b)

Applicant/Patent Owner: KYOWA HAKKO KIRIN CO., LTD.

Application No./Patent No.: 7,718,175 Filed/Issue Date: 05-18-2010

Titled: METHOD OF MODULATING THE ACTIVITY OF FUNCTIONAL IMMUNE MOLECULES TO INTERLEUKIN-5 RECEPTOR PROTEIN

KYOWA HAKKO KIRIN CO., LTD. , a corporation
(Name of Assignee) (Type of Assignee, e.g., corporation, partnership, university, government agency, etc.

states that it is:

1. ☒ the assignee of the entire right, title, and interest in;

2. ☐ an assignee of less than the entire right, title, and interest in (The extent (by percentage) of its ownership interest is _____ %); or

3. ☐ the assignee of an undivided interest in the entirety of (a complete assignment from one of the joint inventors was made) the patent application/patent identified above, by virtue of either:

A. ☐ An assignment from the inventor(s) of the patent application/patent identified above. The assignment was recorded in the United States Patent and Trademark Office at Reel _____, Frame _____, or for which a copy therefore is attached.

OR

B. ☒ A chain of title from the inventor(s), of the patent application/patent identified above, to the current assignee as follows:

1. From: Hanai, N; Nakamura, K; Yamasaki, M; Uchida, K; Shimkawa, T; Imakoma, S; Honda, Y; Hosaka, R; Yamane, N; Amarawa, H To: KYOWA HAKKO KOGYO CO., LTD.

The document was recorded in the United States Patent and Trademark Office at Reel 012301, Frame 0276, or for which a copy thereof is attached.

2. From: KYOWA HAKKO KOGYO CO., LTD. To: KYOWA HAKKO KIRIN CO., LTD.

The document was recorded in the United States Patent and Trademark Office at Reel 022542, Frame 0823, or for which a copy thereof is attached.

3. From: _____ To: _____

The document was recorded in the United States Patent and Trademark Office at Reel _____, Frame _____, or for which a copy thereof is attached.

☐ Additional documents in the chain of title are listed on a supplemental sheet(s).

☒ As required by 37 CFR 3.73(b)(1)(i), the documentary evidence of the chain of title from the original owner to the assignee was, or concurrently is being, submitted for recordation pursuant to 37 CFR 3.11.

[NOTE: A separate copy (*i.e.*, a true copy of the original assignment document(s)) must be submitted to Assignment Division in accordance with 37 CFR Part 3, to record the assignment in the records of the USPTO. See MPEP 302.08]

The undersigned (whose title is supplied below) is authorized to act on behalf of the assignee.

Kenya Shitara
Signature

March 20, 2018.
Date

Kenya Shitara
Printed or Typed Name

Executive Officer and Director, Legal and Intellectual Property Department, Kyowa Hakko Kirin Co., LTD.
Title

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent No.	7,718,175
Issued:	May 18, 2010
To:	Kyowa Hakko Kirin Co., Ltd.
For:	Method of Modulating The Activity of Functional Immune Molecules to Interleukin-5 Receptor Protein

APPLICATION FOR EXTENSION OF PATENT TERM UNDER 35 U.S.C. §156

HAND DELIVERY

Office of Patent Legal Administration
Room MDW 7D55
600 Dulany Street (Madison Building)
Alexandra, VA 22314

Madam:

The Applicant, Kyowa Hakko Kirin Co., Ltd. hereby applies for extension of the term of United States Patent No. 7,718,175 under 35 U.S.C. §156 and provides the following information in accordance with 37 C.F.R. §1.740. Separate applications under 35 U.S.C. §156, which are based on the same regulatory review period for the product, FASENRA™ (benralizumab), are being submitted for United States Patent Nos. 7,179,464 and 8,101,185.

Kyowa Hakko Kirin Co., Ltd. is the assignee of the entire interest in and to U.S. Patent No. 7,718,175 (the '175 patent), and the chain of title is recorded in the USPTO assignment records as follows:

1. Assignment from inventors Nobuo Hanai, Kazuyasu Nakamura, Emi Hosaka, Motoo Yamasaki, Kazuhisa Uchida, Toyohide Shinkawa, Susumu Imabeppu, Yutaka Kanda, Naoko Yamane, Hideharu Anazawa to Kyowa Hakko Kogyo Co., Ltd., recorded at Reel 012301, Frame 0276;
2. Change of name from Kyowa Hakko Kogyo Co., Ltd. to Kyowa Hakko Kirin Co., Ltd., recorded at Reel 022542, Frame 0823.

A Power of Attorney and Statement under 37 C.F.R. §3.73(b) appointing the attorney associated with customer number 1131 to transact all business in the USPTO in connection with the '175 patent is filed concurrently herewith. Copies of these documents are provided as Exhibit E.

BioWa, Inc. was the original Investigational New Drug (IND) applicant for benralizumab (IND Number 100,237), and began the first clinical investigation of benralizumab. On March 7, 2007, BioWa,

Inc. transferred ownership of the IND exemption to MedImmune, Inc. Effective as of April 1, 2008, MedImmune, Inc. became MedImmune, LLC. A copy of the Certificate of Conversion is provided in Exhibit G. AstraZeneca AB, an affiliate of MedImmune, LLC, through AstraZeneca Pharmaceuticals LP as the U.S. agent, was the original marketing authorization applicant for FASENRA™ (benralizumab), submitted on November 16, 2016. AstraZeneca AB is the current holder of BLA No. 761070 for FASENRA™ (benralizumab), which was approved by the U.S. Food and Drug Administration on November 14, 2017. Copies of letters from each of BioWa, Inc., MedImmune, LLC, and AstraZeneca AB authorizing Applicant to rely upon their activities before the U.S. Food and Drug Administration (FDA) are provided in Exhibit F.

1. **Identification of the Approved Product (37 C.F.R. § 1.740(a)(1))**

The name of the approved product is FASENRA™. The active ingredient in FASENRA™ is benralizumab. Benralizumab is a humanized monoclonal antibody (IgG1/κ-class) selective for interleukin-5 receptor alpha subunit (IL5Rα). Benralizumab is produced in Chinese hamster ovary cells by recombinant DNA technology. Benralizumab has a molecular weight of approximately 150 kDa. FASENRA™ (benralizumab) injection is a sterile, preservative-free solution for subcutaneous injection, where each single-dose prefilled syringe delivers 1 mL containing 30 mg benralizumab. FASENRA™ (benralizumab) is indicated for the add-on maintenance treatment of patients with severe asthma aged 12 years and older, and with an eosinophilic phenotype.

2. **Federal Statute Governing Regulatory Approval of the Approved Product (37 C.F.R. § 1.740(a)(2))**

Regulatory approval of the product is governed by the Public Health Service Act (42 U.S.C. § 201 *et seq.*) and the Federal Food, Drug and Cosmetic Act (21 U.S.C. § 355 *et seq.*)

3. **Date of Approval for Commercial Marketing (37 C.F.R. § 1.740(a)(3))**

FASENRA™ (benralizumab) was approved for commercial marketing and use under section 351(a) of the Public Health Service Act on November 14, 2017 (Exhibit A).

4. **Identification of Active Ingredient and Statements Related to Commercial Marketing of the Approved Product (37 C.F.R. §1.740(a)(4))**

The name of the approved product is FASENRA™. The active ingredient in FASENRA™ is benralizumab. Benralizumab is a humanized monoclonal antibody (IgG1/κ-class) selective for interleukin-5 receptor alpha subunit (IL5Rα). Benralizumab is produced in Chinese hamster ovary cells by recombinant DNA technology. Benralizumab has a molecular weight of approximately 150 kDa. FASENRA™ (benralizumab) injection is a sterile, preservative-free solution for subcutaneous injection, where each single-dose prefilled syringe delivers 1 mL containing 30 mg benralizumab. FASENRA™ (benralizumab) is indicated for the add-on maintenance treatment of patients with severe asthma aged 12 years and older, and with an eosinophilic phenotype.

a. Applicant states that FASENRA™ (benralizumab) has been approved for the add-on maintenance treatment of patients with severe asthma aged 12 years and older, and with an eosinophilic phenotype. FASENRA™ (benralizumab) was approved for commercial

marketing and use under Department of Health and Human Services License No. 2059 to AstraZeneca AB, under the provisions of section 351(a) of the Public Health Service Act. (See FASENRA™ (benralizumab) approval letter and product label; Exhibits A & B).

b. Applicant states that benralizumab has not been previously approved for commercial marketing under the Food, Drug and Cosmetic Act, the Public Health Service Act, or the Virus-Serum Toxin Act, either alone or in combination with other ingredients.

5. Statement Regarding Timelines of Submission of Patent Term Extension Request (37 C.F.R. § 1.740(a)(5))

Applicant states that this application for patent term extension is being timely submitted within the sixty day statutory period specified in 35 U.S.C. §156(d)(1). The last day that this application may be timely submitted is January 12, 2018.

6. Complete Identification of the Patent for Which Extension is Being Sought (37 C.F.R. § 1.740(a)(6))

a.	Names of inventors:	Nobuo Hanai, Kazuyasu Nakamura, Emi Hosaka, Motoo Yamasaki, Kazuhisa Uchida, Toyohide Shinkawa, Susumu Imabeppu, Yutaka Kanda, Naoko Yamane, Hideharu Anazawa
b.	Patent Number:	7,718,175
c.	Date of Issue:	May 18, 2010
d.	Date of Expiration:	April 7, 2020

7. Copy of the Patent for Which an Extension is Being Sought (37 C.F.R. § 1.740(a)(7))

A copy of U.S. Patent No. 7,718,175 is provided in Exhibit C.

8. Copies of Disclaimers, Certificates of Correction, Receipt of Maintenance Fee Payment, or Reexamination Certificate (37 C.F.R. §1.740(a)(8))

a. The '175 patent is subject to a terminal disclaimer.

b. A certificate of correction has not been issued for the '175 patent.

c. The maintenance fees for the '175 patent that were due 3.5 and 7.5 years after grant were timely paid (Exhibit D).

d. The '175 patent has not been the subject of a reexamination proceeding.

9. Statement regarding Patent Claims Relative to Approved Product (37 C.F.R. § 1.740(a)(9))

a. Claims 1-3 of the '175 patent claim the approved product or the approved use of the approved product.

b. Pursuant to 37 C.F.R. § 1.740(a)(9) and MPEP § 2753, the following explanation is provided which demonstrates the manner in which claims 1-3 read on the approved product, and the manner in which claims 1-3 read on the manner of using the approved product.

i. Description of the approved product

Benralizumab (previously referred to as MEDI-563) for subcutaneous injection is a humanized afucosylated interleukin-5 receptor alpha-directed cytolytic monoclonal antibody (IgG1, kappa).

ii. Demonstration of the manner in which claims 1-3 of U.S. Patent No. 7,718,175 read on the approved product.

Claims 1-3 of the '175 patent read as follows:

Claims of the '175 patent	**Demonstration of the manner in which each claim reads on the product**
1. An antibody composition comprising antibody molecules, wherein 100% of the antibody molecules comprising an Fc region comprising complex N-glycoside-linked sugar chains bound to the Fc region through N-acetylglucosamines of the reducing terminal of the sugar chains do not contain sugar chains with a fucose bound to the N-acetylglucosamines, and wherein said antibody molecules bind to an interleukin-5 receptor protein.	As discussed in Tan et al., *J Asthma Allergy*, 9: 71-81 (2016) (Exhibit K), eosinophils are a key target in treating inflammatory respiratory diseases, such as asthma and COPD. One therapeutic mechanism by which eosinophils are targeted is through activation of antibody-dependent cellular cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC) by effector cells (such as killer cells, natural killer cells, activated macrophages, or the like). The mechanism of ADCC activity is specifically mediated through an antibody which recruits an effector cell to a target cell. More specifically, as described in the '175 patent (column 2, lines 41-46), ADCC occurs when an Fcγ receptor (FcγR) on the surface of an effector cell binds the Fc region of an antibody bound to the surface of a target cell, resulting in expression of ADCC by the effector cell and lysis of the target cell. Enhancing the ADCC activity of an antibody is critical for developing therapeutics that utilize this pathway. According to the '175 patent (column 2, line 46 to column 3, line 11), it is known that several amino acid residues in the second domain of the antibody hinge region and the C region (the Cγ2 / CH2 domain) and a sugar chain linked to the Cγ2 domain, are important for this binding interaction. As discussed in the '175 patent in column 8, lines 2-26, the sugar chain can be roughly classified into two kinds: a sugar chain which binds to serine, threonine (called an O-glycoside-linked sugar chain), and a sugar chain which binds to asparagine (called N-glycoside-

Claims of the '175 patent	Demonstration of the manner in which each claim reads on the product
	linked sugar chain). The sugar chain terminal which binds to asparagine is called the reducing terminal, and the opposite side is called the non-reducing terminal. The importance of the sugar chain on the antibody is specifically discussed in the '175 patent beginning in column 2, line 52 – column 3, line 10 which states: regarding the sugar chain, Boyd et al. (*Molecular Immunol.*, 32, 1311 (1995)) have examined effects of a sugar chain on the ADCC activity and CDC activity, by treating a human CDR-grafted antibody, CAMPATH-1H (human IgG1 subclass), produced using Chinese hamster ovary cell (CHO cell) or mouse myeloma NS0 cell with various sugar hydrolyzing enzymes, and reported that elimination of sialic acid of the non-reducing terminal does not have influence upon both activities. Further elimination of galactose residue however was reported to exert influence upon only the CDC activity, decreasing about 50% of its activity. Complete elimination of the sugar chain was reported to cause disappearance of both activities. Moreover, Lifely et al. (*Glycobiology*, 5(8): 813-822 (1995)) have analyzed the sugar chain of a human CDR-grafted antibody, CAMPATH-1H (human IgG1 subclass) which was produced using CHO cell, NS0 cell or rat myeloma YO cell, measured its ADCC activity and reported that the CAMPATH-1H derived from YO cell shows the greatest ADCC activity, suggesting that N-acetylglucosamine at the bisecting position is important for the activity. These reports indicate that the structure of the sugar chain plays an important role in the effector functions of human antibodies of the IgG1 subclass and that it is possible to prepare an antibody having higher effector function by changing the structure of the sugar chain. As stated in the '175 patent, column 8, line 23-26, a fucose sugar may be bound to N-acetylglucosamine at the reducing terminal by an α1,3 bond, an α1,6 bond, and the like. The paper by Kolbeck, R., et al., *J. Allergy Clin. Immunol.*, 125(6):1344-1353 (2010) (See Exhibit H), discusses on page 1345 that afucosylation of the oligosaccharide core of human IgG1 has previously been shown to result in a 5- to 50-fold higher affinity to human FcγRIIIa, the main activating Fcγ receptor (FcγR) expressed on natural killer (NK) cells, macrophages, and neutrophils. Furthermore, as stated in Tan,

Claims of the '175 patent	Demonstration of the manner in which each claim reads on the product
	"afucosylation enhances the interaction of benralizumab with its binding site and thus heightens ADCC functions by >1,000-fold over the parental antibody." As further disclosed in Tan, benralizumab (also known as MEDI-563) is a humanized, recombinant, afucosylated IgG1κ monoclonal antibody. In other words, it has been engineered without a fucose sugar residue in the CH2 region (Cγ2 region). Furthermore, as discussed in Tan, many functions of eosinophils (and basophils) are driven by the cytokine interleukin 5 (IL-5), and benralizumab has been designed to specifically bind to the α chain of the IL-5 receptor (IL-5Rα) expressed by mature eosinophils. More specifically, according to the Evaluate™ Press Release (Exhibit L), Benralizumab is an anti-eosinophil monoclonal antibody that depletes eosinophils via ADCC, the process by which natural killer cells are activated to target eosinophils. Benralizumab induces direct, rapid, and near complete depletion of eosinophils in the bone marrow, blood, and target tissue. Additionally, a further discussion regarding the lack of fucosylation of benralizumab can be found in WO 2015/105926 in paragraphs [00859]-[000861] on pages 188-189 which states: "[B]enralizumab, also known as MEDI-563, is a fully humanized IgG1 anti- human IL5-Rα antibody that binds to an epitope on IL-5Rα which is in close proximity to the IL-5 binding site and thus inhibits IL-5 receptor signaling originally developed by MedImmune. Benralizumab induces apoptosis through antibody-dependent cell- mediated cytotoxicity (ADCC) depleting eosinophils, a key target cell in inflammatory respiratory disease. **Benralizumab is also afucosylated. i.e., a fucose sugar residue in the CH2 region of the oligosaccharide core of human IgGI is removed**. This removal results in a 5- to 50-fold higher affinity to the main activating Fcγ receptor (human FcγRIIIa) expressed on natural killer (NK) cells, macrophages and neutrophils. **Therefore, afucosylated benralizumab has an improved receptor-mediated**

Claims of the '175 patent	Demonstration of the manner in which each claim reads on the product
	effector function, resulting in an amplified eosinophil apoptosis *in vitro* via antibody-dependent cell-mediated cytotoxicity (ADCC) by more than 1000-fold over the parental antibody (Kolbeck et al., 2009 and Ferrara et al, 2011, the contents of each of which are incorporated herein by reference in their entirety). Benralizumab is a monoclonal antibody that binds to a distinct epitope within the extracellular domain of recombinant human IL-5Rα, preventing receptor dimerization and subsequent inflammatory signaling (Ghazi et al, 2012). **Benralizumab is afucosylated, and afucosylation is associated with enhanced ADCC.** Benralizumab was found to induce apoptosis in eosinophils and basophils through ADCC (Kolbeck et al, 2009). While anti-IL-5 mAbs act by neutralizing the effects of IL-5, in contrast, Benralizumab targets the effector cells, mainly eosinophils and basophils, and consequently actively depletes these cells. Indeed, in animal models, Benralizumab depleted peripheral blood eosinophils to less than the limit of detection (Ghazi et al, 2012, Busse et al, 2010, Kolbeck et al, 2009) (emphasis added)." Finally, the approved product label for benralizumab under "12.1 Mechanism of Action" portion under "Clinical Pharmacology" in Section 12 states that "[B]enralizumab is a humanized afucosylated, monoclonal antibody (IgG1, kappa) that directly binds to the alpha subunit of the human interleukin-5 receptor (IL-5Rα) with a dissociation consent of 11 pM. The IL-5 receptor is expressed on the surface of eosinophils and basophils. In an *in vitro* setting, the **absence of fucose in the Fc[1] domain of benralizumab facilitates binding (45.5 mM) to** FcγRIII receptors on immune effectors cells, such as natural killer (NK) cells, leading to apoptosis of eosinophils and basophils through antibody-dependent cell-mediated cytotoxicity (ADCC) (emphasis added). As demonstrated by the above recited publications and the approved product label for benralizumab, 100% of

[1] The Fc region of benralizumab comprises N-glycoside-linked sugar chains bound to this region through N-acetylglucosamines.

Claims of the '175 patent	Demonstration of the manner in which each claim reads on the product
	the antibody has an Fc region that does not contain sugar chains with fucose (bound to one or more N-acetylglucosamines). All of the limitations of claim 1 are met by benralizumab.
2. The antibody composition according to claim 1, wherein the antibody molecules are selected from the group consisting of (a), (b) and (c); (a) human antibodies; (b) humanized antibodies; (c) antibody fragments.	Claim 2 depends from claim 1. Benralizumab is an IgG1, kappa humanized monoclonal antibody as shown by the approved product label contained in Exhibit B. All of the limitations of claim 2 are met by benralizumab.
3. The antibody composition according to claim 1, wherein the antibody molecules belong to an IgG class.	Claim 3 depends from claim 1. Benralizumab is an IgG1, kappa humanized monoclonal antibody as shown by the approved product label contained in Exhibit B. All of the limitations of claim 3 are met by benralizumab.

10. Relevant Dates under 35 U.S.C. § 156 for Determination of Applicable Regulatory Review Period (37 C.F.R. § 1.740(a)(10))

U.S. Patent No. 7,718,175 issued on May 18, 2010. The following information is provided pursuant to 37 C.F.R. § 1.740(a)(10)(i)(A)-(C):

a. **The effective date of the investigational new drug application (IND) and the IND number. 37 C.F.R. § 1.740(a)(10)(i)(A)**

The investigational new drug application (IND) was submitted on June 29, 2006, and was effective 30 days later on July 29, 2006. The IND number is 100,237. BioWa, Inc. was the original IND applicant for benralizumab. The IND was transferred by BioWa, Inc. to MedImmune, Inc., effective as of March 7, 2007. Effective as of April 1, 2008, MedImmune, Inc. became MedImmune, LLC.

b. **The date on which a new drug application (NDA) or a Product License Application (PLA) was initially submitted and the NDA or PLA number. 37 C.F.R. § 1.740(a)(10)(i)(B)**

A Biologics License Application (BLA) was initially submitted to FDA pursuant to Section 351(a) of the Public Health Service Act and 505(b)(1) of the Federal Food, Drug, and Cosmetic Act, by AstraZeneca AB, an affiliate of MedImmune LLC, through AstraZeneca Pharmaceuticals LP as the U.S. agent, on November 16, 2016. FDA acknowledged its receipt of the BLA on November 16, 2016 via a BLA Acknowledgment to AstraZeneca AB notifying AstraZeneca AB that the BLA would be filed on January 15, 2017. The BLA was assigned number BLA 761070.

c. **The date on which the NDA was approved or the Product License issued. 37 C.F.R. § 1.740(a)(10)(i)(C)**

The FDA approved BLA 761070, authorizing the marketing of FASENRA™ (benralizumab), on November 14, 2017. FASENRA™ (benralizumab) was approved under Department of Health and Human Services U.S. License No. 2059 (*see* Exhibit A).

11. **Summary of Significant Events During Regulatory Review Period (37 C.F.R. § 1.740(a)(11))**

On June 29, 2006, BioWa, Inc. submitted to FDA an IND for BIW-8405 for asthma.

As of July 29, 2006, BioWa, Inc. had not received any notice from the FDA that the studies proposed under IND 100,237 were under a clinical hold, so the studies under IND 100,237 could be commenced. Effective as of March 7, 2007, BioWa, Inc. transferred ownership of the investigational new drug exemption to MedImmune, Inc. Effective as of April 1, 2008, MedImmune, Inc. became MedImmune, LLC.

Multiple randomized, double-blind, parallel-group, placebo-controlled clinical trials were conducted under IND 100,237, among other studies, including:

A Phase 1, Double-Blind, Placebo-Controlled Study to Evaluate the Safety, Tolerability and Effects of Benralizumab (MEDI-563), A Humanized Anti-Interleukin-5 Receptor Alpha Monoclonal Antibody, on Airway Eosinophils in Adults With Atopic Asthma (Study Start Date: January 2008; Study Completion Date: March 2011);

A Phase 2, Multicenter, Randomized, Double-blind, Placebocontrolled Study to Evaluate the Safety and Efficacy of Intravenously Administered Benralizumab (MEDI-563), A Humanized Anti-interleukin-5 Receptor Alpha Monoclonal Antibody, on Asthma Control Following Acute Exacerbations in Adults (Study Start Date: February 2009; Study Completion Date: March 2011);

A Phase 2b, Dose-ranging Study to Evaluate the Efficacy and Safety of Benralizumab (MEDI-563) in Adults With Uncontrolled Asthma (Study Start Date: December 2010; Study Completion Date: August 2013);

A Multicentre, Randomized, Double-blind, Parallel Group, Placebo-controlled, Phase III Efficacy and Safety Study of Benralizumab (MEDI-563) Added to High-dose Inhaled Corticosteroid Plus Long-Acting β2 Agonist in Patients with Uncontrolled Asthma (Study Start Date: September 19, 2013; Study Completion Date: April 5, 2016);

A Multicentre, Randomized, Double-blind, Parallel Group, Placebo-controlled, Phase III Study to evaluate the Efficacy and Safety of Benralizumab in Asthmatic Adults and Adolescents Inadequately Controlled on Inhaled Corticosteroid Plus Long-Acting β2 Agonist (CALIMA)(Study Start Date: August 2013; Study Completion Date: March 2016); and

A Multicenter, Randomized, Double-blind, Parallel Group, Placebo-controlled, Phase 3 Efficacy and Safety Study of Benralizumab (MEDI-563) to Reduce Oral Corticosteroid Use in Patients With Uncontrolled Asthma on High Dose Inhaled Corticosteroid Plus Long-acting β2 Agonist and Chronic Oral Corticosteroid Therapy (ZONDA)(Study Start Date: April 2014; Study Completion Date: August 2016)

A Multicentre, Double-blind, Randomized, Parallel Group, Phase 3 Safety Extension Study to Evaluate the Safety and Tolerability of Benralizumab (MEDI-563) in Asthmatic Adults and Adolescents on Inhaled Corticosteroid Plus Long-acting β2 Agonist (BORA)(Study Start Date: November 19, 2014; Estimated Study Completion Date: July 9, 2018).

On November 16, 2016, AstraZeneca AB, an affiliate of MedImmune, LLC, through AstraZeneca Pharmaceuticals LP as the U.S. agent, submitted the BLA for benralizumab subcutaneous injection as an add-on maintenance treatment for patients with severe asthma.

FDA acknowledged its receipt of the BLA on November 16, 2016 via BLA Acknowledgment. Number BLA 761070 was assigned to the BLA. The BLA Acknowledgment stated that the BLA would be filed on January 15, 2017.

FASENRA™ (benralizumab) was approved on November 14, 2017 for commercial marketing and use under Department of Health and Human Services U.S. License No. 2059, under the provisions of section 351(a) of the Public Health Service Act (Exhibit A). Approval of the BLA was issued to AstraZeneca AB.

The activities undertaken by the marketing applicants during the regulatory review period are provided in Exhibits I (IND Period) and J (BLA Period).

12. **Statement Concerning Eligibility for and Duration of Extension Sought Under 35 U.S.C. § 156 (37 C.F.R. § 1.740(a)(12))**

    a. In the opinion of the Applicant, the '175 patent is eligible for an extension of patent term under 35 U.S.C. § 156 and 37 C.F.R. § 1.790 because:

        i. One or more claims of the '175 patent claim the approved product or a method of using the approved product. 35 U.S.C. § 156(a) and 37 C.F.R. § 1.720(a).

        ii. The term of the '175 patent has not expired before the submission of this Application. 35 U.S.C. § 156(a)(1) and 37 C.F.R. § 1.720(g).

        iii. The term of the '175 patent has not been extended under 35 U.S.C. § 156. 35 U.S.C. § 156(a)(2) and 37 C.F.R. § 1.720(b).

        iv. This Application is submitted by the owner of record of the '175 patent and in compliance with 35 U.S.C. § 156 and 37 C.F.R. § 1.720. 35 U.S.C. § 156(a)(3) and 37 C.F.R. § 1.720(c), 1.730(b).

        v. The approved product, FASENRA™ (benralizumab), was subject to a regulatory review period before its commercial marketing or use. 35 U.S.C. § 156(a)(4) and 37 C.F.R. § 1.720(d).

        vi. The permission for commercial marketing or use of the approved product after regulatory review is the first permitted commercial marketing or use of the product. 35 U.S.C. § 156(a)(5) and 37 C.F.R. § 1.720(e).

vii. The application for patent term extension is submitted within the 60-day period beginning on the approval date of the approved product. 35 U.S.C. § 156(d)(1) and 37 C.F.R. §1.720(f).

viii. The term of no other patent has been extended pursuant to 35 USC § 156 based on the same regulatory review period for the product, FASENRA™ (benralizumab). 35 U.S.C. § 156(c)(4) and 37 C.F.R. § 1.720(h). Note that separate applications under 35 U.S.C. §156, which are based on the same regulatory review period for the product, FASENRA™ (benralizumab), are being submitted for United States Patent Nos. 7,179,464 and 8,101,185.

b. Period of request extension and explanation of how the length of extension was calculated.

i. Applicant requests that the term of the '175 patent be extended by a period of 4 ¼ years (1,552 days).

ii. The requested period of extension of the term of the '175 patent corresponds to the regulatory review period that is available for extension under 35 U.S.C. § 156, based on the facts and circumstances of the regulatory review period of the approved product, FASENRA™ (benralizumab). The requested period of extension is based on the regulatory review and issue date of the '175 patent and was determined as follows:

1. The relevant dates for determining the period of patent term extension are:

IND became effective:	July 29, 2006
U.S. Patent No. 7,718,175 issued:	May 18, 2010
BLA for FASENRA™ (benralizumab) was submitted:	November 16, 2016
BLA for FASENRA™ (benralizumab) was filed:	January 15, 2017
BLA was approved:	November 14, 2017

2. The '175 patent issued **during** the period specified in 35 U.S.C. § 156(g)(1)(B)(i). The regulatory review period thus includes a period based on the time between the day the '175 patent issued and the date the BLA was submitted. This period is 2,375 days. [May 18, 2010 to November 16, 2016] Pursuant to 35 U.S.C. § 156(c)(2) the calculated regulatory review period includes one-half of the time in the period specified in 35 U.S.C. § 156(g)(1)(B)(i). Accordingly, the regulatory review period includes 1,188 days (one-half of 2,375 days).

3. The '175 patent issued prior to the start of the period specified in 35 U.S.C. § 156(g)(1)(B)(ii). The regulatory review period thus includes a period equal to the number of days from the day the BLA was submitted and the day the BLA was approved. [November 16, 2016 – November 14, 2017] This period is 364 days.
4. The period of patent term extension for the approved product, FASENRA™ (benralizumab), determined according to 35 U.S.C. § 156(b), (c)(2) and (g)(1), and in view of the calculated regulatory review periods set forth above, is 1,552 days (1,188 days plus 364 days).
5. The 14-year limit pursuant to 35 U.S.C. § 156(c)(3) does not apply to this application for the following reasons: The '175 patent will expire on April 7, 2020 and the date that is 14 years after the date of approval of the approved product is November 14, 2031. The period from the date the patent expires to the end of the 14-year period is 4,238 days, which is longer than the calculated period of patent term extension.
6. The '175 patent issued after the enactment of 35 U.S.C. § 156, therefore, the period of extension on the basis of regulatory review may not exceed 5-years pursuant to 35 U.S.C. § 156(g)(6)(A). The requested period of extension, taking into account the 14-year limit pursuant to 35 U.S.C. § 156(c)(3), is 1,552 days, which is about 4 ¼ years. Accordingly, the 5-year limit under 35 U.S.C. § 156(g)(6)(A) does not apply to this extension.

13. **Statement Pursuant to 37 C.F.R. § 1.740(a)(13)**

Applicant acknowledges the duty to disclose to the Director of the United States Patent and Trademark Office and the Secretary of Health and Human Services or the Secretary of Agriculture any information which is material to the determination of entitlement to the extension sought.

14. **Applicable Fee (37 C.F.R. § 1.740(a)(14))**

The fee for this application prescribed in 37 C.F.R. § 1.20(j) is $1120.00. Please charge the fee, and any fees associated and due in this application to our Deposit Account No. 13-3080, referencing the number: 208689-9028.

15. **Name and Address for Correspondence (37 C.F.R. § 1.740(a)(15))**

Please direct all inquiries and correspondence relating to this application for patent term extension to:

Lisa L. Mueller
MICHAEL BEST & FRIEDRICH LLP
444 W. Lake Street, Suite 3200
Chicago, Illinois 60606
312.222.0800 (main)
312.596.5812 (direct)
312.222.0818 (fax)

16. **Copies of this Application (37 C.F.R. § 1.740(b))**

Pursuant to 37 C.F.R. § 1.740(b), two additional copies of this application (for a total of three copies) are filed concurrently herewith.

Respectfully submitted,

/Lisa L. Mueller/ Reg. No. 38,978
Lisa L. Mueller, Reg. No. 38,978

Please recognize our Customer number 105263 as our correspondence address

MICHAEL BEST & FRIEDRICH LLP
444 W. Lake Street, Suite 3200
Chicago, Illinois 60606
312.222.0800 (main)
312.596.5812 (direct)
312.222.0818 (fax)

January 11, 2018

Exhibit A

Copy of the BLA Approval Letter

DEPARTMENT OF HEALTH AND HUMAN SERVICES

Food and Drug Administration
Silver Spring MD 20993

BLA 761070

BLA APPROVAL

AstraZeneca AB
c/o AstraZeneca Pharmaceuticals LP
1800 Concord Pike
Wilmington, Delaware 19803

Attention: Les Thomas
Director, Regulatory Affairs

Dear Mr. Thomas:

Please refer to your Biologics License Application (BLA) dated and received November 16, 2016, submitted under section 351(a) of the Public Health Service Act for Fasenra (benralizumab) 30 mg/mL injection.

LICENSING

We are issuing Department of Health and Human Services U.S. License No. 2059 to AstraZeneca AB, Karlebyhus, Astraallen, Sodertalje, Stockholm County, Sweden, under the provisions of section 351(a) of the Public Health Service Act controlling the manufacture and sale of biological products. The license authorizes you to introduce or deliver for introduction into interstate commerce, those products for which your company has demonstrated compliance with establishment and product standards.

Under this license, you are authorized to manufacture the product Fasenra (benralizumab). Fasenra is indicated for add-on maintenance treatment of patients with severe asthma aged 12 years and older, and with an eosinophilic phenotype.

MANUFACTURING LOCATIONS

Under this license, you are approved to manufacture benralizumab drug substance at AstraZeneca Pharmaceuticals LP Frederick Manufacturing Center in Frederick, Maryland. The final formulated product will be manufactured, filled, labeled, and packaged at (b)(4) (b)(4). You may label your product with the proprietary name, Fasenra, and will market it in 1.0 mL accessorized pre-filled syringe.

DATING PERIOD

The dating period for Fasenra shall be 24 months from the date of manufacture when stored at 2 to 8°C. The date of manufacture shall be defined as the date of final sterile filtration of the formulated drug product. The dating period for your drug substance shall be (b)(4) months from the date of manufacture when stored at (b)(4). We have approved the stability protocols in

your license application for the purpose of extending the expiration dating period of your drug substance and drug product under 21 CFR 601.12.

FDA LOT RELEASE

You are not currently required to submit samples of future lots of Fasenra to the Center for Drug Evaluation and Research (CDER) for release by the Director, CDER, under 21 CFR 610.2. We will continue to monitor compliance with 21 CFR 610.1, requiring completion of tests for conformity with standards applicable to each product prior to release of each lot.

Any changes in the manufacturing, testing, packaging, or labeling of Fasenra, or in the manufacturing facilities, will require the submission of information to your biologics license application for our review and written approval, consistent with 21 CFR 601.12.

APPROVAL & LABELING

We have completed our review of this application, as amended. It is approved, effective on the date of this letter, for use as recommended in the enclosed agreed-upon labeling text.

CONTENT OF LABELING

As soon as possible, but no later than 14 days from the date of this letter, submit, via the FDA automated drug registration and listing system (eLIST), the content of labeling [21 601.14(b)] in structured product labeling (SPL) format, as described at http://www.fda.gov/ForIndustry/DataStandards/StructuredProductLabeling/default.htm. Content of labeling must be identical to the enclosed labeling (text for the package insert and for the patient information insert). Information on submitting SPL files using eLIST may be found in the guidance for industry titled "SPL Standard for Content of Labeling Technical Qs and As" at http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM072392.pdf.

The SPL will be accessible via publicly available labeling repositories.

CARTON AND IMMEDIATE CONTAINER LABELS

Submit final printed carton and container labels that are identical to the enclosed carton and immediate container labels as soon as they are available, but no more than 30 days after they are printed. Please submit these labels electronically according to the guidance for industry titled *Providing Regulatory Submissions in Electronic Format — Certain Human Pharmaceutical Product Applications and Related Submissions Using the eCTD Specifications (May 2015, Revision 3)*. For administrative purposes, designate this submission "**Final Printed Carton and Container Labels for approved BLA 761070.**" Approval of this submission by FDA is not required before the labeling is used.

ADVISORY COMMITTEE

Your application for Fasenra was not referred to an FDA advisory committee because this biologic is not the first in its class.

REQUIRED PEDIATRIC ASSESSMENTS

Under the Pediatric Research Equity Act (PREA) (21 U.S.C. 355c), all applications for new active ingredients, new indications, new dosage forms, new dosing regimens, or new routes of administration are required to contain an assessment of the safety and effectiveness of the product for the claimed indication(s) in pediatric patients unless this requirement is waived, deferred, or inapplicable.

We are waiving the pediatric study requirement for ages zero to 5 years because the necessary studies are impossible or highly impracticable. This is because there are too few children with the condition to study.

We are deferring submission of your pediatric study for ages 6 to 11 years for this application because pediatric studies should be delayed until additional safety or effectiveness data have been collected.

Your deferred pediatric study required by section 505B(a) of the Federal Food, Drug, and Cosmetic Act are required postmarketing study. The status of this postmarketing study must be reported annually according to 21 CFR 601.28 and section 505B(a)(3)(B) of the Federal Food, Drug, and Cosmetic Act. This required study is listed below.

3287-1 Conduct an open-label, pharmacokinetic and pharmacodynamics study of benralizumab in pediatric patients 6 to 11 years of age with a continued safety evaluation out to a minimum of 48 weeks.

Draft Protocol submission date:	June 2018
Final protocol submission date:	October 2018
Study Completion date:	May 2022
Final report submission date:	December 2022

Submit the protocol to your IND 100237, with a cross-reference letter to this BLA.

Reports of this required pediatric postmarketing study must be submitted as a BLA or as a supplement to your approved BLA with the proposed labeling changes you believe are warranted based on the data derived from these studies. When submitting the reports, please clearly mark your submission "**SUBMISSION OF REQUIRED PEDIATRIC ASSESSMENTS**" in large font, bolded type at the beginning of the cover letter of the submission.

We note that you have fulfilled the pediatric study requirement for ages 12 to 17 years for this application.

POSTMARKETING COMMITMENTS NOT SUBJECT TO THE REPORTING REQUIREMENTS UNDER SECTION 506B

We remind you of your postmarketing commitments:

3287-2 Perform a leachable study to evaluate the (b)(4) drug product container closure systems through the end of shelf-life when stored under the recommended conditions. Perform testing at regular intervals and include appropriate methods to detect, identify, and quantify organic non-volatile (e.g., HPLC-UV-MS), volatile (e.g., headspace GC-MS) and semi-volatile (e.g., GCMS) species and metals (e.g., ICP-MS). Update study results in the BLA Annual Report. Submit the complete data and risk evaluation for potential impact of leachables on product safety and quality to the BLA.

The timetable you submitted on October 31, 2017, states that you will conduct this study according to the following schedule:

Final Bulk Product Report Submission:	December 2021
Final Report Submission (Drug Product):	December 2023

Submit clinical protocols to your IND 100237 for this product. Submit nonclinical and chemistry, manufacturing, and controls protocols and all postmarketing final reports to this BLA. In addition, under 21 CFR 601.70 you should include a status summary of each commitment in your annual progress report of postmarketing studies to this BLA. The status summary should include expected summary completion and final report submission dates, any changes in plans since the last annual report, and, for clinical studies/trials, number of patients entered into each study/trial. All submissions, including supplements, relating to these postmarketing commitments should be prominently labeled "**Postmarketing Commitment Protocol**," "**Postmarketing Commitment Final Report**," or "**Postmarketing Commitment Correspondence**."

PROMOTIONAL MATERIALS

You may request advisory comments on proposed introductory advertising and promotional labeling. To do so, submit, in triplicate, a cover letter requesting advisory comments, the proposed materials in draft or mock-up form with annotated references, and the package insert to:

Food and Drug Administration
Center for Drug Evaluation and Research
Office of Prescription Drug Promotion
5901-B Ammendale Road
Beltsville, MD 20705-1266

As required under 21 CFR 601.12(f)(4), you must submit final promotional materials, and the package insert, at the time of initial dissemination or publication, accompanied by a Form FDA 2253. Form FDA 2253 is available at http://www.fda.gov/downloads/AboutFDA/ReportsManualsForms/Forms/UCM083570.pdf. Information and Instructions for completing the form can be found at http://www.fda.gov/downloads/AboutFDA/ReportsManualsForms/Forms/UCM375154.pdf. For more information about submission of promotional materials to the Office of Prescription Drug Promotion (OPDP), see http://www.fda.gov/AboutFDA/CentersOffices/CDER/ucm090142.htm.

REPORTING REQUIREMENTS

You must submit adverse experience reports under the adverse experience reporting requirements for licensed biological products (21 CFR 600.80). You should submit postmarketing adverse experience reports to:

Food and Drug Administration
Center for Drug Evaluation and Research
Central Document Room
5901-B Ammendale Road
Beltsville, MD 20705-1266

Prominently identify all adverse experience reports as described in 21 CFR 600.80.

You must submit distribution reports under the distribution reporting requirements for licensed biological products (21 CFR 600.81).

You must submit reports of biological product deviations under 21 CFR 600.14. You should promptly identify and investigate all manufacturing deviations, including those associated with processing, testing, packing, labeling, storage, holding and distribution. If the deviation involves a distributed product, may affect the safety, purity, or potency of the product, and meets the other criteria in the regulation, you must submit a report on Form FDA-3486 to:

Food and Drug Administration
Center for Drug Evaluation and Research
Division of Compliance Risk Management and Surveillance
5901-B Ammendale Road
Beltsville, MD 20705-1266

Biological product deviations, sent by courier or overnight mail, should be addressed to:

Food and Drug Administration
Center for Drug Evaluation and Research
Division of Compliance Risk Management and Surveillance
10903 New Hampshire Avenue, Bldg. 51, Room 4206
Silver Spring, MD 20903

MEDWATCH-TO-MANUFACTURER PROGRAM

The MedWatch-to-Manufacturer Program provides manufacturers with copies of serious adverse event reports that are received directly by the FDA. New molecular entities and important new biologics qualify for inclusion for three years after approval. Your firm is eligible to receive copies of reports for this product. To participate in the program, please see the enrollment instructions and program description details at http://www.fda.gov/Safety/MedWatch/HowToReport/ucm166910.htm.

POST APPROVAL FEEDBACK MEETING

New molecular entities and new biologics qualify for a post approval feedback meeting. Such meetings are used to discuss the quality of the application and to evaluate the communication process during drug development and marketing application review. The purpose is to learn from successful aspects of the review process and to identify areas that could benefit from improvement. If you would like to have such a meeting with us, call the Regulatory Project Manager for this application.

If you have any questions, call Colette Jackson, Senior Regulatory Health Project Manager, at (301) 796-1230.

Sincerely,

{See appended electronic signature page}

Curtis J. Rosebraugh, MD, MPH
Director
Office of Drug Evaluation II
Office of New Drugs
Center for Drug Evaluation and Research

ENCLOSURES: Content of Labeling
Carton and Container Labeling

This is a representation of an electronic record that was signed electronically and this page is the manifestation of the electronic signature.

/s/

CURTIS J ROSEBRAUGH
11/14/2017

Exhibit B

Approved Product Label

HIGHLIGHTS OF PRESCRIBING INFORMATION
These highlights do not include all the information needed to use FASENRA™ safely and effectively. See full prescribing information for FASENRA.

FASENRA (benralizumab) injection, for subcutaneous use
Initial U.S. Approval: XXXX

INDICATIONS AND USAGE
FASENRA is an interleukin-5 receptor alpha-directed cytolytic monoclonal antibody (IgG1, kappa) indicated for the add-on maintenance treatment of patients with severe asthma aged 12 years and older, and with an eosinophilic phenotype. (1)

Limitations of Use:
- Not for treatment of other eosinophilic conditions. (1)
- Not for relief of acute bronchospasm or status asthmaticus. (1)

DOSAGE AND ADMINISTRATION
- Administer by subcutaneous injection. (2.1)
- Recommended dose is 30 mg every 4 weeks for the first 3 doses, followed by once every 8 weeks thereafter. (2.1)

DOSAGE FORMS AND STRENGTHS
Injection: 30 mg/mL solution in a single-dose prefilled syringe. (3)

CONTRAINDICATIONS
Known hypersensitivity to benralizumab or excipients. (4)

WARNINGS AND PRECAUTIONS
- Hypersensitivity reactions: hypersensitivity reactions (e.g., anaphylaxis, angioedema, urticaria, rash) have occurred after administration of FASENRA. Discontinue in the event of a hypersensitivity reaction. (5.1)
- Reduction in Corticosteroid Dosage: Do not discontinue systemic or inhaled corticosteroids abruptly upon initiation of therapy with FASENRA. Decrease corticosteroids gradually, if appropriate. (5.3)
- Parasitic (Helminth) Infection: Treat patients with pre-existing helminth infections before therapy with FASENRA. If patients become infected while receiving FASENRA and do not respond to anti-helminth treatment, discontinue FASENRA until the parasitic infection resolves. (5.4)

ADVERSE REACTIONS
Most common adverse reactions (incidence greater than or equal to 5%) include headache and pharyngitis. (6.1)

To report SUSPECTED ADVERSE REACTIONS, contact AstraZeneca at 1-800-236-9933 or FDA at 1-800-FDA-1088 or www.fda.gov/medwatch.

See 17 for PATIENT COUNSELING INFORMATION and FDA-approved patient labeling.

Revised: XX/20XX

FULL PRESCRIBING INFORMATION: CONTENTS*

*Sections or subsections omitted from the full prescribing information are not listed.

FULL PRESCRIBING INFORMATION

1 INDICATIONS AND USAGE

FASENRA is indicated for the add-on maintenance treatment of patients with severe asthma aged 12 years and older, and with an eosinophilic phenotype *[see Clinical Studies (14)]*.

Limitations of use:

- FASENRA is not indicated for treatment of other eosinophilic conditions.
- FASENRA is not indicated for the relief of acute bronchospasm or status asthmaticus.

2 DOSAGE AND ADMINISTRATION

2.1 Recommended Dose

FASENRA is for subcutaneous use only.

The recommended dose of FASENRA is 30 mg administered once every 4 weeks for the first 3 doses, and then once every 8 weeks thereafter by subcutaneous injection into the upper arm, thigh, or abdomen.

2.2 Preparation and Administration

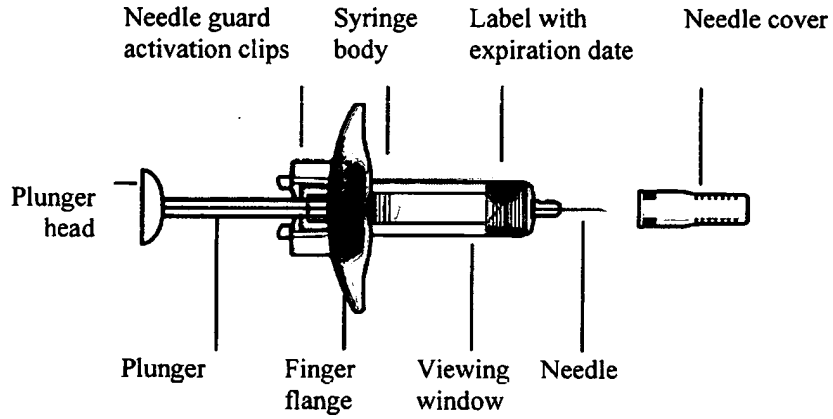
FASENRA should be administered by a healthcare professional. In line with clinical practice, monitoring of patients after administration of biologic agents is recommended *[see Warnings and Precautions (5.1)]*.

Prior to administration, warm FASENRA by leaving carton at room temperature for about 30 minutes. Administer FASENRA within 24 hours or discard into sharps container.

Instructions for Prefilled Syringe with Needle Safety Guard

Refer to **Figure 1** to identify the prefilled syringe components for use in the administration steps.

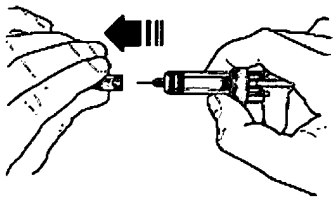
Figure 1



Do not touch the needle guard activation clips to prevent premature activation of the needle safety guard.

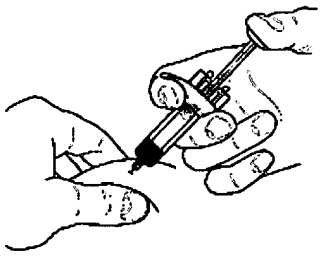
1 **Grasp the syringe body,** not the plunger, to remove prefilled syringe from the tray. Check the expiration date on the syringe. Visually inspect FASENRA for particulate matter and discoloration prior to administration. FASENRA is clear to opalescent, colorless to slightly yellow, and may contain a few translucent or white to off-white particles. Do not use FASENRA if the liquid is cloudy, discolored, or if it contains large particles or foreign particulate matter. The syringe may contain a small air bubble; this is normal. **Do not** expel the air bubble prior to administration.

2



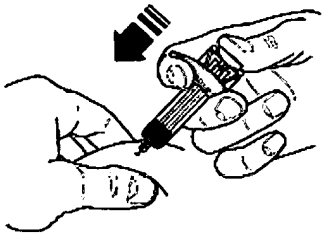
Do not remove needle cover until ready to inject. Hold the syringe body and remove the needle cover by pulling straight off. Do not hold the plunger or plunger head while removing the needle cover or the plunger may move. If the prefilled syringe is damaged or contaminated (for example, dropped without needle cover in place), discard and use a new prefilled syringe.

3 Gently pinch the skin and insert the needle at the recommended



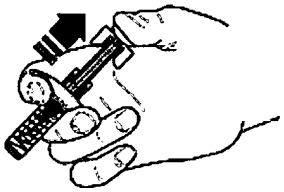
injection site (i.e., upper arm, thigh, or abdomen).

4



Inject all of the medication by pushing in the plunger all the way until the plunger head is **completely between** the needle guard activation clips. **This is necessary to activate the needle guard.**

5



After injection, maintain pressure on the plunger head and remove the needle from the skin. Release pressure on the plunger head to allow the needle guard to cover the needle. **Do not re-cap the prefilled syringe.**

6 Discard the used syringe into a sharps container.

3 DOSAGE FORMS AND STRENGTHS

Injection: 30 mg/mL solution of FASENRA in a single-dose prefilled syringe. FASENRA is a clear to opalescent, colorless to slightly yellow solution and may contain a few translucent or white to off-white particles.

4 CONTRAINDICATIONS

FASENRA is contraindicated in patients who have known hypersensitivity to benralizumab or any of its excipients *[see Warnings and Precautions (5.1)]*.

5 WARNINGS AND PRECAUTIONS

5.1 Hypersensitivity Reactions

Hypersensitivity reactions (e.g., anaphylaxis, angioedema, urticaria, rash) have occurred following administration of FASENRA. These reactions generally occur within hours of administration, but in some instances have a delayed onset (i.e., days). In the event of a hypersensitivity reaction, FASENRA should be discontinued *[see Contraindications (4)]*.

5.2 Acute Asthma Symptoms or Deteriorating Disease

FASENRA should not be used to treat acute asthma symptoms or acute exacerbations. Do not use FASENRA to treat acute bronchospasm or status asthmaticus. Patients should seek medical advice if their asthma remains uncontrolled or worsens after initiation of treatment with FASENRA.

5.3 Reduction of Corticosteroid Dosage

Do not discontinue systemic or inhaled corticosteroids abruptly upon initiation of therapy with FASENRA. Reductions in corticosteroid dose, if appropriate, should be gradual and performed under the direct supervision of a physician. Reduction in corticosteroid dose may be associated with systemic withdrawal symptoms and/or unmask conditions previously suppressed by systemic corticosteroid therapy.

5.4 Parasitic (Helminth) Infection

Eosinophils may be involved in the immunological response to some helminth infections. Patients with known helminth infections were excluded from participation in clinical trials. It is unknown if FASENRA will influence a patient's response against helminth infections.

Treat patients with pre-existing helminth infections before initiating therapy with FASENRA. If patients become infected while receiving treatment with FASENRA and do not respond to anti-helminth treatment, discontinue treatment with FASENRA until infection resolves.

6 ADVERSE REACTIONS

The following adverse reactions are described in greater detail in other sections:

- Hypersensitivity Reactions *[see Warnings and Precautions (5.1)]*

6.1 Clinical Trials Experience

Because clinical trials are conducted under widely varying conditions, adverse reaction rates observed in the clinical trials of a drug cannot be directly compared to rates in the clinical trials of another drug and may not reflect the rates observed in practice.

Across Trials 1, 2, and 3, 1,808 patients received at least 1 dose of FASENRA *[see Clinical Studies (14)]*. The data described below reflect exposure to FASENRA in 1,663 patients, including 1,556 exposed for at least 24 weeks and 1,387 exposed for at least 48 weeks. The safety exposure for FASENRA is derived from two phase 3 placebo-controlled studies (Trials 1 and 2) from 48 weeks duration [FASENRA every 4 weeks (n = 841), FASENRA every 4 weeks for 3 doses, then every 8 weeks (n = 822), and placebo (n = 847)]. While a dosing regimen of FASENRA every 4 weeks was included in clinical trials, FASENRA administered every 4 weeks for 3 doses, then every 8 weeks thereafter is the recommended dose *[see Dosage and Administration (2.1)]*. The population studied was 12 to 75 years of age, of which 64% were female and 79% were white.

Adverse reactions that occurred at greater than or equal to 3% incidence are shown in **Table 1**.

Table 1. Adverse Reactions with FASENRA with Greater than or Equal to 3% Incidence in Patients with Asthma (Trials 1 and 2)

Adverse Reactions	FASENRA (N= 822) %	Placebo (N=847) %
Headache	8	6
Pyrexia	3	2
Pharyngitis*	5	3
Hypersensitivity reactions**	3	3

* Pharyngitis was defined by the following terms: 'Pharyngitis', 'Pharyngitis bacterial', 'Viral pharyngitis', 'Pharyngitis streptococcal'.
** Hypersensitivity Reactions were defined by the following terms: 'Urticaria', 'Urticaria papular', and 'Rash' *[see Warnings and Precautions (5.1)]*.

28-Week Trial

Adverse reactions from Trial 3 with 28 weeks of treatment with FASENRA (n = 73) or placebo (n = 75) in which the incidence was more common in FASENRA than placebo include headache (8.2% compared to 5.3%, respectively) and pyrexia (2.7% compared to 1.3%, respectively) *[see Clinical Studies (14)]*. The frequencies for the remaining adverse reactions with FASENRA were similar to placebo.

Injection site reactions

In Trials 1 and 2, injection site reactions (e.g., pain, erythema, pruritus, papule) occurred at a rate of 2.2% in patients treated with FASENRA compared with 1.9% in patients treated with placebo.

6.2 Immunogenicity

As with all therapeutic proteins, there is potential for immunogenicity. The detection of antibody formation is highly dependent on the sensitivity and specificity of the assay. Additionally, the observed incidence of antibody (including neutralizing antibody) positivity in an assay may be influenced by several factors including assay methodology, sample handling, timing of sample collection, concomitant medications, and underlying disease. For these reasons, comparison of the incidence of antibodies to benralizumab in the studies described below with the incidence of antibodies in other studies or to other products may be misleading.

Overall, treatment-emergent anti-drug antibody response developed in 13% of patients treated with FASENRA at the recommended dosing regimen during the 48 to 56 week treatment period. A total of 12% of patients treated with FASENRA developed neutralizing antibodies. Anti-benralizumab antibodies were associated with increased clearance of benralizumab and increased blood eosinophil levels in patients with high anti-drug antibody titers compared to antibody negative patients. No evidence of an association of anti-drug antibodies with efficacy or safety was observed.

The data reflect the percentage of patients whose test results were positive for antibodies to benralizumab in specific assays.

7 DRUG INTERACTIONS

No formal drug interaction studies have been conducted.

8 USE IN SPECIFIC POPULATIONS

8.1 Pregnancy

Risk Summary

The data on pregnancy exposure from the clinical trials are insufficient to inform on drug-associated risk. Monoclonal antibodies such as benralizumab are transported across the placenta during the third trimester of pregnancy; therefore, potential effects on a fetus are likely to be greater during the third trimester of pregnancy. In a prenatal and postnatal development study conducted in cynomolgus monkeys, there was no evidence of fetal harm with IV administration of benralizumab throughout pregnancy at doses that produced exposures up to approximately 310 times the exposure at the maximum recommended human dose (MRHD) of 30 mg SC [see Data].

In the U.S. general population, the estimated background risk of major birth defects and miscarriage in clinically recognized pregnancies is 2% to 4% and 15% to 20%, respectively.

Clinical Considerations

Disease-associated maternal and/or embryo/fetal risk:

In women with poorly or moderately controlled asthma, evidence demonstrates that there is an increased risk of preeclampsia in the mother and prematurity, low birth weight, and small for gestational age in the neonate. The level of asthma control should be closely monitored in pregnant women and treatment adjusted as necessary to maintain optimal control.

Data

Animal Data

In a prenatal and postnatal development study, pregnant cynomolgus monkeys received benralizumab from beginning on GD20 to GD22 (dependent on pregnancy determination), on GD35, once every 14 days thereafter throughout the gestation period and 1-month postpartum (maximum 14 doses) at doses that produced exposures up to approximately 310 times that achieved with the MRHD (on an AUC basis with maternal IV doses up to 30 mg/kg once every 2 weeks). Benralizumab did not elicit adverse effects on fetal or neonatal growth (including immune function) up to 6.5 months after birth. There was no evidence of treatment-related external, visceral, or skeletal malformations. Benralizumab was not teratogenic in cynomolgus monkeys. Benralizumab crossed the placenta in cynomolgus monkeys. Benralizumab concentrations were approximately equal in mothers and infants on postpartum day 7, but were lower in infants at later time points. Eosinophil counts were suppressed in infant monkeys with gradual recovery by 6 months postpartum; however, recovery of eosinophil counts was not observed for one infant monkey during this period.

8.2 Lactation

Risk Summary

There is no information regarding the presence of benralizumab in human or animal milk, and the effects of benralizumab on the breast fed infant and on milk production are not known. However, benralizumab is a humanized monoclonal antibody (IgG1/κ-class), and immunoglobulin G (IgG) is present in human milk in small amounts. If benralizumab is transferred into human milk, the effects of local exposure in the gastrointestinal tract and potential limited systemic exposure in the infant to benralizumab are unknown. The developmental and health benefits of breastfeeding should be considered along with the mother's clinical need for benralizumab and any potential adverse effects on the breast-fed child from benralizumab or from the underlying maternal condition.

8.4 Pediatric Use

There were 108 adolescents aged 12 to 17 with asthma enrolled in the Phase 3 exacerbation trials (Trial 1: n=53, Trial 2: n=55). Of these, 46 received placebo, 40 received FASENRA every 4 weeks for 3 doses, followed by every 8 weeks thereafter, and 22 received FASENRA every 4 weeks. Patients were required to have a history of 2 or more asthma exacerbations requiring oral or systemic corticosteroid treatment in the past 12 months and reduced lung function at baseline (pre-bronchodilator FEV_1<90%) despite regular treatment with medium or high dose ICS and LABA with or without OCS or other controller therapy. The pharmacokinetics of benralizumab in adolescents 12 to 17 years of age were consistent with adults based on population pharmacokinetic analysis and the reduction in blood eosinophil counts

was similar to that observed in adults following the same FASENRA treatment. The adverse event profile in adolescents was generally similar to the overall population in the Phase 3 studies *[see Adverse Reactions (6.1)]*. The safety and efficacy in patients younger than 12 years of age has not been established.

8.5 Geriatric Use

Of the total number of patients in clinical trials of benralizumab, 13% (n= 320) were 65 and over, while 0.4% (n=9) were 75 and over. No overall differences in safety or effectiveness were observed between these patients and younger patients, and other reported clinical experience has not identified differences in responses between the elderly and younger patients, but greater sensitivity of some older individuals cannot be ruled out.

10 OVERDOSAGE

Doses up to 200 mg were administered subcutaneously in clinical trials to patients with eosinophilic disease without evidence of dose-related toxicities.

There is no specific treatment for an overdose with benralizumab. If overdose occurs, the patient should be treated supportively with appropriate monitoring as necessary.

11 DESCRIPTION

Benralizumab is a humanized monoclonal antibody (IgG1/κ-class) selective for interleukin-5 receptor alpha subunit (IL-5Rα). Benralizumab is produced in Chinese hamster ovary cells by recombinant DNA technology. Benralizumab has a molecular weight of approximately 150 kDa.

FASENRA (benralizumab) injection is a sterile, preservative-free, clear to opalescent, colorless to slightly yellow solution for subcutaneous injection. Since FASENRA is a protein, a few translucent or white to off-white particles may be present in the solution. Each single-dose prefilled syringe delivers 1 mL containing 30 mg benralizumab, L-histidine (1.4 mg); L-histidine hydrochloride monohydrate (2.3 mg); polysorbate 20 (0.06 mg); α,α-trehalose dihydrate (95 mg); and Water for Injection, USP. The single-dose prefilled syringe contains a 1 mL glass syringe with a staked 29 gauge ½ inch stainless steel needle.

12 CLINICAL PHARMACOLOGY

12.1 Mechanism of Action

Benralizumab is a humanized afucosylated, monoclonal antibody (IgG1, kappa) that directly binds to the alpha subunit of the human interleukin-5 receptor (IL-5Rα) with a dissociation constant of 11 pM. The IL-5 receptor is expressed on the surface of eosinophils and basophils. In an *in vitro* setting, the absence of fucose in the Fc domain of benralizumab facilitates binding (45.5 nM) to FcγRIII receptors on immune effectors cells, such as natural killer (NK) cells, leading to apoptosis of eosinophils and basophils through antibody-dependent cell-mediated cytotoxicity (ADCC).

Inflammation is an important component in the pathogenesis of asthma. Multiple cell types (e.g., mast cells, eosinophils, neutrophils, macrophages, lymphocytes) and mediators (e.g., histamine, eicosanoids, leukotrienes, cytokines) are involved in inflammation. Benralizumab, by binding to the IL-5Rα chain, reduces eosinophils through ADCC; however, the mechanism of benralizumab action in asthma has not been definitively established.

12.2 Pharmacodynamics

In the 52-week Phase 2 dose-ranging trial, asthma patients received 1 of 3 doses of benralizumab [2 mg (n=81), 20 mg (n=81), or 100 mg (n=222)] or placebo (n=222). All doses were administered every 4 weeks for the first 3 doses, followed by every 8 weeks thereafter. Median blood eosinophil levels at baseline were 310, 280, 190 and 190 cells/μL in the 2, 20, and 100 mg benralizumab and placebo groups, respectively. Dose-dependent reductions in blood eosinophils

were observed. At the time of the last dose (Week 40), median blood eosinophil counts were 100, 50, 40, 170 cells/μL in the 2, 20, and 100 mg benralizumab and placebo groups, respectively.

A reduction in blood eosinophil counts was observed 24 hours post dosing in a Phase 2 trial.

In Trials 1 and 2, following SC administration of benralizumab at the recommended dose blood eosinophils were reduced to a median absolute blood eosinophil count of 0 cells/μL *[see Clinical Studies (14)]*. This magnitude of reduction was seen at the first observed time point, 4 weeks of treatment, and was maintained throughout the treatment period.

Treatment with benralizumab was also associated with reductions in blood basophils, which was consistently observed across all clinical studies. In the Phase 2 dose-ranging trial, blood basophil counts were measured by flow cytometry. Median blood basophil counts were 45, 52, 46, and 40 cells/μL in the 2 mg, 20 mg and 100 mg benralizumab and placebo groups, respectively. At 52 weeks (12 weeks after the last dose), median blood basophil counts were 42, 18, 17, and 46 cells/μL in the 2 mg, 20 mg and 100 mg benralizumab and placebo groups, respectively.

12.3 Pharmacokinetics

The pharmacokinetics of benralizumab was approximately dose-proportional in patients with asthma following subcutaneous administration over a dose range of 20 to 200 mg.

Absorption

Following subcutaneous administration to patients with asthma, the absorption half-life was approximately 3.6 days. Based on population pharmacokinetic analysis, the estimated absolute bioavailability was approximately 58% and there was no clinically relevant difference in relative bioavailability in the administration to the abdomen, thigh, or arm.

Distribution:

Based on population pharmacokinetic analysis, central and peripheral volume of distribution of benralizumab was 3.2 L and 2.5 L, respectively, for a 70kg individual.

Metabolism:

Benralizumab is a humanized IgG1 monoclonal antibody that is degraded by proteolytic enzymes widely distributed in the body and not restricted to hepatic tissue.

Elimination:

From population pharmacokinetic analysis, benralizumab exhibited linear pharmacokinetics and no evidence of target receptor-mediated clearance pathway. The estimated typical systemic clearance (CL) for benralizumab was 0.29 L/d for a subject weighing 70kg. Following subcutaneous administration, the elimination half-life was approximately 15 days.

Specific populations:

Age:

Based on population pharmacokinetic analysis, age did not affect benralizumab clearance.

Gender, Race:

A population pharmacokinetics analysis indicated that there was no significant effect of gender and race on benralizumab clearance.

Renal impairment:

No formal clinical studies have been conducted to investigate the effect of renal impairment on benralizumab. Based on population pharmacokinetic analysis, benralizumab clearance was comparable in subjects with creatinine clearance values between 30 and 80 mL/min and patients with normal renal function. There are limited data available in subjects with creatinine clearance values less than 30 mL/min; however, benralizumab is not cleared renally.

Hepatic impairment:

No formal clinical studies have been conducted to investigate the effect of hepatic impairment on benralizumab. IgG monoclonal antibodies are not primarily cleared via hepatic pathway; change in hepatic function is not expected to influence benralizumab clearance. Based on population pharmacokinetic analysis, baseline hepatic function biomarkers (ALT, AST, and bilirubin) had no clinically relevant effect on benralizumab clearance.

Drug-Drug Interaction:

No formal drug-drug interaction studies have been conducted.

Cytochrome P450 enzymes, efflux pumps and protein-binding mechanisms are not involved in the clearance of benralizumab. There is no evidence of IL-5Rα expression on hepatocytes and eosinophil depletion does not produce chronic systemic alterations of proinflammatory cytokines.

An effect of benralizumab on the pharmacokinetics of co-administered medications is not expected. Based on the population analysis, commonly co-administered medications had no effect on benralizumab clearance in patients with asthma.

13 NONCLINICAL TOXICOLOGY

13.1 Carcinogenesis, Mutagenesis, Impairment of Fertility

Long-term animal studies have not been performed to evaluate the carcinogenic potential of benralizumab. Published literature using animal models suggests that IL-5 and eosinophils are part of an early inflammatory reaction at the site of tumorigenesis and can promote tumor rejection. However, other reports indicate that eosinophil infiltration into tumors can promote tumor growth. Therefore, the malignancy risk in humans from an antibody that binds to IL-5Rα such as benralizumab is unknown.

Male and female fertility were unaffected based upon no adverse histopathological findings in the reproductive organs from cynomolgus monkeys treated with benralizumab for 9 months at IV doses up to 25 mg/kg or at SC doses of up to 30 mg/kg once every 2 weeks (approximately 400 and 270 times the MRHD on an AUC basis).

14 CLINICAL STUDIES

The asthma development program for FASENRA included one 52-week dose ranging exacerbation trial (NCT01238861) three confirmatory trials, (Trial 1 [NCT01928771], Trial 2 [NCT01914757], Trial 3 [NCT02075255]) and one 12-week lung function trial (NCT02322775).

Dose-Ranging Trial

The Phase 2 randomized, double-blind, placebo-controlled, 52-week dose-ranging trial, enrolled 609 asthmatic patients 18 years of age and older. Patients were treated with benralizumab 2 mg, 20 mg, or 100 mg or placebo administered subcutaneously every 4 weeks for 3 doses followed by every 8 weeks. The primary endpoint was the annual exacerbation rate and forced expiratory volume in 1 second (FEV_1) and ACQ-6 were key secondary endpoints. Patients were required to have a history of 2 or more asthma exacerbations (but no more than 6 exacerbations) requiring systemic corticosteroid treatment in the past 12 months, ACQ-6 score of 1.5 at least twice during screening, and reduced morning lung function at screening [pre-bronchodilator FEV_1 below 90%] despite treatment with medium- or high-dose ICS plus LABA. Patients were stratified by eosinophilic status. The annual exacerbation rate reduction for patients receiving benralizumab 2 mg, 20 mg, and 100 mg were -12% (80% CI: -52, 18), 34% (80% CI: 6, 54), 29% (80% CI: 10, 44), respectively, compared to placebo (rate 0.56).

Results from this trial and exposure-response modelling of exacerbation rate reduction supported the evaluation of benralizumab 30 mg in the subsequent trials *[see Clinical Pharmacology (12.2 and 12.3)]*. FASENRA is not approved at

2 mg, 20 mg, or 100 mg doses, and should only be administered at the recommended dose of 30 mg *[see Dosage and Administration (2.1)]*.

Confirmatory Trials

Trial 1 and Trial 2, were randomized, double-blind, parallel-group, placebo-controlled, exacerbation trials in patients 12 years of age and older and 48 and 56 weeks in duration, respectively. The trials randomized a total of 2510 patients. Patients were required to have a history of 2 or more asthma exacerbations requiring oral or systemic corticosteroid treatment in the past 12 months, ACQ-6 score of 1.5 or more at screening, and reduced lung function at baseline [pre-bronchodilator FEV_1 below 80% in adults, and below 90% in adolescents] despite regular treatment with high dose inhaled corticosteroid (ICS) (Trial 1) or with medium or high dose ICS (Trial 2) plus a long-acting beta agonist (LABA) with or without oral corticosteroids (OCS) and additional asthma controller medications. Patients were stratified by geography, age, and blood eosinophils count (≥300 cells/μL or <300 cells/μL). FASENRA administered once every 4 weeks for the first 3 doses, and then every 4 or 8 weeks thereafter as add-on to background treatment was evaluated compared to placebo.

All subjects continued their background asthma therapy throughout the duration of the trials.

Trial 3 was a randomized, double-blind, parallel-group, OCS reduction trial in 220 asthma patients. Patients were required treatment with daily OCS (7.5 to 40 mg per day) in addition to regular use of high-dose ICS and LABA with or without additional controller(s). The trial included an 8-week run-in period during which the OCS was titrated to the minimum effective dose without losing asthma control. For the purposes of the OCS dose titration, asthma control was assessed by the investigator based on a patient's FEV_1, peak expiratory flow, nighttime awakenings, short-acting bronchodilator rescue medication use or any other symptoms that would require an increase in OCS dose. Baseline median OCS dose was similar across all treatment groups. Patients were required to have blood eosinophil counts greater than or equal to 150 cells/μL and a history of at least one exacerbation in the past 12 months. The baseline median OCS dose was 10 mg (range: 8 to 40 mg) for all 3 treatment groups (placebo, FASENRA every 4 weeks, and FASENRA every 4 weeks for the first 3 doses, and then once every 8 weeks).

While 2 dosing regimens were studied in Trials 1, 2, and 3, the recommended dosing regimen is 30 mg FASENRA administered every 4 weeks for the first 3 doses, then every 8 weeks thereafter *[see Dosage and Administration (2.1)]*.

Table 2. Demographics and Baseline Characteristics of Asthma Trials

	Total Population		
	Trial 1 (N = 1204)	**Trial 2 (N = 1306)**	**Trial 3 (N=220)**
Mean age (yr)	49	49	51
Female (%)	66	62	61
White (%)	73	84	93
Duration of asthma, median (yr)	15	16	12
Never smoked (%)	80	78	79
Mean baseline FEV_1 pre-bronchodilator (L)	1.67	1.76	1.85
Mean baseline % predicted FEV_1	57	58	60
Mean post-SABA FEV_1/FVC (%)	66	65	62
Mean baseline eosinophil count (cells/μL)	472	472	575
Mean number of exacerbations in previous year	3	3	3

Exacerbations

The primary endpoint for Trials 1 and 2 was the rate of asthma exacerbations in patients with baseline blood eosinophil counts of greater than or equal to 300 cells/μL who were taking high-dose ICS and LABA. Asthma exacerbation was defined as a worsening of asthma requiring use of oral/systemic corticosteroids for at least 3 days, and/or emergency department visits requiring use of oral/systemic corticosteroids and/or hospitalization. For patients on maintenance oral corticosteroids, an asthma exacerbation requiring oral corticosteroids was defined as a temporary increase in stable oral/systemic corticosteroids for at least 3 days or a single depo-injectable dose of corticosteroids. In Trial 1, 35% of patients receiving FASENRA experienced an asthma exacerbation compared to 51% on placebo. In Trial 2, 40% of patients receiving FASENRA experienced an asthma exacerbation compared to 51% on placebo **(Table 3)**.

Table 3. Rate of Exacerbations, Trial 1 and 2 (ITT Population) [a]

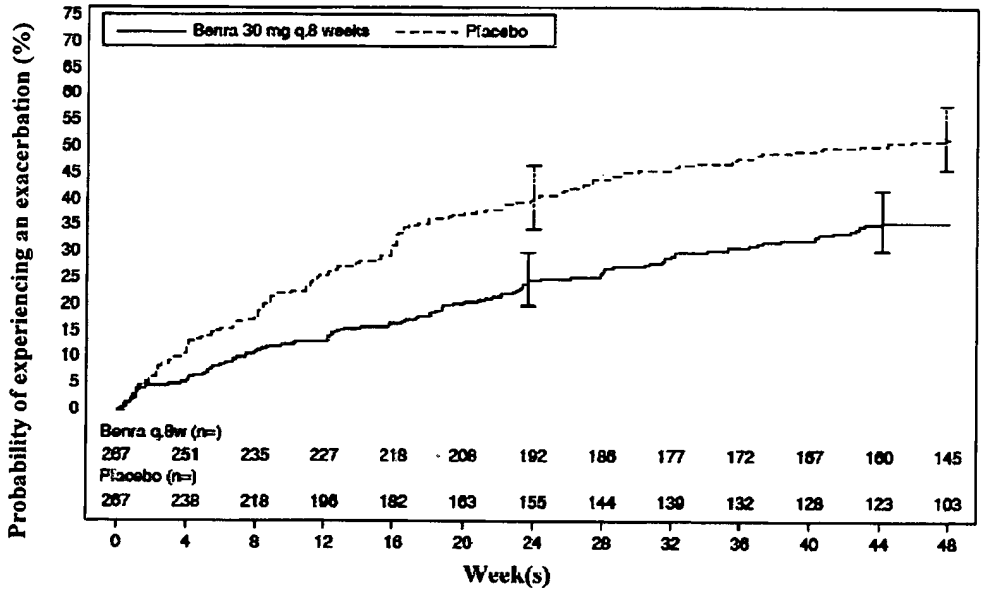
Trial	Treatment	Exacerbations per year		
		Rate	Difference	Rate Ratio (95% CI)
All exacerbations				
Trial 1	FASENRA [b] (n=267)	0.74	-0.78	0.49 (0.37, 0.64)
	Placebo (n=267)	1.52	--	--
Trial 2	FASENRA [b] (n=239)	0.73	-0.29	0.72 (0.54, 0.95)
	Placebo (n=248)	1.01	--	--
Exacerbations requiring hospitalization/emergency room visit				
Trial 1	FASENRA [b] (n=267)	0.09	-0.16	0.37 (0.20, 0.67)
	Placebo (n=267)	0.25	--	--
Trial 2	FASENRA [b] (n=239)	0.12	0.02	1.23 (0.64, 2.35)
	Placebo (n=248)	0.10	--	--
Exacerbations requiring hospitalization				
Trial 1	FASENRA [b] (n=267)	0.07	-0.07	0.48 (0.22, 1.03)
	Placebo (n=267)	0.14	--	--
Trial 2	FASENRA [b] (n=239)	0.07	0.02	1.48 (0.65, 3.37)
	Placebo (n=248)	0.05	--	--

a. Baseline blood eosinophil counts of greater than or equal to 300 cells/μL and taking high-dose ICS

b. FASENRA 30mg administered every 4 weeks for the first 3 doses, and every 8 weeks thereafter

The time to first exacerbation was longer for the patients receiving FASENRA compared with placebo in Trial 1 (**Figure 2**). Similar findings were seen in Trial 2.

Figure 2. Kaplan-Meier Cumulative Incidence Curves for Time to First Exacerbation, Trial 1



Subgroup analyses from Trials 1 and 2 identified patients with a higher prior exacerbation history and baseline blood eosinophil count as potential predictors of improved treatment response. Reductions in exacerbation rates were observed irrespective of baseline peripheral eosinophil counts; however, patients with a baseline blood eosinophil count ≥ 300 cells/μL showed a numerically greater response than those with counts < 300 cells/μL. In both trials patients with a history of 3 or more exacerbations within the 12 months prior to FASENRA randomization showed a numerically greater exacerbation response than those with fewer prior exacerbations.

Oral Corticosteroid Reduction

Trial 3 evaluated the effect of FASENRA on reducing the use of maintenance oral corticosteroids. The primary endpoint was percent reduction from baseline of the final OCS dose during Weeks 24 to 28, while maintaining asthma control (see definition of asthma control in trial description). Compared to placebo, patients receiving FASENRA achieved greater reductions in daily maintenance oral corticosteroid dose, while maintaining asthma control. The median percent reduction in daily OCS dose from baseline was 75% in patients receiving FASENRA (95% CI: 60, 88) compared to 25% in patients receiving placebo (95% CI: 0, 33). Reductions of 50% or higher in the OCS dose were observed in 48 (66%) patients receiving FASENRA compared to those receiving placebo 28 (37%). The proportion of patients with a mean final dose less than or equal to 5 mg at Weeks 24 to 28 was 59% for FASENRA and 33% for placebo (odds ratio 2.74, 95% CI: 1.41, 5.31). Only patients with an optimized baseline OCS dose of 12.5 mg or less were eligible to achieve a 100% reduction in OCS dose during the study. Of those patients, 52% (22 of 42) receiving FASENRA and 19% (8 of 42) on placebo achieved a 100% reduction in OCS dose. Exacerbations resulting in hospitalization and/or ER visit were also assessed as a secondary endpoint. In this 28-week trial, patients receiving FASENRA had 1 event while those on placebo had 14 events (annualized rate 0.02 and 0.32 respectively; rate ratio of 0.07, 95% CI: 0.01, 0.63).

Lung Function

Change from baseline in mean FEV_1 was assessed in Trials 1, 2, and 3 as a secondary endpoint. Compared with placebo, FASENRA provided consistent improvements over time in the mean change from baseline in FEV_1 (**Figure 3** and **Table 4**).

16 HOW SUPPLIED/STORAGE AND HANDLING

FASENRA (benralizumab) injection is a sterile, preservative-free, clear to opalescent, colorless to slightly yellow solution for subcutaneous injection supplied as a single-dose prefilled syringe.

Carton contains one 30 mg/mL single-dose prefilled syringe: NDC 0310-1730-30

Store the prefilled syringe refrigerated at 2°C to 8°C (36°F to 46°F) in the original carton to protect from light. Do not freeze. Do not shake.

17 PATIENT COUNSELING INFORMATION

Advise the patient to read the FDA-approved patient labeling (Patient Information).

Hypersensitivity Reactions

Inform patients that hypersensitivity reactions (e.g., anaphylaxis, angioedema, urticaria, rash) have occurred after administration of FASENRA. These reactions generally occurred within hours of FASENRA administration, but in some instances had a delayed onset (i.e., days). Instruct patients to contact their healthcare professional if they experience symptoms of an allergic reaction *[see Warnings and Precautions (5.1)]*.

Not for Acute Symptoms or Deteriorating Disease

Inform patients that FASENRA does not treat acute asthma symptoms or acute exacerbations. Inform patients to seek medical advice if their asthma remains uncontrolled or worsens after initiation of treatment with FASENRA *[see Warnings and Precautions (5.2)]*.

Reduction of Corticosteroid Dosage

Inform patients to not discontinue systemic or inhaled corticosteroids except under the direct supervision of a physician. Inform patients that reduction in corticosteroid dose may be associated with systemic withdrawal symptoms and/or unmask conditions previously suppressed by systemic corticosteroid therapy *[see Warnings and Precautions (5.3)]*.

Manufactured by
AstraZeneca AB
Södertälje, Sweden SE-15185
US License No. 2059

Distributed by
AstraZeneca Pharmaceuticals LP,
Wilmington, DE 19850

FASENRA is a trademark of the AstraZeneca group of companies.

Patient Information
FASENRA™ (fas-en-rah)
(benralizumab)
injection, for subcutaneous use

What is FASENRA?

FASENRA is a prescription medicine used with other asthma medicines for the maintenance treatment of asthma in people 12 years and older whose asthma is not controlled with their current asthma medicines. FASENRA helps prevent severe asthma attacks (exacerbations) and may improve your breathing. Medicines such as FASENRA reduce blood eosinophils. Eosinophils are a type of white blood cell that may contribute to your asthma.

- FASENRA is not used to treat other problems caused by eosinophils.
- FASENRA is not used to treat sudden breathing problems. Tell your healthcare provider if your asthma does not get better or if it gets worse after you start treatment with FASENRA.

It is not known if FASENRA is safe and effective in children under 12 years of age.

Do not receive FASENRA if you are allergic to benralizumab or any of the ingredients in FASENRA. See the end of this leaflet for a complete list of ingredients in FASENRA.

Before receiving FASENRA, tell your healthcare provider about all of your medical conditions, including if you:

- are taking oral or inhaled corticosteroid medicines. **Do not** stop taking your corticosteroid medicines unless instructed by your healthcare provider. This may cause other symptoms that were controlled by the corticosteroid medicine to come back.
- have a parasitic (helminth) infection.
- are pregnant or plan to become pregnant. It is not known if FASENRA will harm your unborn baby. Tell your healthcare provider if you become pregnant during your treatment with FASENRA.
- are breastfeeding or plan to breastfeed. It is not known if FASENRA passes into your breast milk. You and your healthcare provider should decide if you will receive FASENRA and breastfeed. Talk to your healthcare provider about the best way to feed your baby if you receive FASENRA.

Tell your healthcare provider about all the medicines you take, including prescription and over-the-counter medicines, vitamins, and herbal supplements.

Do not stop taking your other asthma medicines unless your healthcare provider tells you to.

How will I receive FASENRA?

A healthcare provider will inject FASENRA under your skin (subcutaneously) one time every 4 weeks for the first 3 doses, and then every 8 weeks.

What are the possible side effects of FASENRA?

FASENRA may cause serious side effects, including:

- **allergic (hypersensitivity) reactions, including anaphylaxis.** Serious allergic reactions can happen after you get your FASENRA injection. Allergic reactions can sometimes happen hours or days after you get your injection. Tell your healthcare provider or get emergency help right away if you have any of the following symptoms of an allergic reaction:
  - o swelling of your face, mouth and tongue
  - o breathing problems
  - o fainting, dizziness, feeling lightheaded (low blood pressure)
  - o rash
  - o hives

The most common side effects of FASENRA include headache and sore throat.

These are not all the possible side effects of FASENRA.

Call your doctor for medical advice about side effects. You may report side effects to FDA at 1-800-FDA-1088.

General information about the safe and effective use of FASENRA.

Medicines are sometimes prescribed for purposes other than those listed in a Patient Information leaflet. Do not receive FASENRA for a condition for which it was not prescribed. Do not give FASENRA to other people, even if they have the same symptoms you have. It may harm them.

You can ask your doctor or pharmacist for information about FASENRA that is written for health professionals.

What are the ingredients in FASENRA?

Active ingredient: benralizumab

Inactive ingredients: L-histidine, L-histidine hydrochloride monohydrate, polysorbate 20, α,α-trehalose dihydrate, and Water for Injection

Manufactured by: AstraZeneca AB, Södertälje, Sweden SE-15185
U.S. License Number 2059
Distributed by: AstraZeneca Pharmaceuticals LP, Wilmington, DE 19850

For more information, go to www.FASENRA.com or call 1-800-236-9933.

This Patient Information has been approved by the U.S. Food and Drug Administration. Issued: Month Year

Exhibit C

Copy of Patent No. 7,718,175

US007718175B2

(12) **United States Patent**
Hanai et al.

(10) **Patent No.: US 7,718,175 B2**
(45) **Date of Patent: *May 18, 2010**

(54) **METHOD OF MODULATING THE ACTIVITY OF FUNCTIONAL IMMUNE MOLECULES TO INTERLEUKIN-5 RECEPTOR PROTEIN**

(75) Inventors: **Nobuo Hanai**, Machida (JP); **Kazuyasu Nakamura**, Machida (JP); **Emi Hosaka**, Machida (JP); **Motoo Yamasaki**, Machida (JP); **Kazuhisa Uchida**, Machida (JP); **Toyohide Shinkawa**, Machida (JP); **Susumu Imabeppu**, Ube (JP); **Yutaka Kanda**, Machida (JP); **Naoko Yamane**, Machida (JP); **Hideharu Anazawa**, Tokyo (JP)

(73) Assignee: **Kyowa Hakko Kirin Co., Ltd**, Tokyo (JP)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 169 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **11/686,379**

(22) Filed: **Mar. 15, 2007**

(65) **Prior Publication Data**

US 2007/0166300 A1 Jul. 19, 2007

Related U.S. Application Data

(62) Division of application No. 11/126,176, filed on May 11, 2005, now Pat. No. 7,214,775, which is a division of application No. 09/958,307, filed as application No. PCT/JP00/02260 on Apr. 7, 2000, now abandoned.

(30) **Foreign Application Priority Data**

Apr. 9, 1999 (JP) ..... P. 11-103158

(51) **Int. Cl.**
A61K 39/00 (2006.01)
A61K 39/395 (2006.01)

(52) **U.S. Cl.** **424/133.1**; 424/138.1; 424/143.1

(58) **Field of Classification Search** ..... None
See application file for complete search history.

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Primary Examiner—Laura B Goddard
(74) *Attorney, Agent, or Firm*—Sughrue Mion, PLLC

(57) **ABSTRACT**

The invention relates to a method for controlling the activity of an immunologically functional molecule, such as an antibody, a protein, a peptide or the like, an agent of promoting the activity of an immunologically functional molecule, and an immunologically functional molecule having the promoted activity.

3 Claims, 12 Drawing Sheets

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Non-Final Office Action issued Jul. 5, 2007, in U.S. Appl. No. 10/409,598.
1.111 Amendment filed Jan. 4, 2008, in U.S. Appl. No. 10/409,598.
Non-Final Office Action issued Apr. 4, 2008, in U.S. Appl. No. 10/409,598.
1.111 Amendment filed Aug. 4, 2008, in U.S. Appl. No. 10/409,598.
Final Office Action issued Oct. 27, 2008, in U.S. Appl. No. 10/409,598.
1.116 Amendment filed Jan. 30, 2009, in U.S. Appl. No. 10/409,598.
Advisory Action issued Feb. 27, 2009, in U.S. Appl. No. 10/409,598.
Request for Continued Examination filed Mar. 26, 2009, in U.S. Appl. No. 10/409,598.
Notice of Allowance and Issue Fee(s) Due issued May 8, 2009, in U.S. Appl. No. 10/409,598.
Restriction Requirement issued Jul. 13, 2005, in U.S. Appl. No. 10/409,611.
Response to Restriction Requirement filed Aug. 11, 2005, in U.S. Appl. No. 10/409,611.
Non-Final Office Action issued Oct. 12, 2005, in U.S. Appl. No. 10/409,611.
1.111 Amendment (with Declaration and Ids) filed Apr. 11, 2006, in U.S. Appl. No. 10/409,611.
Final Office Action issued Jun. 14, 2006, in U.S. Appl. No. 10/409,611.
Notice of Appeal filed Dec. 14, 2006, in U.S. Appl. No. 10/409,611.
Notice of Abandonment issued Jul. 12, 2007, in U.S. Appl. No. 10/409,611.
Request to Withdraw Abandonment, Request for Continued Examination, 116 Amendment and Information Disclosure Statement filed Jul. 13, 2007, In U.S. Appl. No. 10/409,611.
Communication, Withdrawal of Abandonment issued Jul. 24, 2007, in U.S. Appl. No. 10/409,611.
Decision on Petition issued Dec. 14, 2007, in U.S. Appl. No. 10/409,611.
Non-Final Office Action issued Apr. 21, 2008, in U.S. Appl. No. 10/409,611.
Decision on Petition issued Jul. 9, 2008, in U.S. Appl. No. 10/409,611.
1.111 Amendment filed Jul. 21, 2008, in U.S. Appl. No. 10/409,611.
Supplemental Submission filed Aug. 6, 2008, in U.S. Appl. No. 10/409,611.
Notice of Non-Complaint Amendment issued Nov. 14, 2008, in U.S. Appl. No. 10/409,611.
Response to Notice of Non-Compliant Amendment filed Dec. 15, 2008, in U.S. Appl. No. 10/409,611.
Final Office Action issued Apr. 13, 2009, in U.S. Appl. No. 10/409,611.
Restriction Requirement issued Aug. 22, 2005, in U.S. Appl. No. 10/409,608.
Amendment (Response to Restriction Requirement) filed Nov. 22, 2005, in U.S. Appl. No. 10/409,608.
Restriction Requirement issued Feb. 6, 2006, in U.S. Appl. No. 10/409,608.
Response to Restriction Requirement filed Mar. 27, 2006, in U.S. Appl. No. 10/409,608.
Non-Final Office Action issued May 31, 2006, in U.S. Appl. No. 10/409,608.
1.111 Amendment (with Sequence Listing and Ids) filed Nov. 30, 2006, in U.S. Appl. No. 10/409,608.
Final Office Action issued Feb. 22, 2007, in U.S. Appl. No. 10/409,608.
Notice of Appeal filed Aug. 22, 2007, in U.S. Appl. No. 10/409,608.
Request for Continued Examination and 1.114(c) Amendment filed Mar. 19, 2008, in U.S. Appl. No. 10/409,608.
Non-Final Office Action issued Jun. 6, 2008, in U.S. Appl. No. 10/409,608.
1.111 Amendment filed Sep. 26, 2008, in U.S. Appl. No. 10/409,608.
Final Office Action issued Dec. 31, 2008, in U.S. Appl. No. 10/409,608.
1.116 Amendment filed May 29, 2009, in U.S. Appl. No. 10/409,608.
Advisory Action issued Jun. 3, 2009, in U.S. Appl. No. 10/409,608.
RCE and Terminal Disclaimer filed Jun. 19, 2009, in U.S. Appl. No. 10/409,608.
Non-Final OA issued Oct. 12, 2006 in U.S. Appl. No. 10/803,100.
Non-Final OA issued Apr. 30, 2008 in U.S. Appl. No. 11/783,487.
Non-Final OA issued May 12, 2009 in U.S. Appl. No. 12/261,997.
Restriction Requirement issued Feb. 3, 2009 in U.S. Appl. No. 10/575,261.
Response to Restriction Requirement filed Mar. 3, 2009 in U.S. Appl. No. 10/575,261.
Election of Species Requirement issued May 26, 2009 in U.S. Appl. No. 10/575,261.
Restriction Requirement issued Jun. 20, 2006 in U.S. Appl. No. 10/959,326.
Response to Restriction Requirement filed Jul. 20, 2006 in U.S. Appl. No. 10/959,326.
Supplemental Response to Restriction Requirement and Preliminary Amendment filed Aug. 21, 2006 in U.S. Appl. No. 10/959,326.
Non-Final OA issued Oct. 5, 2006 in U.S. Appl. No. 10/959,326.
Restriction Requirement issued Apr. 8, 2008 in U.S. Appl. No. 11/730,992.
Restriction Requirement issued Oct. 23, 2006 in U.S. Appl. No. 10/959,310.
Response to Restriction Requirement filed Apr. 23, 2007 in U.S. Appl. No. 10/959,310.
Non-Final OA issued Jul. 6, 2007 in U.S. Appl. No. 10/959,310.
Restriction Requirement issued Sep. 14, 2006 in U.S. Appl. No. 10/959,322.
Response to Restriction Requirement filed Oct. 18, 2006 in U.S. Appl. No. 10/959,322.

Non-Final OA issued Mar. 21, 2007 in U.S. Appl. No. 10/959,322.
1.111 Amendment filed Sep. 19, 2007 in U.S. Appl. No. 10/959,322.
Final OA issued Apr. 28, 2008 in U.S. Appl. No. 10/959,322.
1.116 Amendment filed Aug. 28, 2008 in U.S. Appl. No. 10/959,322.
Advisory Action issued Sep. 19, 2008 in U.S. Appl. No. 10/959,322.
RCE and 1.114 Amendment filed Oct. 16, 2008 in U.S. Appl. No. 10/959,322.
Non-Final OA issued Dec. 5, 2008 in U.S. Appl. No. 10/959,322.
1.111 Response filed Apr. 2, 2009 in U.S. Appl. No. 10/959,322.
Restriction Requirement issued Feb. 28, 2007 in U.S. Appl. No. 10/959,309.
Response to Restriction Requirement filed Apr. 2, 2007 in U.S. Appl. No. 10/959,309.
Non-Final OA issued Jun. 14, 2007 in U.S. Appl. No. 10/959,309.
1.111 Amendment filed Dec. 14, 2007 in U.S. Appl. No. 10/959,309.
Final OA issued Apr. 2, 2008 in U.S. Appl. No. 10/959,309.
1.116 Amendment filed Aug. 1, 2008 in U.S. Appl. No. 10/959,309.
Advisory Action issued Aug. 14, 2008 in U.S. Appl. No. 10/959,309.
RCE filed Sep. 2, 2008 in U.S. Appl. No. 10/959,309.
Non-Final OA issued Oct. 10, 2008 in U.S. Appl. No. 10/959,309.
Restriction Requirement issued Jun. 26, 2008 in U.S. Appl. No. 10/575,253.
Response to Restriction Requirement filed Sep. 26, 2008 in U.S. Appl. No. 10/575,253.
Non-Final OA issued Jan. 13, 2009 in U.S. Appl. No. 10/575,253.
Restriction Requirement issued Apr. 1, 2009 in U.S. Appl. No. 10/575,096.
Response to Restriction requirement filed May 29, 2009 in U.S. Appl. No. 10/575,096.
Restriction Requirement issued Jun. 12, 2009 in U.S. Appl. No. 10/575,096.
Restriction Requirement issued Jun. 12, 2007 in U.S. Appl. No. 11/196,503.
Response to Restriction Requirement filed Jul. 12, 2007 in U.S. Appl. No. 11/196,503.
Supplemental Response to Restriction Requirement filed Sep. 6, 2007 in U.S. Appl. No. 11/196,503.
Non-Final OA issued Nov. 13, 2007 in U.S. Appl. No. 11/196,503.
1.111 Amendment filed Feb. 21, 2008 in U.S. Appl. No. 11/196,503.
Final OA issued May 7, 2008 in U.S. Appl. No. 11/196,503.
1.116 Amendment filed Jul. 28, 2008 in U.S. Appl. No. 11/196,503.
Advisory Action issued Aug. 15, 2008 in U.S. Appl. No. 11/196,503.
RCE and 1.114 Amendment filed Sep. 5, 2008 in U.S. Appl. No. 11/196,503.
Non-Final OA issued Oct. 10, 2008 in U.S. Appl. No. 11/196,503.
1.111 Response and Terminal Disclaimer filed Mar. 10, 2009 in U.S. Appl. No. 11/196,503.
Final Office Action issued Jun. 22, 2009, in U.S. Appl. No. 11/196,503.
Restriction Requirement issued Sep. 11, 2007 in U.S. Appl. No. 11/491,501.
Response to Restriction Requirement filed Oct. 11, 2007 in U.S. Appl. No. 11/491,501.
Non-Final Office Action issued Feb. 6, 2008 in U.S. Appl. No. 11/491,501.
1.111 Amendment filed Jul. 7, 2008 in U.S. Appl. No. 11/491,501.
Notice to Comply issued Oct. 28, 2008 in U.S. Appl. No. 11/491,501.
Response to Notice to Comply filed Nov. 13, 2008 in U.S. Appl. No. 11/491,501.
Notice to Comply issued Feb. 25, 2009 in U.S. Appl. No. 11/491,501.
Response to Notice to Comply filed Mar. 18, 2009 in U.S. Appl. No. 11/491,501.
Restriction Requirement issued Jun. 14, 2002 in U.S. Appl. No. 09/796,744.
Response to Restriction Requirement filed Jul. 15, 2002 in U.S. Appl. No. 09/796,744.
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1.111 Amendment filed Mar. 17, 2003 in U.S. Appl. No. 09/796,744.
Non-Final OA issued Jun. 2, 2003 in U.S. Appl. No. 09/796,744.
1.111 Amendment and 1.132 Declaration filed Dec. 2, 2003 in U.S. Appl. No. 09/796,744.
Final OA issued Feb. 25, 2004 in U.S. Appl. No. 09/796,744.
1.116 Amendment and 1.132 Declaration filed Jul. 26, 2004 in U.S. Appl. No. 09/796,744.
Advisory Action issued Aug. 24, 2004 in U.S. Appl. No. 09/796,744.
Notice of Appeal filed Aug. 25, 2004 in U.S. Appl. No. 09/796,744.
RCE filed Sep. 7, 2004 in U.S. Appl. No. 09/796,744.
Non-Final OA issued Nov. 5, 2004 in U.S. Appl. No. 09/796,744.
1.111 Amendment filed Nov. 30, 2004 in U.S. Appl. No. 09/796,744.
Notice of Allowance issued Feb. 22, 2005 in U.S. Appl. No. 09/796,744.
Restriction Requirement issued Feb. 6, 2007 in U.S. Appl. No. 11/094,718.
Response to Restriction Requirement filed Mar. 16, 2007 in U.S. Appl. No. 11/094,718.
Non-Final OA issued May 30, 2007 in U.S. Appl. No. 11/094,718.
1.111 Amendment filed Oct. 29, 2007 in U.S. Appl. No. 11/094,718.
Final OA issued Dec. 21, 2007 in U.S. Appl. No. 11/094,718.
1.116 Amendment filed May 21, 2008 in U.S. Appl. No. 11/094,718.
Advisory Action issued Jun. 13, 2008 in U.S. Appl. No. 11/094,718.
RCE filed Jun. 20, 2008 in U.S. Appl. No. 11/094,718.
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1.111 Amendment filed Dec. 16, 2008 in U.S. Appl. No. 11/094,718.
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Advisory Action issued Jun. 24, 2009 in U.S. Appl. No. 11/094,718.
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Response to Restriction Requirement filed Aug. 12, 2005 in U.S. Appl. No. 10/231,452.
Notice of Non-Compliant Response issued Sep. 28, 2005 in U.S. Appl. No. 10/231,452.
Response to Notice of Non-Compliant Response filed Oct. 25, 2005 in U.S. Appl. No. 10/231,452.
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1.111 Amendment filed Jul. 13, 2006 in U.S. Appl. No. 10/231,452.
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Notice of Appeal filed Mar. 21, 2007 in U.S. Appl. No. 10/231,452.
1.116 Amendment and Terminal Disclaimer filed Apr. 5, 2007 in U.S. Appl. No. 10/231,452.
Advisory Action issued Apr. 24, 2007 in U.S. Appl. No. 10/231,452.
RCE filed May 21, 2007 in U.S. Appl. No. 10/231,452.
Non-Final OA issued Jul. 31, 2007 in U.S. Appl. No. 10/231,452.
Petition to Withdraw Terminal Disclaimer of Apr. 5, 2007 filed Oct. 31, 2007 in U.S. Appl. No. 10/231,452.
1.111 Amendment filed Nov. 30, 2007 in U.S. Appl. No. 10/231,452.
Decision on Petition issued on Jan. 3, 2008 in U.S. Appl. No. 10/231,452.
Final OA issued Feb. 20, 2008 in U.S. Appl. No. 10/231,452.
1.116 Amendment filed Jun. 20, 2008 in U.S. Appl. No. 10/231,452.
Advisory Action issued Aug. 5, 2008 in U.S. Appl. No. 10/231,452.
RCE and 1.114 Amendment filed Aug. 20, 2008 in U.S. Appl. No. 10/231,452.
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Response to Restriction Requirement filed Feb. 12, 2007 in U.S. Appl. No. 11/144,731.
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1.111 Amendment filed Sep. 5, 2007 in U.S. Appl. No. 11/144,731.
Final OA issued Nov. 13, 2007 in U.S. Appl. No. 11/144,731.
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1.116 Amendment filed Sep. 29, 2008 in U.S. Appl. No. 11/144,731.
Advisory Action issued Oct. 15, 2008 in U.S. Appl. No. 11/144,731.
Pre-Appeal Brief Request for Review and Notice of Appeal filed Dec. 1, 2008 in U.S. Appl. No. 11/144,731.
Notice of Panel Decision issued Jan. 13, 2009 in U.S. Appl. No. 11/144,731.
RCE filed Feb. 13, 2009 in U.S. Appl. No. 11/144,731.
Notice of Non-Response Amendment issued Jun. 4, 2009 in U.S. Appl. No. 11/144,731.

Restriction Requirement issued Jul. 13, 2007 in U.S. Appl. No. 10/581,413.
Response to Restriction Requirement filed Aug. 27, 2007 in U.S. Appl. No. 10/581,413.
Non-Final OA issued Oct. 17; 2007 in U.S. Appl. No. 10/581,413.
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Restriction Requirement issued Jul. 28, 2008 in U.S. Appl. No. 10/581,413.
Response to Restriction Requirement filed Aug. 28, 2008 in U.S. Appl. No. 10/581,413.
Final OA issued Dec. 11, 2008 in U.S. Appl. No. 10/581,413.
1.116 Amendment and Statement of Availability filed Apr. 13, 2009 in U.S. Appl. No. 10/581,413.
Advisory Action issued Apr. 27, 2009 in U.S. Appl. No. 10/581,413.
RCE filed May 11, 2009 in U.S. Appl. No. 10/581,413.
Restriction Requirement issued Apr. 25, 2008 in U.S. Appl. No. 10/574,016.
Response to Restriction Requirement filed May 27, 2008 in U.S. Appl. No. 10/574,016.
Non-Final OA issued Aug. 4, 2008 in U.S. Appl. No. 10/574,016.
1.111 Amendment filed Nov. 4, 2008 in U.S. Appl. No. 10/574,016.
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1.111 Amendment and Rule 1.132 Declaration filed May 29, 2009 in U.S. Appl. No. 10/574,016.
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Response to Restriction Requirement filed Apr. 27, 2009 in U.S. Appl. No. 12/019,160.
Non-Final Office Action issued Jun. 24, 2009, in U.S. Appl. No. 12/019,160.
Telephonic Correspondence Record issued Apr. 15, 2009, in Japanese Patent Application No. 2000-611663 and attached Third Party Observation.
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James C. Byrd et al., "Glycoprotein Synthesis in Yeast", The Journal of Biological Chemistry, 1982, 257(24): 14657-14666.
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RCE filed Jul. 18, 2009, in U.S. Appl. No. 11/686,391.
RCE filed Jul. 18, 2009, in U.S. Appl. No. 11/686,404.
RCE filed Jul. 19, 2009, in U.S. Appl. No. 11/686,458.
RCE filed Jul. 20, 2009, in U.S. Appl. No. 11/686,906.
RCE filed Jul. 17, 2009, in U.S. Appl. No. 11/686,911.
RCE filed Jul. 19, 2009, in U.S. Appl. No. 11/686,915.
RCE filed Jul. 18, 2009, in U.S. Appl. No. 11/686,920.
Supplemental Submission under 37 C.F.R. § 1.116 filed Dec. 28, 2004, in U.S. Appl. No. 09/971,773.
Final Office Action issued Jul. 24, 2009, in U.S. Appl. No. 11/240,579.
Request for Continued Examination filed Jul. 29, 2009, in U.S. Appl. No. 10/409,598.
Response to Election of Species Requirement and Preliminary Amendment filed Jun. 25, 2009 in U.S. Appl. No. 10/575,261.
Notice of Allowance issued Jul. 23, 2009, in U.S. Appl. No. 10/959,322.
Non-Final Office Action issued Jul. 22, 2009, in U.S. Appl. No. 10/581,413.
Restriction Requirement issued Jul. 12, 2009, in U.S. Appl. No. 10/575,114.
U.S. Appl. No. 60/572,899, filed May 2004, Mitsuo Satoh.
* cited by examiner

FIG.1

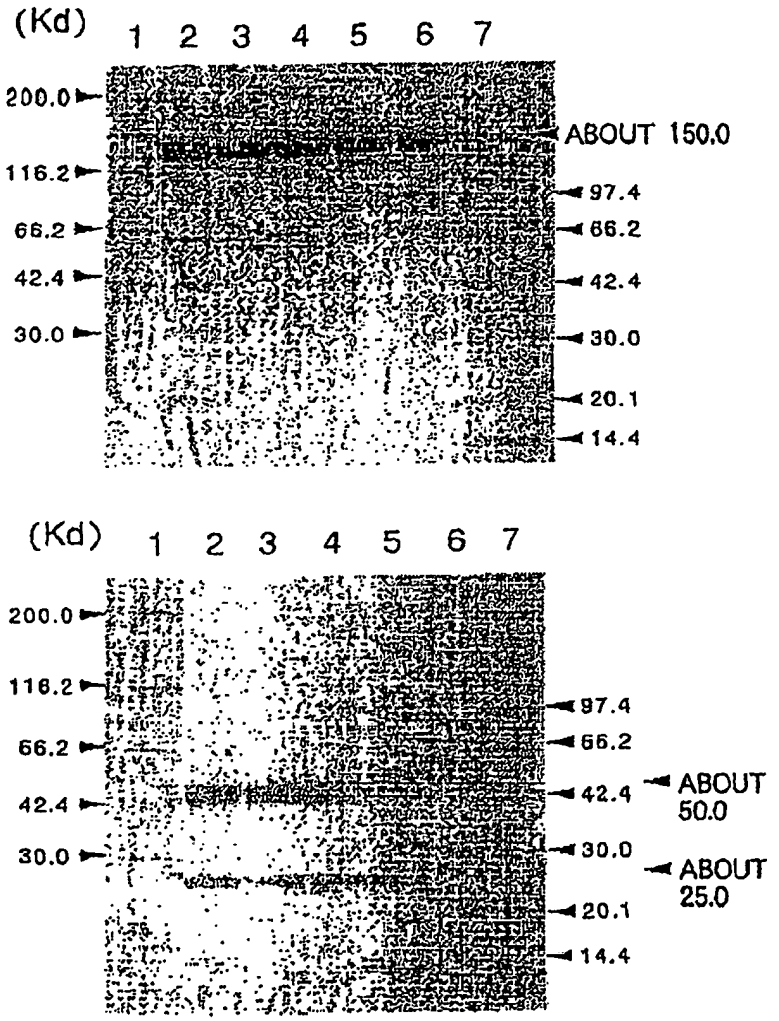

(Kd)
1 2 3 4 5 6 7
200.0
116.2
66.2
42.4
30.0
ABOUT 150.0
97.4
66.2
42.4
30.0
20.1
14.4
(Kd)
1 2 3 4 5 6 7
200.0
116.2
66.2
42.4
30.0
97.4
66.2
42.4
30.0
20.1
14.4
ABOUT 50.0
ABOUT 25.0

FIG.2

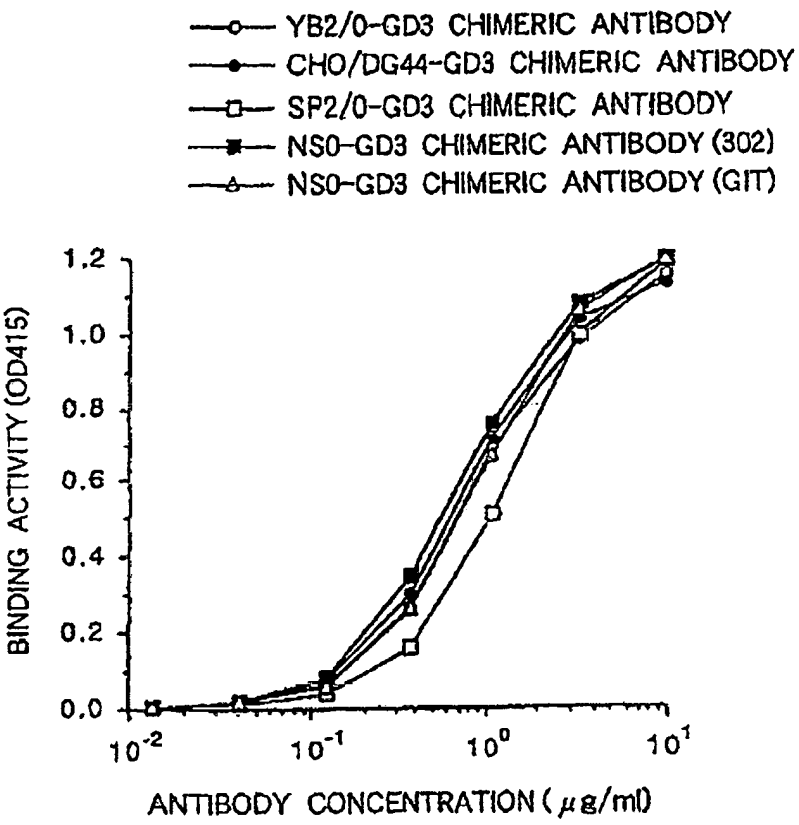

YB2/0-GD3 CHIMERIC ANTIBODY
CHO/DG44-GD3 CHIMERIC ANTIBODY
SP2/0-GD3 CHIMERIC ANTIBODY
NS0-GD3 CHIMERIC ANTIBODY (302)
NS0-GD3 CHIMERIC ANTIBODY (GIT)
BINDING ACTIVITY (OD415)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
10-2
10-1
100
101
ANTIBODY CONCENTRATION (μg/ml)

FIG.3

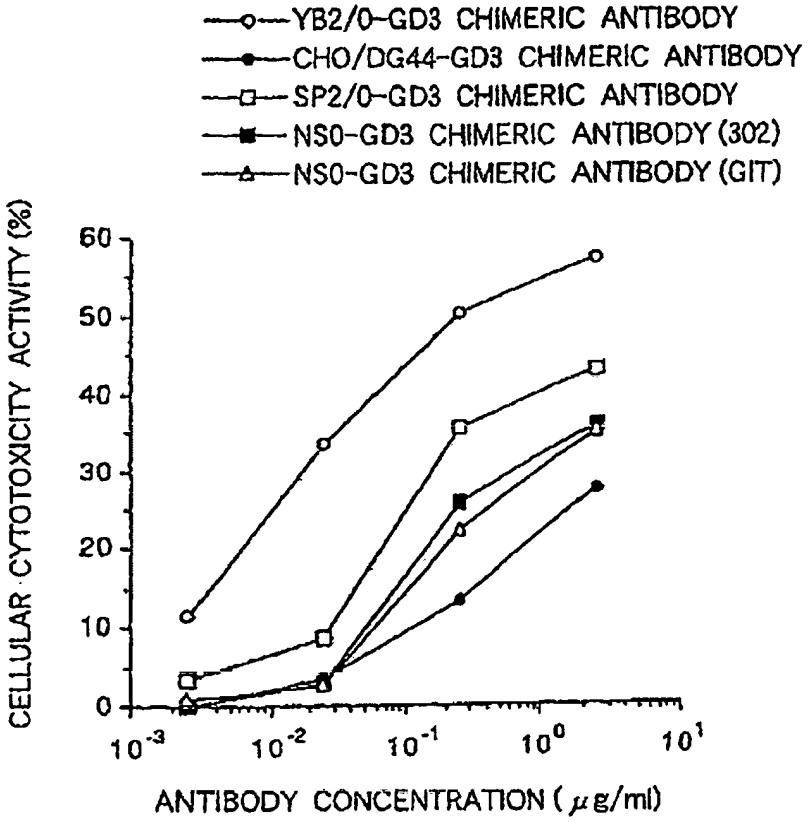

YB2/0-GD3 CHIMERIC ANTIBODY
CHO/DG44-GD3 CHIMERIC ANTIBODY
SP2/0-GD3 CHIMERIC ANTIBODY
NS0-GD3 CHIMERIC ANTIBODY (302)
NS0-GD3 CHIMERIC ANTIBODY (GIT)
CELLULAR CYTOTOXICITY ACTIVITY (%)
60
50
40
30
20
10
0
10^{-3}
10^{-2}
10^{-1}
10^{0}
10^{1}
ANTIBODY CONCENTRATION (μg/ml)

FIG.4

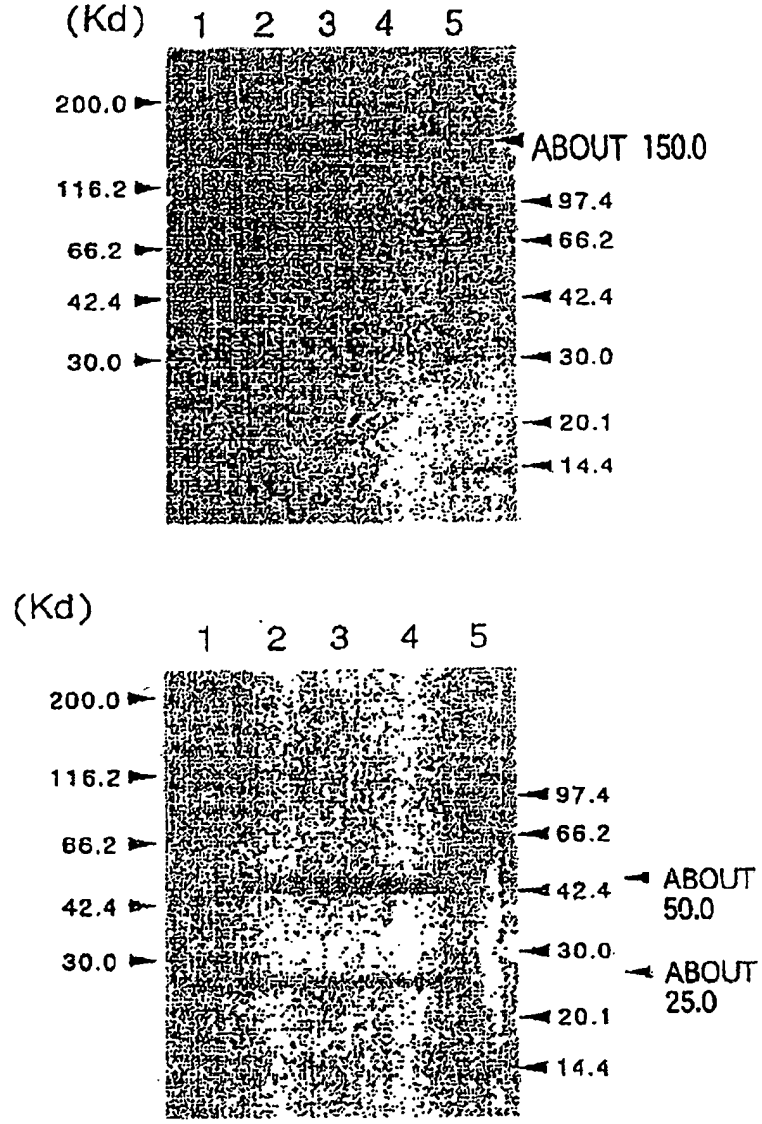

(Kd)
1 2 3 4 5
200.0
116.2
66.2
42.4
30.0
ABOUT 150.0
97.4
66.2
42.4
30.0
20.1
14.4
(Kd)
1 2 3 4 5
200.0
116.2
66.2
42.4
30.0
97.4
66.2
42.4
30.0
20.1
14.4
ABOUT 50.0
ABOUT 25.0

FIG.5

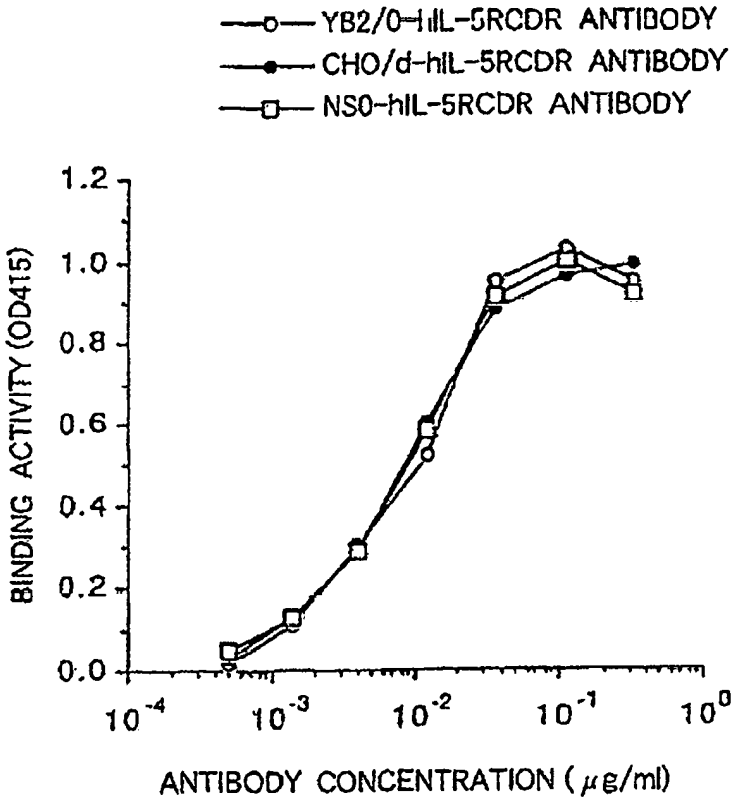

YB2/0-hIL-5RCDR ANTIBODY
CHO/d-hIL-5RCDR ANTIBODY
NS0-hIL-5RCDR ANTIBODY
BINDING ACTIVITY (OD415)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
10^-4
10^-3
10^-2
10^-1
10^0
ANTIBODY CONCENTRATION (μg/ml)

FIG.6

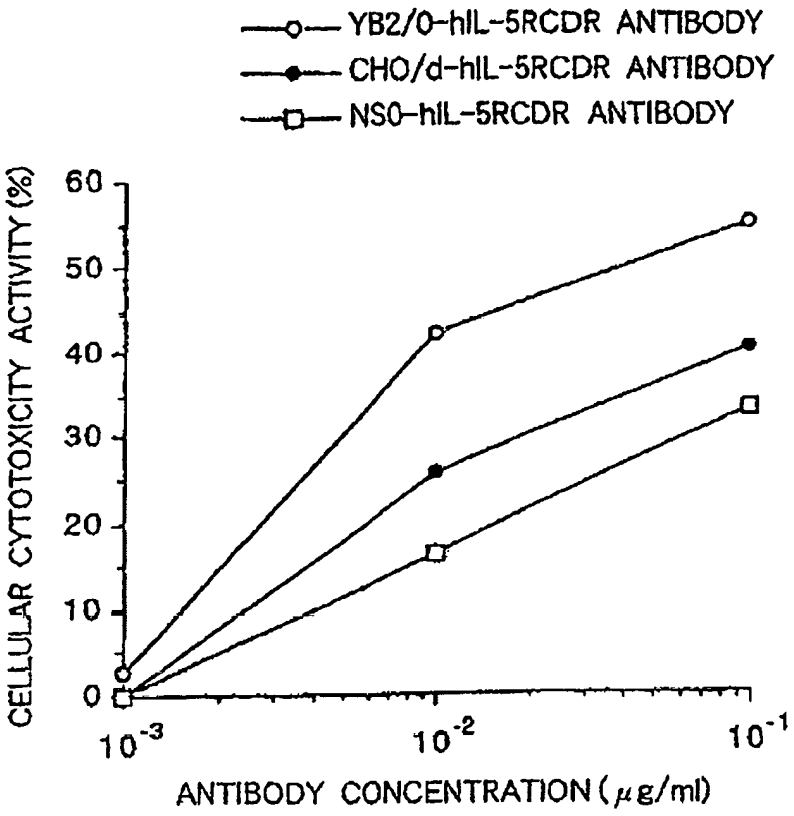

YB2/0-hIL-5RCDR ANTIBODY
CHO/d-hIL-5RCDR ANTIBODY
NS0-hIL-5RCDR ANTIBODY
CELLULAR CYTOTOXICITY ACTIVITY (%)
60
50
40
30
20
10
0
10^{-3}
10^{-2}
10^{-1}
ANTIBODY CONCENTRATION (μg/ml)

FIG.7

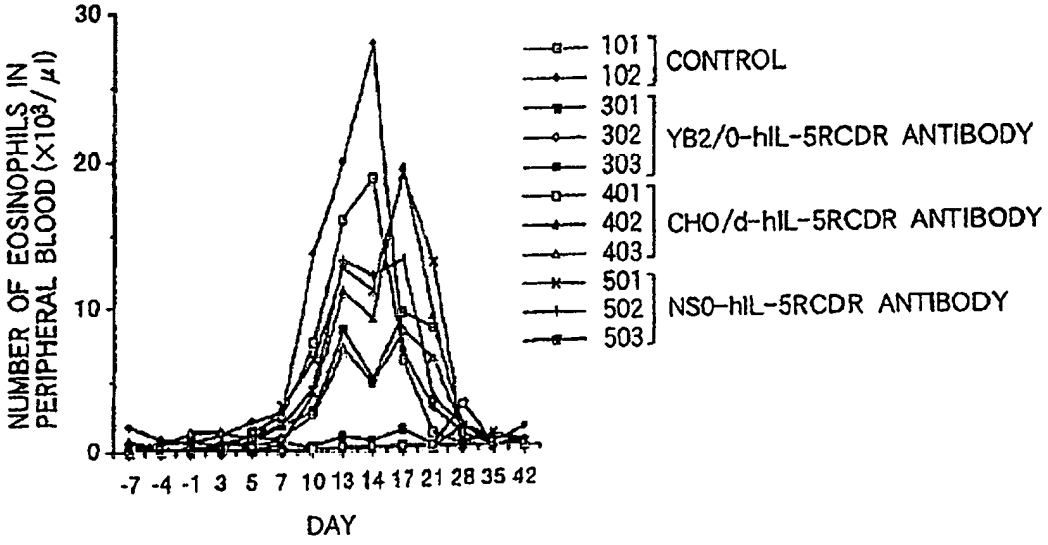
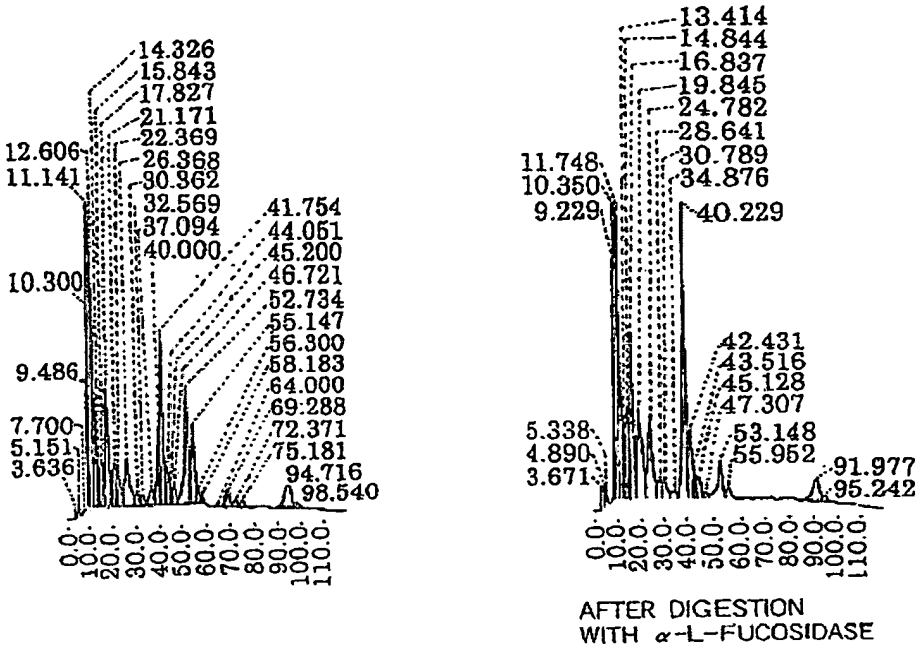

NUMBER OF EOSINOPHILS IN PERIPHERAL BLOOD (X10³/μl)
30
20
10
0
-7 -4 -1 3 5 7 10 13 14 17 21 28 35 42
DAY
101
102
CONTROL
301
302
303
YB2/0-hIL-5RCDR ANTIBODY
401
402
403
CHO/d-hIL-5RCDR ANTIBODY
501
502
503
NS0-hIL-5RCDR ANTIBODY

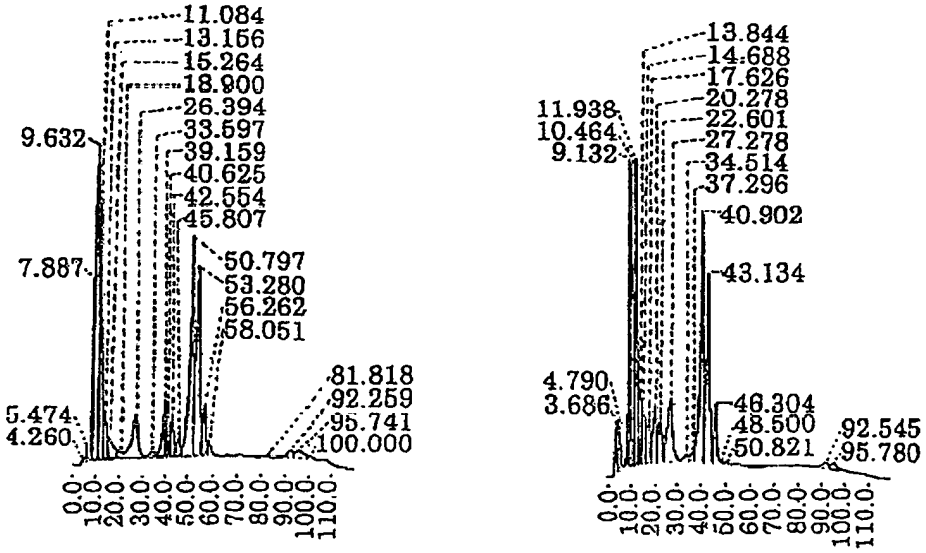
FIG.8

YB2/0-hIL-5RCDR ANTIBODY



AFTER DIGESTION WITH α-L-FUCOSIDASE

NS0-hIL-5RCDR ANTIBODY



AFTER DIGESTION WITH α-L-FUCOSIDASE

FIG.9

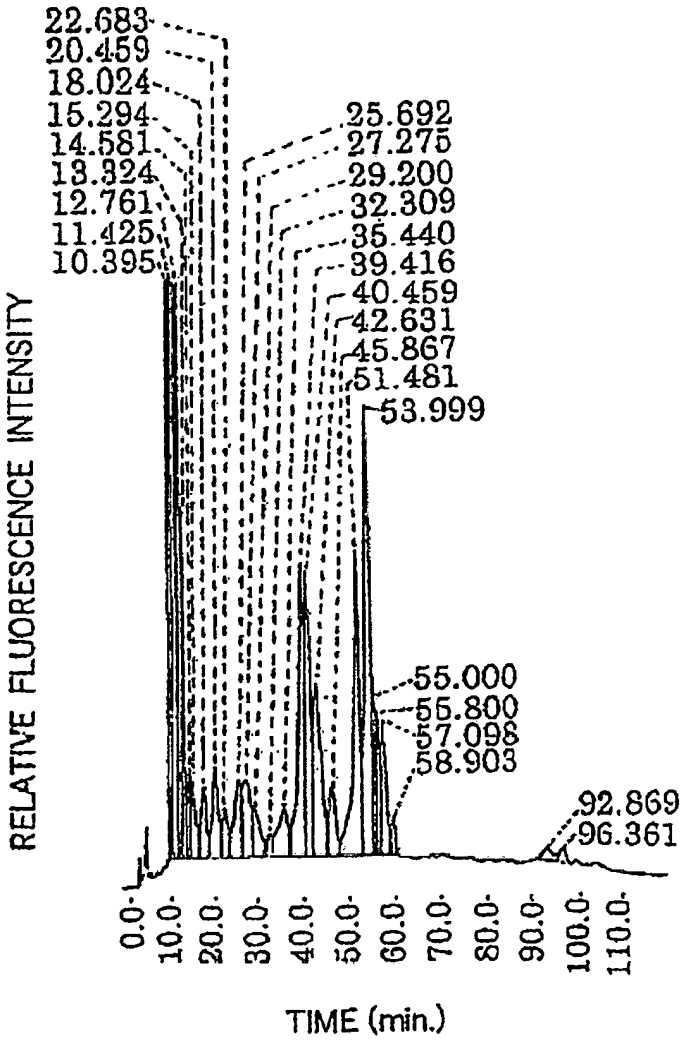

22.683
20.459
18.024
15.294
14.581
13.324
12.761
11.425
10.395
25.692
27.275
29.200
32.309
35.440
39.416
40.459
42.631
45.867
51.481
53.999
55.000
55.800
57.098
58.903
92.869
96.361
RELATIVE FLUORESCENCE INTENSITY
0.0
10.0
20.0
30.0
40.0
50.0
60.0
70.0
80.0
90.0
100.0
110.0
TIME (min.)

FIG.10

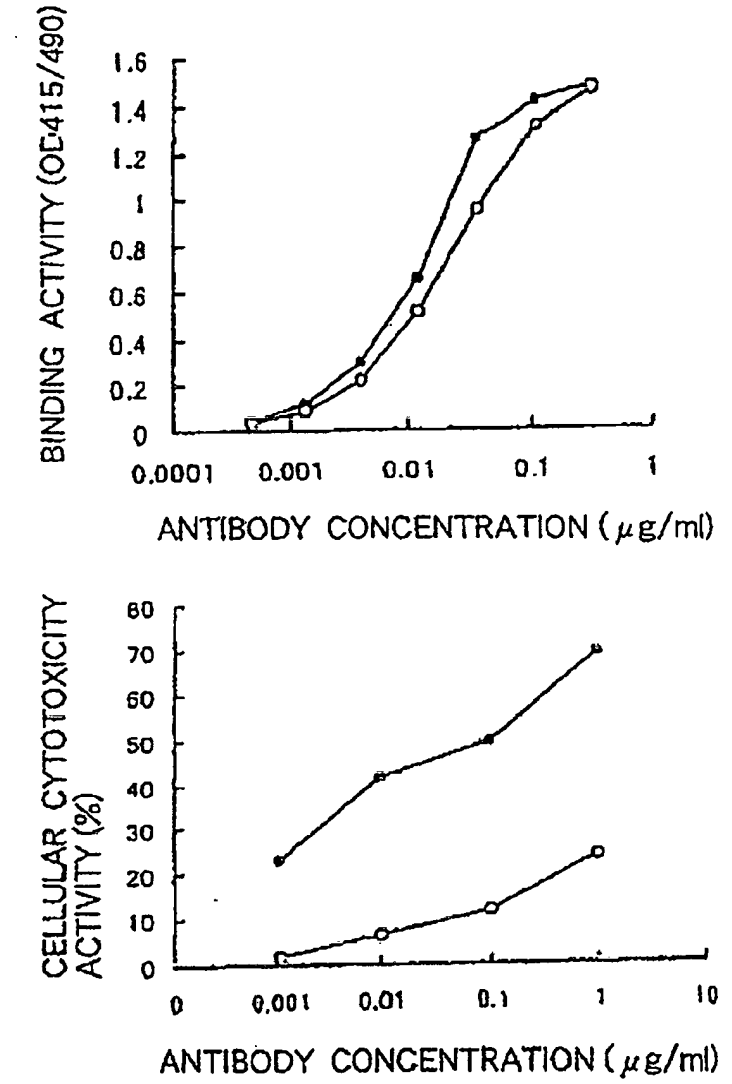

BINDING ACTIVITY (OD415/490)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0.0001
0.001
0.01
0.1
1
ANTIBODY CONCENTRATION (μg/ml)
CELLULAR CYTOTOXICITY ACTIVITY (%)
80
70
60
50
40
30
20
10
0
0
0.001
0.01
0.1
1
10
ANTIBODY CONCENTRATION (μg/ml)

FIG.11

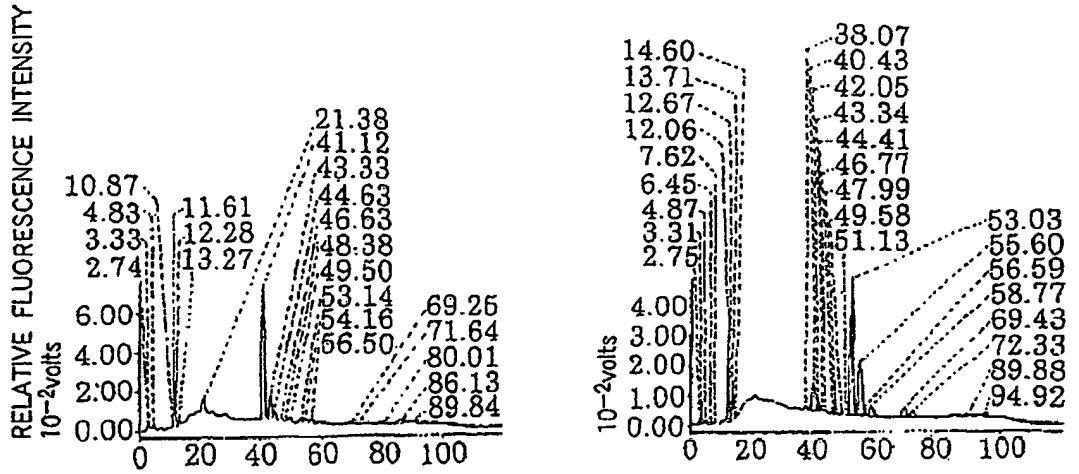
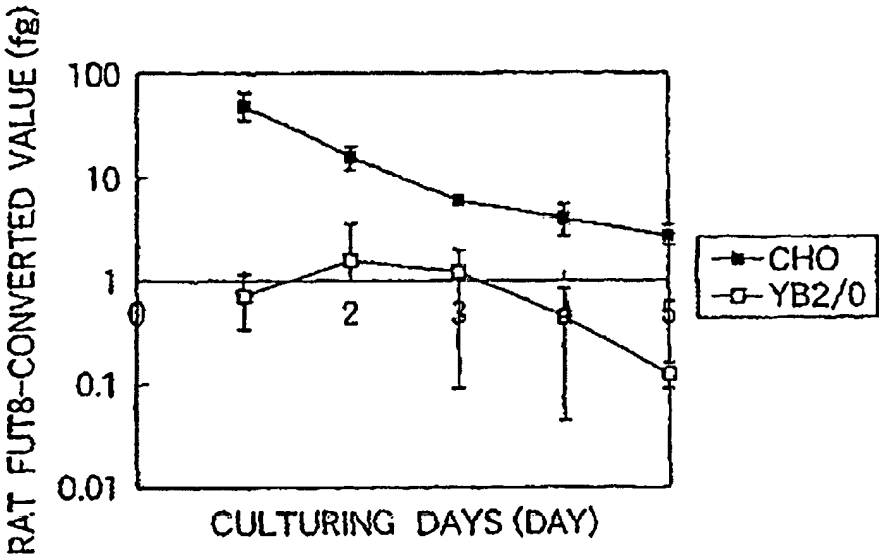

RELATIVE FLUORESCENCE INTENSITY
10⁻²volts
10.87
4.83
3.33
2.74
11.61
12.28
13.27
21.38
41.12
43.33
44.63
46.63
48.38
49.50
53.14
54.16
56.50
69.25
71.64
80.01
86.13
89.84
6.00
4.00
2.00
0.00
0 20 40 60 80 100
14.60
13.71
12.67
12.06
7.62
6.45
4.87
3.31
2.75
38.07
40.43
42.05
43.34
44.41
46.77
47.99
49.58
51.13
53.03
55.60
56.59
58.77
69.43
72.33
89.88
94.92
4.00
3.00
2.00
1.00
0.00
0 20 40 60 80 100

FIG.12


RAT FUT8-CONVERTED VALUE (fg)
100
10
1
0.1
0.01
0
2
3
5
CULTURING DAYS (DAY)
CHO
YB2/0

METHOD OF MODULATING THE ACTIVITY OF FUNCTIONAL IMMUNE MOLECULES TO INTERLEUKIN-5 RECEPTOR PROTEIN

The present application is a divisional of U.S. Ser. No. 11/126,176, filed May 11, 2005 (now U.S. Pat. No. 7,214,775), which is a divisional of U.S. Ser. No. 09/958,307, filed Oct. 9, 2001 (abandoned), which is a 371 of U.S. national phase of PCT/JP00/02260, filed Apr. 7, 2000, which claims benefit of JP 11-103158, filed Apr. 9, 1999, the entire contents of each application are hereby incorporated by reference.

TECHNICAL FIELD

The present invention relates to a method for controlling the activity of an immunologically functional molecule, such as an antibody, a protein, a peptide or the like, an agent of promoting the activity of an immunologically functional molecule, and an immunologically functional molecule having the promoted activity.

BACKGROUND ART

Since antibodies have high binding activity, binding specificity and high stability in blood, their applications to the diagnosis, prevention and treatment of various human diseases have been attempted (*Monoclonal Antibodies: Principles and Applications*, Wiley-Liss, Inc., Chapter 2.1 (1995)). However, an antibody derived from an animal other than human, such as a mouse antibody, is recognized as a foreign material when administered to a human, which thereby induces a human antibody against the mouse antibody (human anti mouse antibody: hereinafter referred to as "HAMA") in the human body, and it is known that the HAMA causes side effects by reaction with the administered mouse antibody (*J. Clin. Oncol.*, 2, 881 (1984); *Blood*, 65, 1349 (1985); *J. Natl. Cancer Inst.*, 80, 932 (1988); *Proc. Natl. Acad. Sci. U.S.A.*, 82, 1242 (1985)), promotes disappearance of the administered mouse antibody from blood (*J. Nuc. Med.*, 26, 1011 (1985); *Blood*, 65, 1349 (1985); *J. Natl. Cancer Inst.*, 80, 937 (1988)) and reduces diagnostic, preventive and therapeutic effects of the mouse antibody (*J. Immunol.*, 135, 1530 (1985); *Cancer Res.*, 46, 6489 (1986)).

For the purpose of solving these problems, attempts have been made to convert an antibody derived from an animal other than human into a humanized antibody, such as a human chimeric antibody or a human complementarity determining region (hereinafter referred to as "CDR")-grafted antibody, using gene recombination techniques. The human chimeric antibody is an antibody in which its antibody variable region (hereinafter referred to as "V region") is of an antibody of an animal other than human and its constant region (hereinafter referred to as "C region") is of a human antibody (*Proc. Natl. Acad. Sci. U.S.A.*, 81, 6851 (1984)). It has been reported that administration of such chimeric antibodies to humans eliminate serious side effects and the half-life in blood was prolonged about 6 times compared to a mouse antibody (*Proc. Natl. Acad. Sci. U.S.A.*, 86, 4220 (1989)). The human CDR-grafted antibody is an antibody in which CDR of a human antibody is replaced by CDR of an antibody other than human (*Nature*, 321, 522 (1986)). It has been reported that, in an experimentation using monkey, the immunogenicity of a human CDR-grafted antibody was reduced and its half-life in blood was prolonged 4 to 5 times compared to a mouse antibody (*J. Immunol.*, 147, 1352 (1991)). These reports show that a humanized antibody is expected to have sufficient effects, as an antibody to be applied to the diagnosis, prevention and treatment of various human diseases, even though it is not a completely human antibody. Actually, clinical tests have been performed with anti-tumor antibodies, such as an anti-CD20 human chimeric antibody, Rituxan (IDEC, Inc.), and an anti-HER2/neu human CDR-grafted antibody, Herceptin (Genentech, Inc.). The safety and therapeutic effects of the anti-CD20 human chimeric antibody and of the anti-HER2/neu human CDR-grafted antibody, to a certain degree, have been confirmed in B lymphoma and breast cancer, respectively (*J. Clin. Oncol.*, 16, 2825 (1998); *J. National Cancer Institute*, 90, 882 (1998)). Moreover, a fragment (Fab') of an anti-GPIIb/IIIa human chimeric antibody, ReoPro (Centocor, Inc.), is commercially available in Europe and America as a secondary disease preventing drug after percutaneous transluminal coronary angioplasty. Currently, a large number of clinical tests are being conducted with other humanized antibodies. Most of these humanized antibodies have been prepared using gene recombination techniques and produced using appropriate animal cells.

It has been revealed that five classes of antibodies, i.e., IgM, IgD, IgG, IgA and IgE, are present in mammals. Antibodies of human IgG class are mainly used in the diagnosis, prevention and treatment of various human diseases because of their long half-life in blood and functional characteristics, such as various effector functions and the like (*Monoclonal Antibodies: Principles and Applications*, Wiley-Liss, Inc., Chapter 2.1 (1995)). The human IgG class antibody is further classified into the following 4 subclasses: IgG1, IgG2, IgG3 and IgG4. A large number of studies have so far been carried out for the antibody-dependent cellular cytotoxicity activity (hereinafter referred to as "ADCC activity") and complement-dependent cytotoxicity activity (hereinafter referred to as "CDC") as effector functions of the IgG class antibody, and it has been reported that antibodies of the IgG1 subclass have the greatest ADCC activity and CDC activity among the human IgG class antibodies (*Chemical Immunology*, 65, 88 (1997)). Therefore, most of the anti-tumor humanized antibodies which require a high effector function are antibodies of human IgG1 subclass, including the above Rituxan and Herceptin.

Expression of ADCC activity and CDC activity of human IgG1 subclass antibodies requires binding of the Fc region of antibody to an antibody receptor existing on the surface of an effector cell, such as a killer cell, a natural killer cell, an activated macrophage or the like (hereinafter referred to as "FcγR") and various complement components. It has been suggested that several amino acid residues in the second domain of the antibody hinge region and C region (hereinafter referred to as "Cγ2 domain") (*Eur. J. Immunol.*, 23, 1098 (1993), *Immunology*, 86, 319 (1995), *Chemical Immunology*, 65, 88 (1997)) and a sugar chain linked to the Cγ2 domain are also important for this binding reaction (*Chemical Immunology*, 65, 88 (1997)). Regarding the sugar chain, Boyd et al. have examined effects of a sugar chain on the ADCC activity and CDC activity, by treating a human CDR-grafted antibody, CAMPATH-1H (human IgG1 subclass), produced using Chinese hamster ovary cell (CHO cell) or mouse myeloma NS0 cell with various sugar hydrolyzing enzymes, and reported that elimination of sialic acid of the non-reducing terminal does not have influence upon both activities. Further elimination of galactose residue however was reported to exert influence upon only the CDC activity, decreasing about 50% of its activity. Complete elimination of the sugar chain was reported to cause disappearance of both activities (*Molecular Immunol.*, 32, 1311 (1995)). Moreover, Lifely et al. have analyzed the sugar chain of a human CDR-grafted antibody, CAMPATH-1H (human IgG1 subclass) which was produced

using CHO cell, NS0 cell or rat myeloma YO cell, measured its ADCC activity and reported that the CAMPATH-1H derived from YO cell shows the greatest ADCC activity, suggesting that N-acetylglucosamine at the bisecting position is important for the activity (*Glycobiology*, 5, 813 (1995): WO 99/54342). These reports show that the structure of sugar chain plays an important role in the effector function of human IgG1 subclass antibodies, and that it may be possible to prepare an antibody having greater effector function by changing the sugar chain structure. Actually, however, structures of sugar chains are complex and vary greatly. There exists a need therefore to further study the structure in order to obtain greater effector function.

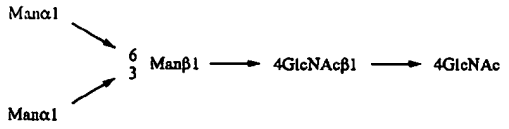
DISCLOSURE OF THE INVENTION

An object of the present invention is to specify a sugar chain which increases the ADCC activity, by analyzing sugar chains of human IgG1 subclass antibodies produced by various animal cells, and to thereby also provide a method for controlling the activity of an immunologically functional molecule. Since the ADCC activity is improved in such antibodies, increase in the therapeutic effect for various human diseases can be expected by use of not only anti-tumor antibodies but also anti-other diseases antibodies, as well as proteins or peptides against various diseases. Particularly, in the clinical application of anti-tumor antibodies, the anti-tumor effect of an antibody alone is insufficient in many of current cases. The insufficiencies of known antibodies have required the concomitant use of chemotherapy (*Science*, 280, 1197, 1998). The dependency on chemotherapy however will be reduced, with a reduction of side effects, it a stronger anti-tumor effect of an antibody alone is provided by the improvement of ADCC activity. The present inventors have evaluated in vitro activity of various humanized antibodies of human IgG1 subclass produced by two kinds of Chinese hamster ovary cells, CHO/dhFr cell (ATCC CRL 9096) and CHO/DG44 cell (*Somatic Cell and Molecular Genetics*, 12, 555 (1986)), mouse myeloma NS0 cell (RCB 0213, *BIO/TECHNOLOGY*, 10, 169 (1992)), mouse myeloma SP2/0-Ag14 cell (hereinafter referred to as "SP2/0 cell"; ATCC CRL 1581) and rat myeloma YB2/3HL.P2.G11.16Ag.20 cell (hereinafter referred to as "YB2/0 cell"; ATCC CRL 1662) and have discovered, as a result, that the ADCC activity of a humanized antibody produced by the rat myeloma YB2/0 cell is considerably higher than that of the humanized antibodies produced by other cells. Further, as a result of an in vivo activity evaluation using *Macaca faseicularis*, it has been discovered that the humanized antibody produced by YB2/0 cell shows the greatest effect, suggesting the utility of an antibody having elevated ADCC activity in a human clinical application. In addition, a sugar chain having the ability to increase the ADCC activity has been identified by analyzing and comparing structures of the sugar chains of humanized antibodies produced by various animal cells in detail, and the present invention has been accomplished.

More specifically, the present invention relates to the following (1) to (62).

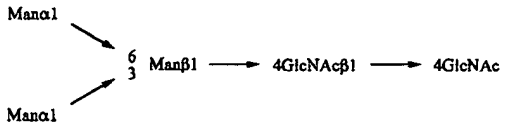
(1) A method for controlling the activity of an immunologically functional molecule, which comprises regulating the presence or absence of binding of fucose to N-acetylglucosamine of the reducing terminal of an N-glycoside-linked sugar chain which binds to the immunologically functional molecule.

(2) The method according to (1), wherein the N-glycoside-linked sugar chain which binds to the immunologically functional molecule comprises:



(3) A method for enhancing the activity of an immunologically functional molecule, which comprises binding a sugar chain in which fucose is not present in N-acetylglucosamine of the reducing terminal of an N-glycoside-linked sugar chain to the immunologically functional molecule.

(4) The method according to (3), wherein the sugar chain comprises:



(5) The method according to (3), wherein the sugar chain is synthesized in a cell which has a low enzyme activity of adding fucose to N-acetylglucosamine of the reducing terminal or does not have said enzyme activity.

(6) The method according to (5), wherein the enzyme which adds fucose to N-acetylglucosamine of the reducing terminal is a fucosyltransferase.

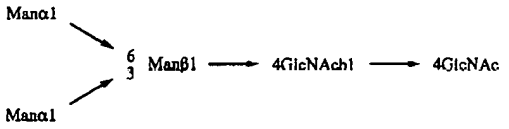
(7) The method according to (6), wherein the fucosyltransferase is α1,6-fucosyltransferase.

(8) The method according to (3), wherein the sugar chain is synthesized in a rat myeloma cell.

(9) The method according to (8), wherein the rat myeloma cell is YB2/3HL.P2.G11.16Ag.20 cell (ATCC CRL 1662).

(10) A method for inhibiting the activity of an immunologically functional molecule, which comprises binding a sugar chain in which fucose is present in N-acetylglucosamine of the reducing terminal of an N-glycoside-linked sugar chain to an immunologically functional molecule.

(11) The method according to (10), wherein the sugar chain comprises:



(12) The method according to (10), wherein the sugar chain is synthesized in a cell which has a high enzyme activity of adding fucose to N-acetylglucosamine of the reducing terminal.

(13) The method according to (12), wherein the enzyme which adds fucose to N-acetylglucosamine of the reducing terminal is a fucosyltransferase.

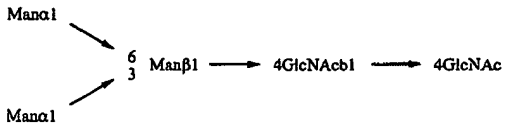
(14) The method according to (13), wherein the fucosyltransferase is α1,6-fucosyltransferase.

(15) The method according to (1) to (14), wherein the immunologically functional molecule is an antibody, a protein or a peptide.

(16) An agent of promoting the activity of an immunologically functional molecule, comprising a sugar chain in which

fucose is not present in N-acetylglucosamine of the reducing terminal of an N-glycoside-linked sugar chain.

(17) The agent of promoting the activity of an immunologically functional molecule according to (16), wherein the sugar chain comprises:



(18) The agent of promoting the activity of an immunologically functional molecule according to (16), wherein the sugar chain is synthesized in a cell which has a low enzyme activity of adding fucose to N-acetylglucosamine of the reducing terminal or does not have said enzyme activity.

(19) The agent of promoting the activity of an immunologically functional molecule according to (18), wherein the enzyme which adds fucose to N-acetylglucosamine of the reducing terminal is a fucosyltransferase.

(20) The agent of promoting the activity of an immunologically functional molecule according to (19), wherein the fucosyltransferase is α1,6-fucosyltransferase.

(21) The agent of promoting the activity of an immunologically functional molecule according to (16), wherein the sugar chain is synthesized in a rat myeloma cell.

(22) The agent of promoting the activity of an immunologically functional molecule according to (21), wherein the rat myeloma cell is YB2/3HL.P2.G11.16Ag.20 cell (ATCC CRL 1662).

(23) The agent of promoting the activity of an immunologically functional molecule according to any one of (16) to (22), wherein the immunologically functional molecule is an antibody, a protein or a peptide.

(24) An immunologically functional molecule having a promoted immunologically functional activity, to which molecule a sugar chain in which fucose is not present in N-acetylglucosamine of the reducing terminal of an N-glycoside-linked sugar chain is bound.

(25) An immunologically functional molecule having an inhibited immunologically functional activity, to which molecule a sugar chain in which fucose is present in N-acetylglucosamine of the reducing terminal of an N-glycoside-linked sugar chain is bound.

(26) The immunologically functional molecule according to (24), wherein the immunologically functional molecule is an antibody, a protein or a peptide.

(27) The immunologically functional molecule according to (25), wherein the immunologically functional molecule is an antibody, a protein or a peptide.

(28) A method for producing the immunologically functional molecule according to (24), which comprises using a cell which has a low enzyme activity of adding fucose to N-acetylglucosamine of the reducing terminal or does not have said enzyme activity.

(29) The method according to (28), wherein the enzyme which adds fucose to N-acetylglucosamine of the reducing terminal is a fucosyltransferase.

(30) The method according to (29), wherein the fucosyltransferase is α1,6-fucosyltransferase.

(31) A method for producing the immunologically functional molecule according to (24), wherein a rat myeloma cell is used in the method for producing an immunologically functional molecule having a promoted immunologically functional activity.

(32) The method according to (31), wherein the rat myeloma cell is YB2/3HL.P2.G11.16Ag.20 cell.

(33) A method for producing the immunologically functional molecule according to (25), wherein a cell having a high enzyme activity of adding fucose to N-acetylglucosamine of the reducing terminal is used.

(34) The method according to (33), wherein the enzyme which adds fucose to N-acetylglucosamine of the reducing terminal is a fucosyltransferase.

(35) The method according to (34), wherein the fucosyltransferase is α1,6-fucosyltransferase.

(36) The immunologically functional molecule according to (26), wherein the antibody recognizes a tumor-related antigen.

A tumor-related antigen of the present invention is an antigen which is expressed in a tumor cell in greater amount in comparison with normal cells. Examples include ganglioside GD2, GD3 and GM2 (*Cancer Immunol. Immunother.*, 43, 152 (1996)), HER2 (*J. Surgical Research*, 77, 85 (1998)), CD52 (*Leukemia Research*, 22, 185 (1998)), MAGE (*APMIS*, 106, 665 (1998)) and the like. In addition, a factor which induces growth of a tumor cell and its receptor are also tumor-related antigens. Examples include a basic fibroblast growth factor and its receptor (*Pancreas*, 17, 169 (1998)), a vascular endothelial cell growth factor and its receptor (*Pathology International*, 48, 499 (1998)) and the like.

(37) The immunologically functional molecule according to (36), wherein the tumor-related antigen is ganglioside GD3.

(38) The immunologically functional molecule according to (36), wherein the antibody is produced by 7-9-51 (FERM BP-6691).

(39) The immunologically functional molecule according to (26), wherein the antibody recognizes an antigen related to an allergy or inflammation.

An antigen related to an allergy or inflammation according to the present invention is an antigen which induces an allergy or inflammation and an antigen which is induced accompanied by an allergy or inflammation. Examples include interleukin 5 and its receptor (*International Archives. Allergy. Immunol.*, 117, 11 (1998)), a tumor necrosis factor and its receptor (*Cytokine*, 8, 651 (1996)) and the like.

(40) The immunologically functional molecule according to (39), wherein the antigen related to an allergy or inflammation is human interleukin 5 receptor α chain.

(41) The immunologically functional molecule according to (39), wherein the antibody is produced by No. 3 (FERM BP-6690).

(42) The immunologically functional molecule according to (26), wherein the antibody recognizes an antigen related to a cardiovascular disease.

An antigen related to a cardiovascular disease according to the present invention is an antigen which is concerned in a cardiovascular disease induced by thrombus, vascular re-stricture or the like. Examples include platelet GpIIb/IIIa (*Thrombosis Research*, 89, 129 (1998)), a platelet-derived growth factor and its receptor (*American J. Physiology*, 269, 1641 (1995)), a blood coagulation factor (*Thrombosis. Haemostasis*, 79, 14 (1998)) and the like.

(43) The immunologically functional molecule according to (27), wherein the antibody recognizes an antigen related to an autoimmune disease.

An antigen related to an autoimmune disease according to the present invention is an autoantigen which induces an

immune response as the cause of a disease and an antigen that enhances the response. Examples include auto-DNA (*Rheumatology International*, 17, 223 (1998)), CD4 (*Rheumatic Diseases Clinics. North America*, 24, 567 (1998)) and the like.

(44) The immunologically functional molecule according to (26), wherein the antibody recognizes an antigen related to a viral or bacterial infection.

An antigen related to a viral or bacterial infection according to the present invention is an antigen related to its infection and growth in a viral or bacterial target cell and also includes a viral or bacterial product. Examples include gp120 (*Virology*, 248, 394 (1998)), CXCR4 (*J. Virology*, 72, 8453 (1998)), Vero toxin (*J. Clinical Microbiology*, 34, 2053 (1996)) and the like.

(45) An agent for diagnosing a cancer, comprising the immunologically functional molecule according to (36) as an active ingredient.

(46) An agent for treating a cancer, comprising the immunologically functional molecule according to (36) as an active ingredient.

(47) An agent for preventing a cancer, comprising the immunologically functional molecule according to (36) as an active ingredient.

(48) An agent for diagnosing an allergy or inflammation, comprising the antibody according to (39) as an active ingredient.

(49) An agent for treating an allergy or inflammation, comprising the antibody according to (39) as an active ingredient.

(50) An agent for preventing an allergy or inflammation, comprising the antibody according to (39) as an active ingredient.

(51) An agent for diagnosing a cardiovascular disease, comprising the antibody according to (42) as an active ingredient.

(52) An agent for treating a cardiovascular disease, comprising the antibody according to (42) as an active ingredient.

(53) An agent for preventing a cardiovascular disease, comprising the antibody according to (42) as an active ingredient.

(54) An agent for diagnosing an autoimmune disease, comprising the antibody according to (43) as an active ingredient.

(55) An agent for treating an autoimmune disease, comprising the antibody according to (43) as an active ingredient.

(56) An agent for preventing an autoimmune disease, comprising the antibody according to (43) as an active ingredient.

(57) An agent for diagnosing a viral or bacterial infection, comprising the antibody according to (44) as an active ingredient.

(58) An agent for treating a viral or bacterial infection, comprising the antibody according to (44) as an active ingredient.

(59) An agent for preventing a viral or bacterial infection, comprising the antibody according to (44) as an active ingredient.

(60) An agent for diagnosing various diseases, comprising the peptide or protein according to (26) or (27) as an active ingredient.

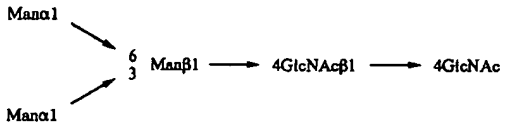
Examples of the various diseases according to the present invention include a cancer, an allergic disease, an inflammatory disease, a cardiovascular disease, an autoimmune disease, a viral or bacterial infection and the like.

(61) An agent for treating various diseases, comprising the peptide or protein according to (60) as an active ingredient.

(62) An agent for preventing various diseases, comprising the peptide or protein according to (60) as an active ingredient.

Based on the binding form of immunologically functional molecules, the sugar chain is roughly classified into two kinds, namely a sugar chain which binds to asparagine (called N-glycoside-linked sugar chain) and a sugar chain which binds to serine, threonine and the like (called O-glycoside-linked sugar chain).

The N-glycoside-linked sugar chain according to the present invention has various structures (*Biochemical Experimentation Method* 23—*Method for Studying Glycoprotein Sugar Chains* (Gakkai Shuppan Center), edited by Reiko Takahashi (1989)), but each case has the following common basic core structure.



In the above structure, the sugar chain terminal which binds to asparagine is called a reducing terminal, and the opposite side is called a non-reducing terminal. The fucose may be bound to N-acetylglucosamine of the reducing terminal by, for example, an α1,3 bond, an α1,6 bond or the like.

Examples of the N-glycoside-linked sugar chains include a high mannose type, in which only mannose binds to the non-reducing terminal of the core structure; a complex type, in which the non-reducing terminal side of the core structure has one or more branches of galactose-N-acetylglucosamine (hereinafter referred to as "Gal-GlcNAc") and the non-reducing terminal side of Gal-GlcNAc further has a structure such as a sialic acid, bisecting N-acetylglucosamine or the like; a hybrid type, in which the non-reducing terminal side of the core structure has both branches of the high mannose N-glycoside-linked sugar chain and complex N-glycoside-linked sugar chain; and the like.

An immunologically functional molecule is a molecule which is originally derived from the living body and is involved in various immune responses. Specifically, it includes antibodies, proteins, peptides and the like.

An antibody is a protein which is produced in vivo by an immune response as a result of the stimulation by a foreign antigen and has an activity to specifically bind to the antigen. Examples of the antibody include an antibody secreted by a hybridoma cell prepared from spleen cells of an immunized animal after immunization of the animal with an antigen, as well as an antibody prepared by gene recombination techniques, namely an antibody obtained by introducing an antibody encoding gene-inserted antibody expression vector into a host cell. Examples include an antibody produced by a hybridoma, a humanized antibody, a human antibody and the like.

A hybridoma is a cell which produces a monoclonal antibody having a desired antigen specificity and is obtained by cell fusion of a B cell prepared by immunizing a mammal other than human with an antigen, with a mycloma cell derived from a mouse or the like.

Humanized antibodies includes a human chimeric antibody, a human complementarity determining region (hereinafter referred to as "CDR")-grafted antibody and the like.

A human chimeric antibody is an antibody comprising an antibody heavy chain variable region (hereinafter referred also to as "HV" or "VH", wherein the heavy chain is an H chain and the variable region is a V region) and an antibody light chain variable region (hereinafter referred to also as

"LV" or "VL", wherein the light chain is an L chain) derived from an animal other than human, a heavy chain constant region (hereinafter referred to also as "CH", wherein the constant region is a C region) of a human antibody and a light chain constant region (hereinafter referred to also as "CL") of a human antibody. Animals other than human may be any of mouse, rat, hamster, rabbit and the like, so long as a hybridoma can be prepared from the same.

The human chimeric antibody can be produced by obtaining cDNAs encoding VH and VL from a hybridoma which produces a monoclonal antibody, inserting each of the cDNAs into an expression vector for a host cell having a gene encoding human antibody CH and human antibody CL to construct a human chimeric antibody expression vector, and then introducing the vector into a host cell to express the antibody.

Any CH of the human chimeric antibody may be used, so long as it belongs to a human immunoglobulin (hereinafter referred to as "hIg"), but those of the hIgG class are preferred and any of subclasses belonging to the hIgG class, such as hIgG1, hIgG2, hIgG3 and hIgG4, can be used. Moreover, any CL of the human chimeric antibody may be used, so long as it belongs to hIg, and those of κ class or λ class can be used.

A human CDR-grafted antibody is an antibody in which amino acid sequences of CDRs of the VH and VL of an antibody derived from an animal other than human are grafted to appropriate positions of the VH and VL of a human antibody.

The human CDR-grafted antibody can be produced by constructing cDNAs encoding V regions in which CDR sequences of the VH and VL of an antibody derived from an animal other than human are grafted to CDR sequences of the VH and VL of a human antibody, inserting each of the cDNAs into an expression vector for a host cell having a gene encoding the CH of a human antibody and the CL of a human antibody to construct a human CDR-grafted antibody expression vector, and introducing the expression vector into a host cell to express the human CDR-grafted antibody.

The CH of the human CDR-grafted antibody may be any region which belongs to hIg, but those of the hIgG class are preferred. Any of subclasses belonging to the hIgG class such as hIgG1, hIgG2, hIgG3, hIgG4 and the like can be used. Also, the CL of the human CDR-grafted antibody may be any region which belongs to hIg, and those of κ class or λ class can be used.

A human antibody is originally meant to be an antibody naturally existing in the human body, but it also includes antibodies obtained from a human antibody phage library and a human antibody-producing transgenic animal or a human antibody-producing transgenic plant, which are prepared based on recent advances in genetic engineering, cell engineering and developmental engineering techniques.

The antibody existing in the human body can be obtained, for example, by isolating a human peripheral blood lymphocyte, immortalizing it by its infection with EB virus or the like, followed by cloning, culturing a lymphocyte capable of producing the antibody, and purifying the antibody from the culture mixture.

The human antibody phage library is a library in which an antibody fragment, such as Fab, a single chain antibody or the like, is expressed on the phage surface by inserting an antibody gene prepared from human B cell into a phage gene. A phage expressing an antibody fragment having the desired antigen binding activity can be recovered from this library, using its activity to bind to an antigen-immobilized substrate as the marker. The antibody fragment can be converted further into a human antibody molecule comprising two full H chains and two full L chains by genetic engineering techniques.

A human antibody-producing transgenic non-human animal is an animal in which a human antibody-encoding gene is integrated into cells. Specifically, a human antibody-producing transgenic animal can be prepared by introducing a human antibody-encoding gene into a mouse ES cell, transplanting the ES cell into an early stage embryo of another mouse, and developing an animal. The human antibody may be prepared and accumulated in a culture mixture of the human antibody-producing transgenic animal by obtaining a human antibody-producing hybridoma according to a hybridoma preparation method usually carried out in mammals other than human and then culturing the hybridoma.

An activity of antibodies of the present invention includes ADCC activity.

ADCC activity as used herein refers to an activity to injury a tumor cell or the like by activating an effector cell via the binding of the Fc region of an antibody to an Fc receptor existing on the surface of an effector cell such as a killer cell, a natural killer cell, an activated macrophage or the like (*Monoclonal Antibodies: Principles and Applications*, Wiley-Liss, Inc., Chapter 2.1 (1995)).

Any protein and peptide can be used, so long as they can activate various immune response. Examples include interferon molecules, such as interleukin-2 (IL-2) (*Science*, 193, 1007 (1976)) and interleukin-12 (IL-12) (*J. Leuc. Biol.*, 55, 280 (1994)); colony-stimulating factors, such as granulocyte colony-stimulating factor (G-CSF) (*J. Biol. Chem.*, 258, 9017 (1983)), macrophage colony-stimulating factor (M-CSF) (*J. Exp. Med.*, 173, 269 (1992)) and granulocyte macrophage colony-stimulating factor (MG-CSF) (*J. Biol. Chem.*, 252, 1998 (1977)); growth factors, such as erythropoietin (EPO) (*J. Biol. Chem.*, 252, 5558 (1977)) and thrombopoietin (TPO) (*Nature*, 369, 533 (1994)); and the like.

The activities of protein and peptide of the present invention are activities of various immunocompetent cells including lymphocytes (T cell, B cell and the like) and macrophage, or various immune response reactions, when the sugar chain-containing protein and peptide are administered into the living body.

The promotion of activities of protein and peptide of the present invention includes activation of NK cell and T cell by IL-2 and IL-12, promotion activities of erythrocyte production by EPO and the like which are further increased,

1. Method for Analyzing Sugar Chain of Immunologically Functional Molecule

(1) Compositional analysis of neutral sugar and aminosugar

As described above, the sugar chain of IgG comprises a neutral sugar, such as galactose, mannose, fucose or the like, an aminosugar, such as N-acetylglucosamine or the like, and an acidic sugar, such as sialic acid or the like.

Regarding compositional analysis of the sugar chain of an antibody, the compositional ratio can be analyzed by releasing neutral sugars or amino sugars by acid hydrolysis of the sugar chain.

Specific methods include a method using a sugar composition analyzer (BioLC) manufactured by Dionex. The BioLC is an apparatus for analyzing sugar composition by HPAEC-PAD (high performance anion-exchange chromatography-pulsed amperometric detection) method (*J. Liq. Chromatogr.*, 6, 1577 (1983)).

The compositional ratio can also be analyzed by a fluorescence labeling method using 2-aminopyridine. Specifically, the compositional ratio can be calculated by fluorescence-

labeling an acid-hydrolyzed sample with 2-aminopyridine in accordance with a known method (*Agric. Biol. Chem.*, 55 (1), 283-284 (1991)) and carrying out HPLC analysis.

(2) Sugar chain structure analysis

The structure of the sugar chain of an antibody can be analyzed by a two-dimensional sugar chain mapping method (*Anal. Biochem.*, 171, 73 (1988), *Biochemical Experimentation Method* 23—*Method for Studying Glycoprotein Sugar Chains* (Gakkai Shuppan Center), edited by Reiko Takahashi (1989)). The two-dimensional sugar chain mapping method is a method in which the sugar chain structure is estimated, for example, by plotting the retention time or eluting position of the sugar chain by reverse phase chromatography as the X axis and the retention time or eluting position of the sugar chain by a normal phase chromatography as the Y axis, and comparing the results with those of known sugar chains.

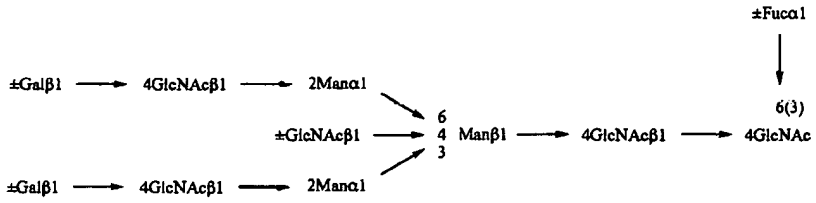
Specifically, the sugar chain is released from the antibody by hydrazinolysis of the antibody, fluorescence labeling of the sugar chain with 2-aminopyridine (hereinafter referred to as "PA") (*J. Biochem.*, 95, 197 (1984)) is carried out, and then the sugar chain is separated from an excess PA reagent and the like by gel filtration and subjected to reverse phase chromatography. Subsequently, each peak of the fractionated sugar chain is analyzed by normal phase chromatography. Based on these results, the sugar chain structure can be estimated by plotting the spots on a two-dimensional sugar chain map and comparing them with those of sugar chain standards (manufactured by Takara Shuzo) or a reference (*Anal. Biochem.*, 171, 73 (1988)).

In addition, the structure estimated by the two-dimensional sugar chain mapping method can be confirmed by mass spectrometry, such as MALDI-TOF-MS or the like, of each sugar chain.

2. Method for Controlling the Activity of Immunologically Functional Molecule

The method of the present invention for controlling the activity of an immunologically functional molecule is described below using immunoglobulin G (hereinafter referred to as "IgG") as an example.

The N-glycoside-linked sugar chain which binds to IgG is a biantennary composite sugar chain mainly having the following structure (hereinafter referred to as "biantennary").



The present invention also includes similar sugar chains wherein an acidic sugar, sialic acid, is further added to Gal of the non-reducing terminal of N-glycoside-linked sugar chain or a bisecting N-acetylglucosamine is added to the N-glycoside-linked sugar chain.

In an IgG type, an N-glycoside-linked sugar chain is bound to one position in the Fc region. Since an IgG type comprises two H chains, the Fc moiety is present at two positions in one antibody molecule. Accordingly, the sugar chain binding region is also present at two positions.

The activity of IgG changes depending on the number of N-glycoside-linked sugar chain in which fucose is not bound to N-acetylglucosamine, to be added to the above two sugar chain binding regions. That is, when the N-glycoside-linked sugar chain in which fucose is not bound to N-acetylglucosamine is added to at least one of the sugar chain binding regions, the activity of the immunologically functional molecule is increased. As an example, the degree of the activity of IgG will be as follows: F0 antibody>F1 antibody>F2 antibody, wherein the F0 antibody designates an antibody in which the N-glycoside-linked sugar chain in which fucose is not bound to N-acetylglucosamine is added to both of the two sugar chain binding regions; the F1 antibody designates an antibody in which the N-glycoside-linked sugar chain in which fucose is not bound to N-acetylglucosamine is added to one of the sugar chain binding regions; and the F2 antibody designates an antibody in which the N-glycoside-linked sugar chain in which fucose is bound to N-acetylglucosamine is added to both of the sugar chain binding regions.

The produced antibody may not always have a single sugar chain structure, and the F0 antibody, F1 antibody and F2 antibody may be present as a mixture when the presence or absence of fucose is taken into consideration. In order to control ADCC activity of the produced antibody, the sugar chain bound to the antibody is analyzed using the above method for analyzing the sugar chain of an immunologically functional molecule, and using the analyzed result as an index.

ADCC activity of the produced antibody may be promoted by increasing the existing ratio of the F1 antibody and F0 antibody. Specifically, the F1 antibody and F0 antibody may be purified, or expression in a host cell may be regulated in such a manner that the N-glycoside-linked sugar chain in which fucose is not bound to N-acetylglucosamine is added to the immunologically functional molecule.

ADCC activity of the produced antibody may be inhibited by increasing the existing ratio of the F2 antibody. Specifically, the F2 antibody may be purified, or expression in a host cell may be regulated in such a manner that the N-glycoside-linked sugar chain in which fucose is bound to N-acetylglucosamine is added to the immunologically functional molecule.

As described above, strength of the desired activity can be controlled by regulating the existing ratio of F0 antibody, F1 antibody and F2 antibody.

3. Method for Producing Immunologically Functional Molecule

A method for producing an immunologically functional molecule having an N-glycoside-linked sugar chain in which fucose is not bound to N-acetylglucosamine or an immunologically functional molecule having an N-glycoside-linked sugar chain in which fucose is bound to N-acetylglucosamine is described below.

In order to bind a desired sugar chain to an antibody, a peptide or a protein, it can be produced by introducing a gene encoding the antibody, peptide or protein of interest into a host cell and culturing the resulting cell. Alternatively, it can also be produced by introducing a gene encoding the antibody, peptide or protein of interest into an animal or a plant and culturing the resulting animal or plant.

The host cell, animal or plant useful in the production of an immunologically functional molecule having an N-glycoside-linked sugar chain in which fucose is not bound to N-acetylglucosamine may be any cell, animal or plant, so long as, for example, it has a low enzyme activity of adding fucose to the N-acetylglucosamine which binds to the Fc region of an antibody or does not have the enzyme activity. Examples of the cell which has a low enzyme activity of adding fucose to the N-acetylglucosamine that binds to the Fc region of the antibody or does not have the enzyme activity include a rat myeloma cell, YB2/3HL.P2.G11.16Ag.20 cell (ATCC CRL 1662 (hereinafter referred to as "YB2/0 cell")), and the like.

Also, a cell, animal or plant having a low or no enzyme activity related to an α1,6 bond may be made, for example, by deleting a gene encoding the α1,6 bond-related enzyme in the host cell, animal or plant or by adding a mutation to the gene to reduce or eliminate the enzyme activity, and may be used as a host cell, animal or plant. The α1,6 bond-related enzyme includes fucosyltransferases, and is preferably α1,6-fucosyltransferase (hereinafter referred to as "FUT8").

The host cell, animal or plant for use in the production of an immunologically functional molecule having an N-glycoside-linked sugar chain in which fucose is bound to N-acetylglucosamine may be any cell, animal or plant, so long as, for example, it has a high enzyme activity of adding fucose to the N-acetylglucosamine which binds to the Fc region of an antibody.

Also, a cell, animal or plant which has a high enzyme activity related to an α1,6 bond can be prepared by introducing a gene encoding the α1,6 bond-related enzyme in the host cell, animal or plant or by adding a mutation to the gene to increase the enzyme activity, and may be used as a host cell, animal or plant. The α1,6 bond-related enzyme includes fucosyltransferases, and is preferably FUT8.

Host cells may be any of bacteria, yeast, animal cells, insect cells, plant cells and the like, so long as they can express the gene of interest.

Examples of bacterial host cells include microorganisms belonging to the genus *Escherichia*, the genus *Serratia*, the genus *Bacillus*, the genus *Brevibacterium*, the genus *Corynebacterium*, the genus *Microbacterium*, the genus *Pseudomonas* and the like, such as *Escherichia coli* XL1-Blue, *Escherichia coli* XL2-Blue, *Escherichia coli* DH1, *Escherichia coli* MC1000, *Escherichia coli* KY3276, *Escherichia coli* W1485, *Escherichia coli* JM109, *Escherichia coli* HB101, *Escherichia coli* No. 49, *Escherichia coli* W3110, *Escherichia coli* NY49, *Escherichia coli* GI698, *Escherichia coli* TB1, *Serratia ficaria, Serratia fonticola, Serratia liquefaciens, Serratia marcescens, Bacillus subtilis, Bacillus amyloliquefaciens, Brevibacterium ammoniagenes, Brevibacterium immariophilum* ATCC 14068, *Brevibacterium saccharolyticum* ATCC 14066, *Brevibacterium flavum* ATCC 14067, *Brevibacterium lactofermentum* ATCC 13869, *Corynebacterium glutamicum* ATCC 13032, *Corynebacterium glutamicum* ATCC 13869, *Corynebacterium acetoacidophilum* ATCC 13870, *Microbacterium ammoniaphilum* ATCC 15354, *Pseudomonas putida, Pseudomonas* sp. D-0110 and the like.

Examples of yeast host cells includes microorganisms belonging to the genus *Saccharomyces*, the genus *Schizosaccharomyces*, the genus *Kluyveromyces*, the genus *Trichosporon*, the genus *Schwanniomyces*, the genus *Pichia*, the genus *Candida* and the like, such as *Saccharomyces cerevisiae, Schizosaccharomyces pombe, Kluyveromyces lactis, Trichosporon pullulans, Schwanniomyces alluvius, Candida utilis* and the like.

Examples of animal host cells include mouse myeloma cells, such as NS0 cell and SP2/0 cell; Chinese hamster ovary cells, such as CHO/dhfr$^-$ cell and CHO/DG44 cell; rat myeloma cells, such as YB2/0 cell and IR983F cell; monkey cells, such as COS cell; human myeloma cells, such as Namalwa cell; and the like. Preferably, Chinese hamster ovary cells, such as CHO/DG44 cell and the like, can be used.

Examples of insect host cells include *Spodoptera frugiperda* ovary cells, such as Sf9 and Sf21 (*Baculovirus Expression Vectors, A Laboratory Manual*, W.H. Freeman and Company, New York (1992)); a *Trichoplusia ni* ovary cell such as High 5 (manufactured by Invitrogen); and the like.

Examples of plant host cells include plant cells of tobacco, potato, tomato, carrot, soybean, rape, alfalfa, rice, wheat, barley and the like.

An immunologically functional molecule can be produced by culturing the obtained transformant in a medium to form and accumulate the immunologically functional molecule in the resulting culture, and then recovering it from the culture.

In addition, an immunologically functional molecule can also be produced by constructing a transgenic animal or plant and culturing the resulting animal or plant.

The animal or plant for the production of an immunologically functional molecule having an N-glycoside-linked sugar chain in which fucose is not bound to N-acetylglucosamine may be any animal or plant, so long as, for example, it has a low enzyme activity of adding fucose to the r-acetylglucosamine which binds to the Fc region of an antibody or does not have the enzyme activity.

Also, a knockout non-human animal or knockout plant having a low or no enzyme activity related to an α1,6 bond may be prepared by deleting a gene encoding the α1,6 bond-related enzyme in the animal or plant or by adding a mutation to the gene to reduce or eliminate the enzyme activity, and may be used. The α1,6 bond-related enzyme includes fucosyltransferases, and is preferably FUT8.

Any animal or plant may be used as an animal or plant for use in the production of an immunologically functional molecule having an N-glycoside-linked sugar chain in which fucose is bound to N-acetylglucosamine, so long as, for example, with regard to an antigen, it has a high enzyme activity of adding fucose to the N-acetylglucosamine which binds to the Fc region of the antibody.

Also, a transgenic non-human animal or transgenic plant which has a high enzyme activity related to an α1,6 bond may be prepared by introducing a gene encoding the α1,6 bond-related enzyme in the animal or plant or by adding a mutation to the gene to increase the enzyme activity, and may be used. The α1,6 bond-related enzyme includes fucosyltransferases, and is preferably FUT8.

The transgenic non-human animal can be obtained by directly injecting a desired gene into a fertilized egg (*Proc. Natl. Acad. Sci. USA.*, 77, 7380 (1980)).

The transgenic non-human animals include mouse, rat, rabbit, fowl, goat, cattle and the like.

Also, a transgenic non-human animal or knockout non-human animal having a desired gene can be obtained by introducing the desired gene into an embryonic stem cell and preparing the animal by an aggregation chimera method or

injection chimera method (*Manipulating the Mouse Embryo, A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press (1994); *Gene Targeting, A Practical Approach*, IRL Press at Oxford University Press (1993); *Biomaterial Series* 8, Gene Targeting, Preparation of mutation mouse using ES cell, Yodo-sha (1995)).

Examples of the embryonic stem cell include embryonic stem cells of mouse (*Nature*, 292, 154 (1981)), rat, fowl, pig, monkey, goat, cattle and the like.

In addition, the transgenic non-human animal or knockout non-human animal can also be prepared using a clonal technique in which a nucleus into which a desired gene is introduced is transplanted into an enucleated egg (*Science*, 280, 1256, (1998); *Science*, 278, 824, (1997)).

An immunologically functional molecule can be produced by introducing DNA encoding the immunologically functional molecule into an animal prepared by the above method to thereby form and accumulate the immunologically functional molecule in the animal, and then collecting the immunologically functional molecule from the animal. The immunologically functional molecule may be made to be formed and accumulated in the milk (Japanese Published Unexamined Patent Application No. 309192/88), egg or the like of the animal.

The method for producing a transgenic plant is described, for example, in a reference (*Biol. Chem.*, 380, 825 (1999)) and the like. The method for producing a knockout plant is described, for example, in a reference (*Plant Journal*, 11, 1195 (1997)).

Regarding the method for producing an immunologically functional molecule using a plant, the immunologically functional molecule can be produced, for example, by culturing a transgenic plant into which DNA encoding the immunologically functional molecule is introduced, in accordance with a known method (*Tissue Culture*, 20, (1994); *Tissue Culture*, 21, (1995); *Trends in Biotechnology*, 15, 45 (1997)) to thereby form and accumulate the immunologically functional molecule in the plant, and then collecting the immunologically functional molecule from the plant.

In addition, a gene-modified animal capable of producing an immunologically functional molecule having an N-glycoside-linked sugar chain in which fucose is not bound to N-acetylglucosamine or an immunologically functional molecule having an N-glycoside-linked sugar chain in which fucose is bound to N-acetylglucosamine can be obtained by crossing a transgenic non-human animal or knockout non-human animal of a fucosyltransferase, preferably FUT8, with a homologous but different line of the transgenic animal of a desired immunologically functional molecule. The crossing method includes natural crossing, in vitro fertilization and the like.

Also, it is possible to carry out mass production of the sugar chain by introducing a group of genes encoding the isolated enzymes and the like into yeast, *E. coli* and the like (*Nature Biotechnology*, 16, 847 (1998)). Further, the produced enzyme can be used in the modification of an antibody, peptide or protein with the sugar chain or production thereof.

In addition, a sugar chain which promotes the activity of an immunologically functional molecule, according to the present invention, can be substituted with a peptide (*J. Immunol.*, 160, 293 (1998)). Such a peptide has utility in the above method for utilizing sugar chains and is also excellent in view of convenience, because it can be easily fused with an immunologically functional molecule.

A method for producing an immunologically functional molecule having a promoted immunologically functional activity is described below. While a method for producing a humanized antibody is described herein as an example, other immunologically functional molecules can be prepared by the above-mentioned method or in accordance with a method similar thereto.

4. Method for Producing Humanized Antibody

(1) Construction of vector for humanized antibody expression

The vector for humanized antibody expression is an expression vector for use in an animal cell into which genes encoding the heavy chain (hereinafter referred to as "H chain") and light chain (hereinafter referred to as "L chain") C regions of a human antibody are inserted, and can be constructed by cloning each of genes encoding the H chain and L chain C regions of a human antibody into an expression vector for animal cell.

The C regions of a human antibody may be H chain and L chain C regions of a suitable human antibody, and examples include the C region of an IgG1 subclass of a human antibody H chain (hereinafter referred to as "hCγ1"), the C region of an κ class of a human antibody L chain (hereinafter referred to as "hCκ") and the like.

The genes encoding the H chain and L chain C regions of a human antibody may be a chromosomal DNA comprising exon and intron, or a cDNA.

The expression vector for animal cell may be any vector, so long as a gene encoding the C region of a human antibody can be inserted and expressed. Examples include pAGE107 (*Cytotechnology*, 3, 133 (1990)), pAGE103 (*J. Biochem.*, 101, 1307 (1987)), pHSG274 (*Gene*, 27, 223 (1984)), pKCR (*Proc. Natl. Acad. Sci. USA*, 78, 1527 (1981), pSG1 β d2-4 (*Cytotechnology*, 4, 173 (1990)) and the like. The promoter and enhancer to be used in the expression vector for animal cell include SV40 early promoter and enhancer (*J. Biochem.*, 101, 1307 (1987)), Moloney mouse leukemia virus LTR (*Biochem. Biophys. Res. Comun.*, 149, 960 (1987)), immunoglobulin H chain promoter (*Cell*, 41, 479 (1985)) and enhancer (*Cell*, 33, 717 (1983)) and the like.

The vector for humanized antibody expression may be any of a vector in which the antibody H chain and L chain are present on separate vectors or a vector in which they are present on the same vector (hereinafter referred to as "tandem vector"); however, a tandem vector for humanized antibody expression is preferable because such tandem humanized antibody expression vectors are easily constructed and introduced into an animal cell and expression amounts of the antibody H chain and L chain in the animal cell can be balanced (*J. Immunol. Methods*, 167, 271 (1994)).

The constructed vector for humanized antibody expression can be used for the expression of a human chimeric antibody and a human CDR-grafted antibody in animal cells.

(2) Preparation of cDNA Encoding V Region of Antibody Derived from Animal Other than Human

cDNA encoding the H chain and L chain V regions of an antibody derived from an animal other than human, such as a mouse antibody, can be obtained as described below.

cDNA is synthesized by extracting mRNA from a hybridoma cell capable of producing the mouse antibody of interest. The synthesized cDNA is cloned into a vector, such as a phage, a plasmid or the like, to prepare a cDNA library. A recombinant phage or recombinant plasmid containing a cDNA encoding the H chain V region and a recombinant phage or recombinant plasmid containing a cDNA encoding the L chain V region are respectively isolated from the library using a C region moiety or V region moiety of a known mouse antibody as the probe. Complete nucleotide sequences of the mouse antibody H chain and L chain V regions of interest on

the recombinant phage or recombinant plasmid are determined, and full amino acid sequences of the H chain and L chain V regions are deduced from the nucleotide sequences.

The animal other than human may be any animal, such as mouse, rat, hamsters rabbit or the like, so long as a hybridoma cell can be produced therefrom.

The method for preparing total RNA from a hybridoma cell includes a guanidine thiocyanate-cesium trifluoroacetate method (*Methods in Enzymol.*, 154, 3 (1987)). The method for preparing mRNA from total RNA includes an oligo (dT) immobilized cellulose column method (*Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Lab. Press, New York, 1989) and the like. Also, Fast Track mRNA Isolation Kit (manufactured by Invitrogen), Quick Prep mRNA Purification Kit (manufactured by Pharmacia) and the like can be exemplified as a kit for preparing mRNA from a hybridoma cell.

Examples of the method for synthesizing cDNA and preparing a cDNA library include conventional methods (*Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Lab. Press, New York, 1989, *Current Protocols in Molecular Biology*, Supplement 1-34), a method which uses a commercially available kit, such as Super Script™ Plasmid System for cDNA Synthesis and Plasmid Cloning (manufactured by GIBCO BRL or ZAP-cDNA Kit (manufactured by Stratagene) and the like.

The vector into which the cDNA synthesized using mRNA extracted from a hybridoma cell is inserted in preparing a cDNA library may be any vector, so long as the cDNA can be inserted. Examples include ZAP Express (*Strategies*, 5, 58 (1992)), pBluescript II SK(+) (*Nucleic Acids Research*, 17, 9494 (1989)), λzapII (manufactured by Stratagene), λgt10 and λgt11 (*DNA Cloning: A Practical Approach*, I, 49 (1985)), Lambda BlueMid (manufactured by Clontech), λEx-Cell and pT7T3 18U (manufactured by Pharmacia), pcD2 (*Mol. Cell. Biol.*, 3, 280 (1983)), pUC18 (*Gene*, 33, 103 (1985)) and the like.

The *E. coli* to be used for introducing the cDNA library constructed by a phage or plasmid vector may be any strain, so long as the cDNA library can be introduced, expressed and maintained. Examples include XL1-Blue MRF' (*Strategies*, 5, 81 (1992)), C600 (*Genetics*, 39, 440 (1954)), Y1088 and Y1090 (*Science*, 222, 778 (1983)), NM522 (*J. Mol. Biol.*, 166, 1 (1983)), K802 (*J. Mol. Biol.*, 16, 118 (1966)), JM105 (*Gene*, 38, 275 (1985)) and the like.

A colony hybridization or plaque hybridization method which uses an isotope- or fluorescence-labeled probe may be used for selecting a cDNA clone encoding the H chain and L chain V regions of an antibody derived from an animal other than human from the cDNA library (*Molecular Cloning; A Laboratory Manual*, Cold Spring Harbor Lab. Press, New York, 1989). Also, the cDNA encoding the H chain and L chain V regions can be prepared through polymerase chain reaction (hereinafter referred to as "PCR"; *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Lab. Press, New York, 1989; *Current Protocols in Molecular Biology*, Supplement 1-34) by preparing primers and using cDNA prepared from mRNA or a cDNA library as the template.

The nucleotide sequence of the cDNA selected by the above method can be determined by digesting the cDNA with appropriate restriction enzymes and the like, cloning the fragments into a plasmid, such as pBluescript SK(−) (manufactured by Stratagene) or the like, carrying out the reaction by a usually used nucleotide analyzing method, such as the dideoxy method of Sanger et al. (*Proc. Natl. Acad. Sci. USA*, 74, 5463 (1977)) or the like, and then analyzing the sequence using an automatic nucleotide sequence analyzer such as A.L.F. DNA sequencer (manufactured by Pharmacia) or the like.

Whether the obtained cDNA encodes the full amino acid sequence of the H chain and L chain V regions of the antibody containing a secretion signal sequence can be confirmed by estimating the full amino acid sequence of the H chain and L chain V regions from the determined nucleotide sequence and comparing it with full amino acid sequences of the H chain and L chain V regions of known antibodies (*Sequences of Proteins of Immunological Interest*, US Dept. Health and Human Services, 1991).

(3) Analysis of V Region Amino Acid Sequence of Antibody Derived from Animal Other than Human

Regarding the full amino acid sequence of the H chain and L chain V regions of the antibody comprising a secretion signal sequence, the length and N-terminal amino acid sequence of the secretion signal sequence can be estimated and subgroups to which they belong can be known by comparing it with full amino acid sequences of the H chain and L chain V regions of known antibodies (*Sequences of Proteins of Immunological Interest*, US Dept. Health and Human Services, 1991). Each CDR amino acid sequence of the H chain and L chain V regions can also be identified by comparing it with amino acid sequences of the H chain and L chain V regions of known antibodies (Sequences of Proteins of Immunological Interest, US Dep. Health and Human Services, 1991)

(4) Construction of Human Chimeric Antibody Expression Vector

A human chimeric antibody expression vector can be constructed by cloning cDNA encoding the H chain and L chain V regions of an antibody derived from an animal other than human into the upstream of a gene encoding the H chain and L chain C regions of a human antibody on the humanized antibody expression vector described in the item 4(1). For example, a human chimeric antibody expression vector can be produced by connecting cDNAs encoding the H chain and L chain V regions derived from an antibody of an animal other than human respectively with a synthetic cDNA which comprises a 3'-terminal side nucleotide sequences of the H chain and L chain V regions of an antibody derived from an animal other than human, a 5'-terminal side nucleotide sequence of the H chain and L chain C regions derived from a human antibody and appropriate restriction enzyme recognizing sequences on both termini, and cloning them into the upstream of a gene encoding the H chain and L chain C regions of a human antibody on the humanized antibody expression vector described in the item 4(1) in such a manner that they are expressed in a suitable form.

(5) Construction of cDNA Encoding V Region of Human CDR-Grafted Antibody

The cDNA encoding the H chain and L chain V regions derived from a human CDR-grafted antibody can be obtained as described below. First, the amino acid sequence of the framework (hereinafter referred to as "FR") of the H chain and L chain V regions of a human antibody for grafting CDR of the H chain and L chain V regions of an antibody derived from an animal other than human is selected. Any sequence can be used as the amino acid sequence of FR of the H chain and L chain v regions of a human antibody, so long as it is derived from a human antibody. For example, amino acid sequences of FR of the H chain and L chain V regions of human antibodies registered at a data base, such as Protein Data Bank or the like, an amino acid sequence common in each subgroup of FR of the H chain and L chain V regions of human antibodies (*Sequences of Proteins of Immunological*

Interest, US Dept. Health and Human Services, 1991) and the like can be used, but in order to prepare a human CDR-grafted antibody having sufficient activity, it is desirable to select an amino acid sequence having a high homology (at least 60% or more) with the objective amino acid sequence of the H chain and L chain v regions of an antibody derived from an animal other than human.

Next, the objective amino acid sequence of CDR of the H chain and L chain V regions of an antibody derived from an animal other than human is grafted to the selected amino acid sequence of FR of the H chain and L chain v regions a human antibody, and amino acid sequences of the H chain and L chain V regions of the human CDR-grafted antibody are designed. Taking the codon usage found in nucleotide sequence of the antibody gene (*Sequences of Proteins of Immunological Interest*, US Dept. Health and Human Services, 1991) into consideration, the designed amino acid sequences are converted into DNA sequences and the DNA sequences encoding the amino acid sequences of the H chain and L chain V regions of the human CDR-grafted antibody are designed. Based on the designed DNA sequences, several synthetic DNA fragments having a length about 100 bases are synthesized, and PCR is carried out using these fragments. In this case, based on the reaction efficiency in the PCR and the length of DNA which can be synthesized, it is desirable to design 6 synthetic DNA fragments for each of the H chain and L chain.

Also, cloning into the vector for humanized antibody expression constructed in the item 4(1) can be easily carried out by introducing appropriate restriction enzyme recognizing sequences into 5'-termini of the synthetic DNA positioned at both termini. After the PCR, a plasmid having a DNA sequence encoding the amino acid sequence of the H chain and L chain v regions of the desired human CDR-grafted antibody is obtained by cloning the amplified product into plasmid, such as pBluescript SK(−) (manufactured by Stratagene) or the like, and determining the nucleotide sequence by the method described in the item 4(2).

(6) Modification of Amino Acid Sequence of V Region of Human CDR-Grafted Antibody

It is known that when only the CDR of the H chain and L chain V regions of an antibody derived from an animal other than human of interest is simply grafted to the FR of the H chain and L chain V regions of a human antibody, the antigen binding activity of the human CDR-grafted antibody is reduced in comparison with the activity of the original antibody derived from an animal other than human (*BIO/TECHNOLOGY*, 9, 266 (1991)). As the cause of this, it is considered that not only amino acid sequences of CDR but also several amino acid sequences of FR in the H chain and L chain V regions of the original antibody derived from an animal other than the human are directly or indirectly related to the antigen binding activity, and these amino acid residues are changed into different amino acid residues of FR of the H chain and L chain v regions of the human antibody accompanied by the CDR grafting. In order to resolve this problem, in human CDR-grafted antibodies, attempts have been made to identify, among amino acid sequences of FR of the H chain and L chain V regions of a human antibody, an amino acid residue directly related to the binding to the antibody, an amino acid residue interacting with an amino acid residue of CDR and/or an amino acid residue which keeps three-dimensional structure of the antibody and is directly related to its binding to the antigen, and to increase the reduced antigen binding activity by changing these amino acid residues into amino acid residues found in the original antibody derived from an animal other than human (*BIO/TECHNOLOGY*, 9, 266 (1991)).

In preparing a human CDR-grafted antibody, it is preferable to efficiently identify these FR amino acid residues related to the antigen binding activity, such that construction and analysis of the three-dimensional structure of antibodies is preferably carried out using an x-ray crystal analysis (*J. Mol. Biol.*, 112, 535 (1977)), computer modeling (*Protein Engineering*, 7, 1501 (1994)) and the like. Although information on these three-dimensional structures of antibodies has provided useful information for the preparation of human CDR-grafted antibodies, a method for producing a human CDR-grafted antibody applicable to every antibody has not yet been established. It is preferable therefore to carry out various trial and error experiments on individual antibody, e.g., by preparing several modified products thereof and examining their correlation to the respective antigen binding activities.

Modification of the amino acid residues of the FR of the H chain and L chain V regions of a human antibody can be achieved by the PCR described in the item 4(5) using synthetic DNA for further modification. Achievement of the objective modification is confirmed by determining nucleotide sequence of the amplified fragment after PCR, by the method described in the item 4(2).

(7) Construction of Human CDR-Grafted Antibody Expression Vector

A human CDR-grafted antibody expression vector can be constructed by cloning the cDNA encoding the H chain and L chain V regions of human CDR-grafted antibody constructed in the items 4(5) and 4(6) into the upstream of the gene encoding the H chain and L chain C regions of a human antibody in the humanized antibody expression vector described in the item 4(1). For example, among the synthetic DNA fragments used in constructing the H chain and L chain v regions of human CDR-grafted antibody in (5) and (6) of the item 4, appropriate restriction enzyme recognizing sequences are introduced into 5'-termini of a synthetic DNA fragment positioned at both termini, and cloned into the upstream of the gene encoding the H chain and L chain C regions of a human antibody in the vector for humanized antibody expression described in the item 4(1), in such a manner that they can be expressed in a suitable form to thereby construct a human CDR-grafted antibody expression vector.

(8) Stable Production of Humanized Antibody

A transformant capable of producing a humanized antibody stably can be obtained by introducing the humanized antibody expression vector described in the items 4(4) and 4(7) into an appropriate animal cell.

The method for introducing an expression vector into an animal cell includes an electroporation method (Japanese Published unexamined Patent Application No. 257891/90, Cytotechnology, 3, 133 (1990)) and the like.

The animal cell into which a humanized antibody expression vector is introduced may be any cell, so long as it is an animal cell which can produce the humanized anti-body. Preferred examples include a cell which has a low enzyme activity of adding fucose to N-acetylglucosamine to be bound to the Fc region of the produced antibody and a cell which has no such enzyme activity.

The cell which has a low enzyme activity of adding fucose to N-acetylglucosamine to be bound to the Fc region of the antibody or has no such enzyme activity is a cell having less or no enzymes related to the α1,6-bond. Examples include a cell which has a low fucosyltransferase activity, preferably FUT8 activity, and a cell which has no such activity.

Examples of the cell which has a low enzyme activity of adding fucose to N-acetylglucosamine to be bound to the Fc region of the antibody or has no enzyme activity include a rat

myeloma cell, YB2/0 cell, and the like A cell in which a gene involved in the α1,6 bond-related enzyme is deleted or the enzyme activity is reduced or eliminated by adding a mutation to the gene can also be used as an antibody producing cell.

Specific examples include mouse myeloma cells, such as NS0 cell and SP2/0 cell; Chinese hamster ovary cells, such as CHO/dhfr⁻ cell and CHO/DG44 cell; rat myeloma cells, such as YB2/0 cell and IR983F cell; human myeloma cells, such as Namalwa cell; and the like. Preferably, Chinese hamster ovary cells, such as CHO/DG44 cell and the like, can be used.

After introduction of the expression vector, the transformant capable of stably producing the humanized antibody can be selected using a medium for animal cell culture containing a drug, such as G418 sulfate (hereinafter referred to as "G418"; manufactured by SIGMA) or the like by the method disclosed in Japanese Published Unexamined Patent Application No. 257891/90. The medium for animal cell culture includes RPMI 1640 medium (manufactured by Nissui Pharmaceutical), GIT medium (manufactured by Nippon Pharmaceutical), EX-CELL 302 medium (manufactured by JRH), IMDM medium (manufactured by GIBCO BRL), Hybridoma-SFM medium (manufactured by GIBCO BRL), or a medium prepared by adding various additives, such as fetal bovine serum (hereinafter referred to as "FBS") and the like, to each of these media, and the like. The humanized antibody can be produced by culturing the obtained transformant in a medium, and accumulated in a culture supernatant. The produced amount and antigen binding activity of the humanized antibody in the culture supernatant can be measured by enzyme-linked immunosorbent assay (hereinafter referred to as "ELISA", *Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory*, Chapter 14, 1998; *Monoclonal Antibodies: Principles and Practice*, Academic Press Limited, 1996) and the like Also, production of the humanized antibody by the transformant can be increased using a DHFR gene amplification system or the like by the method disclosed in Japanese Published Unexamined Patent Application No. 257891/90.

The humanized antibody can be purified from a transformant-containing culture supernatant using a protein A column (*Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, Chapter 8, 1988, *Monoclonal Antibodies: Principles and Practice*, Academic Press Limited, 1996). Also, other purification methods generally used for the purification of protein can be used. For example, it can be purified by a combination of gel filtration, ion exchange chromatography, ultrafiltration and the like. The molecular weight of the H chain, L chain or whole antibody molecule of the purified humanized antibody can be measured by polyacrylamide gel electrophoresis (hereinafter referred to as "SDS-PAGE"; *Nature*, 227, 580 (1970)), Western blotting method (*Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, Chapter 12, 1988; *Monoclonal Antibodies: Principles and Practice*, Academic Press Limited, 1996) and the like.

An antibody production method has been shown in the above using an animal cell as the host, and as described in the above item 3, it can also be produced by a bacterium, a yeast, an insect cell, a plant cell, an animal or a plant.

(9) Activity Evaluation of Humanized Antibody

The activity of the purified humanized antibody to bind to an antigen or to an antigen-positive cultured cell line can be measured by the ELISA and fluorescence antibody method (*Cancer Immunol. Immunother.*, 36, 373 (1993)) and the like. The cytotoxic activity for antigen-positive cultured cell lines can be evaluated by measuring its CDC activity, ADCC activity and the like (*Cancer Immunol. Immunother.*, 36, 373 (1993)). In addition, safety and therapeutic effects of the humanized antibody in humans can be evaluated using an appropriate model of an animal species relatively close to human, such as *Macaca faseicularis* or the like,

5. Application Method of Immunologically Functional Molecule

As shown in the humanized antibody described in the above item 4, an antibody having high ADCC activity is useful in the prevention and treatment of various diseases including a cancer, an allergy, a cardiovascular disease and a viral or bacterial infection.

In cancer, namely a malignant tumor, cancer cells proliferate. Conventional anticancer agents have a characteristic in inhibiting proliferation of cancer cells. On the other hand, since an antibody having high ADCC activity can treat cancers by injuring proliferation of the cancer cells through its cytotoxic effect, it is more effective as a therapeutic drug than conventional anticancer agents.

Since the allergic reaction is induced by the release of a mediator molecule from immune cells, the allergic reaction can be inhibited by removing the immune cells using an antibody having high ADCC activity.

The cardiovascular disease includes arteriosclerosis and the like. Arteriosclerosis is currently treated by balloon catheter, but cardiovascular diseases can be prevented and treated by inhibiting proliferation of arterial cells in re-stricture after the treatment, by using an antibody having high ADCC activity.

Various diseases including viral or bacterial infections can be prevented and treated by inhibiting proliferation of the virus- or bacterium-infected cells using an antibody having high ADCC activity.

Also, an antibody having inhibited ADCC activity is useful in the prevention and treatment of autoimmune diseases. The antibody having inhibited ADCC activity is also useful in the prevention and treatment of autoimmune diseases from the viewpoint of suppressing the immune response promoted in autoimmune diseases.

The medicament containing the antibody according to the present invention can be administered as a therapeutic drug alone, but generally, it is desirable to provide it as a pharmaceutical preparation produced by an appropriate method well known in the technical field of manufacturing pharmacy, by mixing it with one or more pharmaceutically acceptable carriers.

It is desirable to select a route of administration which is most effective in carrying out a treatment. Examples include oral administration and parenteral administration, such as buccal, airway, rectal, subcutaneous, intramuscular, intravenous or the like. In an antibody preparation, intravenous administration is preferred.

The dosage form includes sprays, capsules, tablets, granules, syrups, emulsions, suppositories, injections, ointments, tapes and the like.

Liquid preparations, such as emulsions and syrups, can be produced using, as additives, water; saccharides, such as sucrose, sorbitol, fructose etc.; glycols, such as polyethylene glycol, propylene glycol, etc.; oils, such as sesame oil, olive oil, soybean oil, etc.; antiseptics, such as p-hydroxybenzoic acid esters, etc., flavors, such as strawberry flavor, peppermint, etc.; and the like.

Capsules, tablets, powders, granules and the like can be produced using, as additive, fillers, such as lactose, glucose, sucrose, mannitol, etc., disintegrating agents, such as starch, sodium alginate, etc.; lubricants, such as magnesium stearate, talc, etc.; binders, such as polyvinyl alcohol, hydroxypropy-

lcellulose, gelatin, etc.; surfactants, such as fatty acid ester, etc.; plasticizers, such as glycerol, etc.; and the like.

Examples of the pharmaceutical preparation suitable for parenteral administration include injections, suppositories, sprays and the like.

Injections may be prepared using a carrier, such as a salt solution, a glucose solution, a mixture of both thereof or the like. Alternatively, powdered injections can be prepared by freeze-drying the humanized antibody in the usual way and adding sodium chloride thereto.

Suppositories may be prepared using a carrier such as cacao butter, a hydrogenated fat or carboxylic acid.

Also, sprays may be prepared using the compound as such or using a carrier which does not stimulate the buccal or airway mucous membrane of the patient and can facilitate absorption of the compound by dispersing it as fine particles.

Examples of the carrier include lactose, glycerol and the like. Depending on the properties of the compound and the carrier to be used, it is possible to produce pharmaceutical preparations such as aerosols, dry powders and the like. In addition, the components exemplified as additive agents for oral preparations can also be added to these parenteral preparations.

Although the clinical dose or the frequency of administration varies depending on the objective therapeutic effect, administration method, treating period, age, body weight and the like, it is usually from 10 μg/kg to 20 mg/kg per day and per adult.

Also, regarding the method for examining antitumor effect of the antibody on various tumor cells, in vitro tests include CDC activity measuring method, ADCC activity measuring method and the like, and in vivo tests include an antitumor experiment using a tumor system in an experimental animal such as a mouse or the like.

CDC activity and ADCC activity measurements and antitumor experiments can be carried out in accordance with the methods described in references (*Cancer Immunology Immunotherapy*, 36, 373 (1993); *Cancer Research*, 54, 1511 (1994)) and the like.

6. Method for Promoting or Inhibiting Activity of Immunologically Functional Molecule

The activity of an immunologically functional molecule can be promoted by producing an antibody, peptide or protein to which a fucose-free sugar chain is bound by the above method.

When the immunologically functional molecule having the promoted activity is administered to the living body, various immune cells including cells such as killer cells, natural killer cells, activated macrophages and the like as effector cells relating to the ADCC activity are activated in the living body, so that it becomes possible to control various immune responses.

Also, the activity of an immunologically functional molecule can be inhibited by producing an antibody, a peptide or a protein to which a fucose-existing sugar chain is bound by the above method.

When the immunologically functional molecule having the inhibited activity is administered to the living body, activities of various immune cells involved in the ADCC activity are weakened in the living body, so that it becomes possible to control various immune responses.

Examples of the present invention are shown below, but the scope of the present invention is not limited thereto.

BRIEF EXPLANATION OF THE DRAWINGS

FIG. **1** is a graph showing electrophoresis patterns of SDS-PAGE of five purified anti-GD3 chimeric antibodies (using gradient gel from 4 to 15%). The upper drawing and the lower drawing show a result of the electrophoresis under non-reducing conditions and that under reducing conditions, respectively. Lanes 1 to 7 show an electrophoresis pattern of high molecular weight markers, an electrophoresis pattern of YB2/0-GD3 chimeric antibody, an electrophoresis pattern of CHO/DG44-GD3 chimeric antibody, an electrophoresis pattern of SP2/0-GD3 chimeric antibody, an electrophoresis pattern of NS0-GD3 chimeric antibody (302), an electrophoresis pattern of NS0-GD3 chimeric antibody (GIT), and an electrophoresis pattern of low molecular weight markers, respectively.

FIG. **2** is a graph showing the activity of five purified anti-GD3 chimeric antibodies to bind to GD3, measured by changing the antibody concentration. The axis of ordinates and the axis of abscissas show the binding activity with GD3 and the antibody concentration, respectively. Open circles, closed circles, open squares, closed squares, and open triangles show the activity of YB2/0-GD3 chimeric antibody, the activity of CHO/DG44-GD3 chimeric antibody, the activity of SP2/0-GD3 chimeric antibody, the activity of NS0-GD3 chimeric antibody (302), and the activity of NS0-GD3 chimeric antibody (GIT), respectively.

FIG. **3** is a graph showing the ADCC activity of five purified anti-GD3 chimeric antibodies for a human melanoma cell line G-361. The axis of ordinates and the axis of abscissas show the cytotoxic activity and the antibody concentration, respectively. Open circles, closed circles, open squares, closed squares, and open triangles show the activity of YB2/0-GD3 chimeric antibody, the activity of CHO/DG44-GD3 chimeric antibody, the activity of SP2/0-GD3 chimeric antibody, the activity of NS0-GD3 chimeric antibody (302), and the activity of NS0-GD3 chimeric antibody (GIT), respectively.

FIG. **4** is a graph showing electrophoresis patterns of SDS-PAGE of three purified anti-hIL-5Rα CDR-grafted antibodies (using gradient gel from 4 to 15%). The upper drawing and the lower drawing show results of the electrophoresis carried out under non-reducing conditions and those under reducing conditions, respectively. Lanes 1 to 5 show an electrophoresis pattern of high molecular weight markers, an electrophoresis pattern of YB2/0-hIL 5RCDR antibody, an electrophoresis pattern of CHO/d-hIL-5RCDR antibody, an electrophoresis pattern of NS0-hIL-5RCDR antibody, and an electrophoresis pattern of low molecular weight markers, respectively.

FIG. **5** is a graph showing the activity of three purified anti-hIL-5Rα CDR-grafted antibodies to bind to hIL-5Rα, measured by changing the antibody concentration. The axis of ordinates and the axis of abscissas show the binding activity with hIL-5Rα and the antibody concentration, respectively. Open circles, closed circles, and open squares show the activity of YB2/0-hIL-5RαCDR antibody, the activity of CHO/d-hIL5RCDR antibody, and the activity of NS0-hIL-5RCDR antibody, respectively.

FIG. **6** is a graph showing the ADCC activity of three purified anti-hIL-5Rα CDR-grafted antibodies for an hIL-5R expressing mouse T cell line CTLL-2(h5R). The axis of ordinates and the axis of abscissas show the cytotoxic activity and the antibody concentration, respectively. Open circles, closed circles, and open squares show the activity of YB2/0-hIL-

5Rα CDR antibody, the activity of CHO/d-hIL-5RCDR antibody, and the activity of NS0-hIL-5RCDR antibody, respectively.

FIG. **7** is a graph showing the inhibition activity of three purified anti-hIL-5Rα CDR-grafted antibodies in an hIL-5-induced eosinophil increasing model of *Macaca faseicularis*. The axis of ordinates and the axis of abscissas show the number of eosinophils in peripheral blood and the number of days (the day of the commencement of antibody and hIL-5 administration was defined as 0 day). Results in the antibody non-administration group are shown by 101 and 102, results in the YB2/0-hIL-5RCDR antibody administered group are shown by 301, 302 and 303, results in the CHO/d-hIL-5RCDR antibody administered group are shown by 401, 402 and 403, and results in the NS0-hIL-5RCDR antibody administered group are shown by 501, 502 and 503.

FIG. **8** is a graph showing an elution pattern of reverse phase HPLC elution of a PA-treated sugar chain (left side), and an elution pattern obtained by treating the PA-treated sugar chain with α-L-fucosidase and then analyzed by reverse phase HPLC (right side), of the purified anti-hIL-5Rα CDR-grafted antibody produced by YB2/0 (upper side) and the purified anti-hIL-5Rα CDR-grafted antibody produced by NS0 (lower side). The axis of ordinates and the axis of abscissas show relative the fluorescence intensity and the elution time, respectively.

FIG. **9** is a graph showing an elution pattern obtained by preparing a PA-treated sugar chain from the purified anti-hIL-5Rα CDR-grafted antibody produced by CHO/d cell and analyzing it by reverse phase HPLC. The axis of ordinates and the axis of abscissas show the relative fluorescence intensity and the elution time, respectively.

FIG. **10** is a graph showing the GD3-binding activity of non-adsorbed fraction and a part of adsorbed fraction, measured by changing the antibody concentration. The axis of ordinates and the axis of abscissas show the binding activity with GD3 and the antibody concentration, respectively. Closed circles and open circles show the non-adsorbed fraction and a part of the adsorbed fraction, respectively. The lower graph shows the ADCC activity of non-adsorbed fraction and a part of adsorbed fraction for a human melanoma line G-361. The axis of ordinates and the axis of abscissas show the cytotoxic activity and the antibody concentration, respectively. Closed circles and open circles show the non-adsorbed fraction and a part of the adsorbed fraction, respectively.

FIG. **11** is a graph showing elution patterns obtained by analyzing PA-treated sugar chains prepared from non-adsorbed fraction and a part of adsorbed fraction by a reverse HPLC. The left side drawing and the right side drawing show an elution pattern of the non-adsorbed fraction and an elution pattern of a part of the adsorbed fraction, respectively. The axis of ordinates and the axis of abscissas show the relative fluorescence strength and the elution time, respectively.

FIG. **12** is a graph showing the amount of FUT8 transcription product by respective host cell lines when a rat FUT8 sequence is used as the standard internal control. Closed circle and open circles show the result when CHO cell line was used and the result when YB2/0 cell line was used, as the host cell, respectively.

BEST MODE FOR OUT THE INVENTION

Example 1

Production of Anti-Ganglioside GD3 Human Chimeric Antibody:

1. Construction of Tandem Expression Vector, pChiLHGM4, for Anti-Ganglioside GD3 Human Chimeric Antibody

A plasmid, pChi641LGM40, was constructed by ligating a fragment of about 4.03 kb containing an L chain cDNA, obtained by digesting an L chain expression vector, pChi641LGM4 (*J. Immunol. Methods*, 167, 271 (1994)) for anti-ganglioside GD3 human chimeric antibody (hereinafter referred to as "anti-GD3 chimeric antibody") with restriction enzymes, MuII (manufactured by Takara Shuzo) and SalI (manufactured by Takara Shuzo), with a fragment of about 3.40 kb containing a G418-resistant gene and a splicing signal, obtained by digesting an expression vector pAGE107 (*Cytotechnology*, 3, 133 (1990)) for animal cell with restriction enzymes, MuII (manufactured by Takara Shuzo) and SalI (manufactured by Takara Shuzo), using DNA Ligation Kit (manufactured by Takara Shuzo), and then transforming *E. coli* HB101 (*Molecular Cloning; A Laboratory Manual*, Cold Spring Harbor Lab. Press, New York, 1989) with the ligated product using DNA Ligation Kit (manufactured by Takara Shuzo).

Next, a fragment of about 5.68 kb containing an L chain cDNA, obtained by digesting the constructed plasmid pChi641LGM40 with a restriction enzyme, ClaI (manufactured by Takara Shuzo), blunt-ending it using DNA Blunting Kit (manufactured by Takara Shuzo) and further digesting it with MluI (manufactured by Takara Shuzo), was ligated with a fragment of about 8.40 kb containing an H chain cDNA, obtained by digesting an anti-GD3 chimeric antibody H chain expression vector, pChi641HGM4 (*J. Immunol. Methods*, 167, 271 (1994)) with a restriction enzyme, XhoI (manufactured by Takara Shuzo), blunt-ending it using DNA Blunting Kit (manufactured by Takara Shuzo) and further digesting it with MluI (manufactured by Takara Shuzo), using DNA Ligation Kit (manufactured by Takara Shuzo), and then *E. coli* HB101 (*Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Lab. Press, New York, 1989) was transformed with the ligated product to thereby construct a tandem expression vector, pChi641LHGM4, for anti-GD3 chimeric antibody.

2. Production of Cells Stably Producing Anti-GD3 Chimeric Antibody

Using the tandem expression vector, pChi641LHGM4, for anti-GD3 chimeric antibody constructed in the item 1 of Example 1, cells capable of stably producing an anti-GD3 chimeric antibody were prepared as described below.

(1) Production of Producer Cell Using Rat Myeloma YB2/0 Cell

After introducing 5 μg of the anti-GD3 chimeric antibody expression vector, pChi641LHGM4, into 4×10^6 cells of rat myeloma YB2/0 by electroporation (*Cytotechnology*, 3, 133 (1990)), the cells were suspended in 40 ml of RPMI1640-FBS (10) (RPMI1640 medium containing 10% FBS (manufactured by GIBCO BRL)) and dispensed in 200 μl/well into a 96 well culture plate (manufactured by Sumitomo Bakelite). Twenty-four hours after culturing at 37° C. in a 5% CO_2 incubator, G418 was added to a concentration of 0.5 mg/ml, followed by culturing for 1 to 2 weeks. The culture supernatant was recovered from respective well in which colonies of transformants showing G418 resistance were formed and growth of colonies was observed, and the antigen binding activity of the anti-GD3 chimeric antibody in the supernatant was measured by the ELISA shown in the item 3 of Example 1.

Regarding the transformants in wells in which production of the anti-GD3 chimeric antibody was observed in culture supernatants, in order to increase amount of the antibody

production using a DHFR gene amplification system, each of them was suspended in the RPMI1640-FBS(10) medium containing 0.5 mg/ml of G418 and 50 nM DHFR inhibitor, methotrexate (hereinafter referred to as "MTX"; manufactured by SIGMA) to give a density of 1 to 2×10^5 cells/ml, and the suspension was dispensed in 2 ml into wells of a 24 well plate (manufactured by Greiner). Transformants showing 50 nM MTX resistance were induced by culturing at 37° C. for 1 to 2 weeks in a 5% CO_2 incubator. The antigen binding activity of the anti-GD3 chimeric antibody in culture supernatants in wells in which growth of transformants was observed was measured by the ELISA shown in the item 3 of Example 1. Regarding the transformants in wells in which production of the anti-GD3 chimeric antibody was observed in culture supernatants, the MTX concentration was increased to 100 nM and then to 200 nM, and a transformant capable of growing in the RPMI1640-FBS(10) medium containing 0.5 mg/ml of G418 and 200 nM MTX and of producing the anti-GD3 chimeric antibody in a large amount was finally obtained by the same method as described above. The obtained transformant was made into a single cell (cloning) by limiting dilution twice.

The obtained anti-GD3 chimeric antibody-producing transformed cell clone 7-9-51 has been deposited on Apr. 5, 1999, as FERM BP-6691 in National Institute of Bioscience and Human Technology, Agency of Industrial Science and Technology (Higashi 1-1-3, Tsukuba, Ibaraki, Japan).

(2) Production of Producer Cell Using CHO/DG44 Cell

After introducing 4 μg of the anti-GD3 chimeric antibody expression vector, pChi641LHGM4, into 1.6×10^6 cells of CHO/DG44 by electroporation (*Cytotechnology*, 3, 133 (1990)), the cells were suspended in 10 ml of IMDM-FBS (10) (IMDM medium containing 10% FBS and 1× concentration of HT supplement (manufactured by GIBCO BRL)) and dispensed in 200 μl/well into a 96 well culture plate (manufactured by Iwaki Glass). Twenty-four hours after culturing at 37° C. in a 5% CO_2 incubator, G418 was added to a concentration of 05 mg/ml, followed by culturing for 1 to 2 weeks. The culture supernatant was recovered from respective well in which colonies of transformants showing G418 resistance were formed and growth of colonies was observed, and the antigen binding activity of the anti-GD3 chimeric antibody in the supernatant was measured by the ELISA shown in the item 3 of Example 1.

Regarding the transformants in wells in which production of the anti-GD3 chimeric antibody was observed in culture supernatants, in order to increase amount of the antibody production using a DHFR gene amplification system, each of them was suspended in an IMDM-dFBS(10) medium (IMDM medium containing 10% dialyzed fetal bovine serum (hereinafter referred to as "dFBS"; manufactured by GIBCO BRL)) containing 0.5 mg/ml of G418 and 10 nM MTX to give a density of 1 to 2×10^5 cells/ml, and the suspension was dispensed in 0.5 ml into wells of a 24 well plate (manufactured by Iwaki Glass). Transformants showing 10 nM MTX resistance were induced by culturing at 37° C. for 1 to 2 weeks in a 5% CO_2 incubator. Regarding the transformants in wells in which their growth was observed, the MTX concentration was increased to 100 ml, and a transformant capable of growing in the IMDM-dFBS(10) medium containing 0.5 mg/ml of G418. and 100 nM MTX and of producing the anti-GD3 chimeric antibody in a large amount was finally obtained by the same method as described above The obtained transformant was made into a single cell (cloning) by limiting dilution twice.

(3) Production of Producer Cell Using Mouse Myeloma NS0 Cell

After introducing 5 μg of the anti-GD3 chimeric antibody expression vector pChi641LHGM4 into 4×10^6 cells of mouse myeloma NS0 by electroporation (*Cytotechnology*, 3, 133 (1990)), the cells were suspended in 40 ml of EX-CELL302-FBS(10) (EX-CELL302 medium containing 10% FBS and 2 mM L-glutamine (hereinafter referred to as "L-Gln"; manufactured by GIBCO BRL)) and dispensed in 200 μl/well into a 96 well culture plate (manufactured by Sumitomo Bakelite). Twenty-four hours after culturing at 37° C. in a 5% CO_2 incubator, G418 was added to a concentration of 0-5 mg/ml, followed by culturing for 1 to 2 weeks. The culture supernatant was recovered from respective well in which colonies of transformants showing G418 resistance were formed and growth of colonies was observed, and the antigen binding activity of the anti-GD3 chimeric antibody in the supernatant was measured by the ELISA shown in the item 3 of Example 1.

Regarding the transformants in wells in which production of the anti-GD3 chimeric antibody was observed in culture supernatants, in order to increase amount of the antibody production using a DHFR gene amplification system, each of them was suspended in an EX-CELL302-dFBS(10) medium (EX-CELL302 medium containing 10% dFBS and 2 mM L-Gln) containing 0.5 mg/ml of G418 and 50 nM MTX to give a density of 1 to 2×10^5 cells/ml, and the suspension was dispensed in 2 ml into wells of a 24 well plate (manufactured by Greiner). Transformants showing 50 nM MTX resistance were induced by culturing at 37° C. for 1 to 2 weeks in a 5% CO_2 incubator. The antigen binding activity of the anti-GD3 chimeric antibody in culture supernatants in wells in which growth of transformants was observed was measured by the ELISA shown in the item 3 of Example 1. Regarding the transformants in wells in which production of the anti-GD3 chimeric antibody was observed in culture supernatants, the MTX concentration was increased to 100 nM and then to 200 nM, and a transformant capable of growing in the Ex-CELL302-dFBS(10) medium containing 0.5 mg/ml of G418 and 200 nM MTX and of producing the anti-GD3 chimeric antibody in a large amount was finally obtained by the same method as described above. The obtained transformant was made into a single cell (cloning) by limiting dilution twice.

3. Measurement of Binding Activity of Antibody to GD3 (ELISA)

The binding activity of the antibody to GD3 was measured as described below.

In 2 ml of ethanol solution containing 10 μg of dipalmitoylphosphatidylcholine (manufactured by SIGMA) and 5 μg of cholesterol (manufactured by SIGMA), 4 nmol of GD3 was dissolved. Into each well of a 96 well plate for ELISA (manufactured by Greiner), 20 μl of the solution (40 pmol/well in final concentration) was dispensed, followed by air-drying, 1% bovine serum albumin (hereinafter referred to as "BSA"; manufactured by SIGMA)-containing PBS (hereinafter referred to as "1% BSA-PBS") was dispensed in 100 μl/well, and then the reaction was carried out at room temperature for 1 hour for blocking remaining active groups. After discarding 1% BSA-PBS, a culture supernatant of a transformant or a diluted solution of a human chimeric antibody was dispensed in 50 μl/well to carry out the reaction at room temperature for 1 hour. After the reaction, each well was washed with 0.05% Tween 20 (manufactured by Wako Pure Chemical Industries)-containing PBS (hereinafter referred to as "Tween-PBS"), a peroxidase-labeled goat anti-human IgG

(H & L) antibody solution (manufactured by American Qualex) diluted 3,000 times with 1% BSA-PBS was dispensed in 50 μl/well as a secondary antibody solution, and then the reaction was carried out at room temperature for 1 hour. After the reaction and subsequent washing with Tween-PBS, ABTS substrate solution (a solution prepared by dissolving 0.55 g of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) ammonium salt in 1 liter of 0.1 M citrate buffer (pH 4.2) and adding 1 μl/ml of hydrogen peroxide to the solution just before use) was dispensed in 50 μl/well for color development, and then absorbance at 415 nm (hereinafter referred to as "OD415") was measured.

4. Purification of Anti-GD3 Chimeric Antibody

(1) Culturing of YB2/0 Cell-Derived Producer Cell and Purification of Antibody

The anti-GD3 chimeric antibody-producing transformed cell clone obtained in the above item 2(1) of Example 1 was suspended in the Hybridoma-SFM medium containing 0.2% BSA, 200 nM MTX and 100 nM triiodothyronine (hereinafter referred to as "T3"; manufactured by SIGMA) to give a density of 3×10^5 cells/ml and cultured using a 2.0 liter capacity spinner bottle (manufactured by Iwaki Glass) under agitating at a rate of 50 rpm. Ten days after culturing at 37° C. in a temperature-controlling room, the culture supernatant was recovered. The anti-GD3 chimeric antibody was purified from the culture supernatant using a Prosep-A (manufactured by Bioprocessing) column in accordance with the manufacture's instructions. The purified anti-GD3 chimeric antibody was named YB2/O-GD3 chimeric antibody.

(2) Culturing of CHO/DG44 Cell-Derived Producer Cell and Purification of Antibody

The anti-GD3 chimeric antibody-producing transformed cell clone obtained in the above item 2(2) of Example 1 was suspended in the EX-CELL302 medium containing 3 mM L-Gln, 0.5% fatty acid concentrated solution (hereinafter referred to as "CDLC"; manufactured by GIBCO BRL) and 0.3% Pluronic F68 (hereinafter referred to as "PF68"; manufactured by GIBCO BRL) to give a density of 1×10^6 cells/ml, and the suspension was dispensed in 50 ml into 175 mm^2 flasks (manufactured by Greiner). Four days after culturing at 37° C. in a 5% CO_2 incubator, the culture supernatant was recovered. The anti-GD3 chimeric antibody was purified from the culture supernatant using a Prosep-A (manufactured by Bioprocessing) column in accordance with the manufacture's instructions. The purified anti-GD3 chimeric antibody was named CHO/DG44-GD3 chimeric antibody.

(3) Culturing of NS0 Cell-Derived Producer Cell and Purification of Antibody

The anti-GD3 chimeric antibody-producing transformed cell clone obtained in the above item 2(3) of Example 1 was suspended in the EX-CELL302 medium containing 2 mM L-Gln, 0.5 mg/ml of G418, 200 nM MTX and 1% FBS, to give a density of 1×10^6 cells/ml, and the suspension was dispensed in 200 ml into 175 mm^2 flasks (manufactured by Greiner). Four days after culturing at 37° C. in a 5% CO_2 incubator, the culture supernatant was recovered. The anti-GD3 chimeric antibody was purified from the culture supernatant using a Prosep-A (manufactured by Bioprocessing) column in accordance with the manufacture's instructions. The purified anti-GD3 chimeric antibody was named NS0-GD3 chimeric antibody (302). Also, the transformed cell clone was suspended in the GIT medium containing 0.5 mg/ml of G418 and 200 nM MTX to give a density of 3×10^5 cells/ml, and the suspension was dispensed in 200 ml into 175 mm^2 flasks (manufactured by Greiner). Ten days after culturing at 37° C. in a 5% CO_2 incubator, the culture supernatant was recovered. The anti-GD3 chimeric antibody was purified from the culture supernatant using a Prosep-A (manufactured by Bioprocessing) column in accordance with the manufacture's instructions. The purified anti-GD3 chimeric antibody was named NS0-GD3 chimeric antibody (GIT).

(4) Culturing of SP2/0 Cell-Derived Producer Cell and Purification of Antibody

The anti-GD3 chimeric antibody-producing transformed cell clone described in Japanese Published Unexamined Patent Application No. 304989/93 was suspended in the GIT medium containing 0.5 mg/ml of G418 and 200 nm MTX to give a density of 3×10^5 cells/ml, and the suspension was dispensed in 200 ml into 175 mm^2 flasks (manufactured by Greiner). Eight days after culturing at 37° C. in a 5% CO_2 incubator, the culture supernatant was recovered. The anti-GD3 chimeric antibody was purified from the culture supernatant using a Prosep-A (manufactured by Bioprocessing) column in accordance with the manufacture's instructions. The purified anti-GD3 chimeric antibody was named SP2/0-GD3 chimeric antibody.

5. Analysis of the Purified Anti-GD3 Chimeric Antibodies

In accordance with a known method (*Nature*, 227, 680, 1970), 4 μg of each of the five anti-GD3 chimeric antibodies produced by and purified from respective animal cells, obtained in the above item 4 of Example 1, was subjected to SDS-PAGE to analyze the molecular weight and purification degree. The results are shown in FIG. 1. As shown in FIG. 1, a single band of about 150 kilodaltons (hereinafter referred to as "Kd") in molecular weight was found under non-reducing conditions, and two bands of about 50 Kd and about 25 Kd under reducing conditions, in each of the purified anti-GD3 chimeric antibodies. These molecular weights almost coincided with the molecular weights deduced from the cDNA nucleotide sequences of H chain and L chain of the antibody (H chain; about 49 Kd, L chain; about 23 Kd, whole molecule; about 144 Kd), and also coincided with the reports stating that the IgG antibody has a molecular weight of about 150 Kd under non-reducing conditions and is degraded into H chains having a molecular weight of about 50 Kd and L chains having a molecular weight of about 25 Kd under reducing conditions due to cutting of the disulfide bond (hereinafter referred to as "S—S bond") in the molecule (*Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, Chapter 14, 1998; *Monoclonal Antibodies: Principles and Practice*, Academic Press Limited, 1996), so that it was confirmed that each anti-GD3 chimeric antibody was expressed and purified as an antibody molecule having the true structure.

Example 2

Activity Evaluation of Anti-GD3 Chimeric Antibody:

1. Binding Activity of Anti-GD3 Chimeric Antibodies to GD3 (ELISA)

The activity of the five purified anti-GD3 chimeric antibodies obtained in the above item 4 of Example 1 to bind to GD3 (manufactured by Snow Brand Milk Products) was measured by the ELISA shown in the item 3 of Example 1. FIG. 2 shows a result of the examination of the binding activity measured by changing the concentration of the anti-GD3 chimeric antibody to be added. As shown in FIG. 2, the five anti-GD3 chimeric antibodies showed almost the same binding activity to GD3. This result shows that antigen binding activities of these antibodies are constant independently of the antibody producing animal cells and their culturing methods. Also, it was suggested from the comparison of the NS0-

GD3 chimeric antibody (302) with the NS0-GD3 chimeric antibody (GIT) that the antigen binding activities are constant independently of the media used in the culturing.

2. In Vitro Cytotoxic Activity (ADCC Activity) of Anti-GD3 Chimeric Antibody

In order to evaluate in vitro cytotoxic activity of the five purified anti-GD3 chimeric antibodies obtained in the above item 4 of Example 1, the ADCC activity was measured in accordance with the following method.

(1) Preparation of Target Cell Solution

A human melanoma cultured cell line G-361 (ATCC CRL 1424) was cultured using the RPMI1640-FBS(10) medium to prepare 1×10^6 cells, and the cells were radioisotope-labeled by reacting them with 3.7 MBq equivalents of a radioactive substance $Na_2{}^{51}CrO_4$ at 37° C. for 1 hour. After the reaction, the cells were washed three times through their suspension in the RPMI1640-FBS(10) medium and centrifugation, re-suspended in the medium and then allowed to stand at 4° C. for 30 minutes in ice for spontaneous dissolution of the radioactive substance. After centrifugation, the precipitate was adjusted to 2×10^5 cells/ml by adding 5 ml of the RPMI1640-FBS(10) medium and used as the target cell solution.

(2) Preparation of Effector Cell Solution

From a healthy person, 50 ml of vein blood was collected, and gently mixed with 0.5 ml of heparin sodium (manufactured by Takeda Pharmaceutical). The mixture was centrifuged to isolate a mononuclear cell layer using Lymphoprep (manufactured by Nycomed Pharma AS) in accordance with the manufacture's instructions. After washing with the RPMI1640-FBS(10) medium by centrifugation three times, the resulting precipitate was re-suspended to give a density of 2×10^6 cells/ml using the medium and used as the effector cell solution.

(3) Measurement of ADCC Activity

Into each well of a 96 well U-shaped bottom plate (manufactured by Falcon), 50 μl of the target cell solution prepared in the above (1) (1×10^4 cells/well) was dispensed. Next, 100 μl of the effector cell solution prepared in the above (2) was added thereto (2×10^5 cells/well, the ratio of effector cells to target cells becomes 20:1). Subsequently, each of the anti-GD3 chimeric antibodies was added to give a final concentration from 0.0025 to 2.5 μg/ml, followed by reaction at 37° C. for 4 hours. After the reaction, the plate was centrifuged, and the amount of ^{51}Cr in the supernatant was measured using a γ-counter. The amount of spontaneously released ^{51}Cr was calculated by the same operation using only the medium instead of the effector cell solution and the antibody solution and measuring the amount of ^{51}Cr in the supernatant. The amount of total released ^{51}Cr was calculated by the same operation using only the medium instead of the antibody solution and adding 1 N hydrochloric acid instead of the effector cell solution, and measuring the amount of ^{51}Cr in the supernatant. The ADCC activity was calculated from the following equation.

$$ADCC\ activity\ (\%) = \frac{{}^{51}Cr\ in\ sample\ supernatant - spontaneously\ released\ {}^{51}Cr}{total\ released\ {}^{51}Cr - spontaneously\ released\ {}^{51}Cr} \times 100$$

The results are shown in FIG. 3. As shown in FIG. 3, among the five anti-GD3 chimeric antibodies, the YB2/0-GD3 chimeric antibody showed the highest ADCC activity, followed by the SP2/0-GD3 chimeric antibody, NS0-GD3 chimeric antibody and CHO-GD3 chimeric antibody in that order. No difference in the ADCC activity was found between the NS0-GD3 chimeric antibody (302) and NS0-GD3 chimeric antibody (GIT) prepared using different media in the culturing. The above results show that the ADCC activity of antibodies greatly varies depending on the animal cells to be used in their production. As its mechanism, since their antigen binding activities were identical, it was considered that it is caused by a difference in the structure of the antibody Fc region.

Example 3

Production of Anti-Human Interleukin 5 Receptor α Chain Human CDR-Grafted Antibody:

1. Production of Cells Stably Producing Anti-Human Interleukin 5 Receptor a Chain Human CDR-Grafted Antibody

(1) Production of Producer Cell Using Rat Myeloma YB2/0 Cell

Using the anti-human interleukin 5 receptor α chain human CDR-grafted antibody (hereinafter referred to as "anti-hIL-5R α CDR-grafted antibody") expression vector, pKANTEX1259 HV3LV0, described in WO 97/10354, cells capable of stably producing anti-hIL-5R α CDR-grafted antibody were prepared as described below.

After introducing 5 μg of the anti-hIL-5R α CDR-grafted antibody expression vector, pKANTEX1259HV3LV0, into 4×10^6 cells of rat myeloma YB2/0 by electroporation (*Cytotechnology*, 3, 133 (1990)), the cells were suspended in 40 ml of RPMI1640-FBS(10) and dispensed in 200 μl/well into a 96 well culture plate (manufactured by Sumitomo Bakelite). Twenty-four hours after culturing at 37° C. in a 5% CO_2 incubator, G418 was added to give a concentration of 0.5 mg/ml, followed by culturing for 1 to 2 weeks. The culture supernatant was recovered from respective well in which colonies of transformants showing G418 resistance were formed and growth of colonies was observed, and the antigen binding activity of the anti-hIL-5R α CDR-grafted antibody in the supernatant was measured by the ELISA shown in the item 2 of Example 3.

Regarding the transformants in wells in which production of the anti-hIL-5R α CDR-grafted antibody was observed in culture supernatants, in order to increase amount of the antibody production using a DUFF gene amplification system, each of the them was suspended in the RPMI1640-FBS(10) medium containing 0.5 mg/ml of G418 and 50 nM MTX to give a density of 1 to 2×10^5 cells/ml, and the suspension was dispensed in 2 ml into wells of a 24 well plate (manufactured by Greiner). Transformants showing 50 nM MTX resistance were induced by culturing at 37° C. for 1 to 2 weeks in a 5% CO_2 incubator. The antigen binding activity of the anti-hIL-5R α CDR-grafted antibody in culture supernatants in wells in which growth of transformants was observed was measured by the ELISA shown in the item 2 of Example 3. Regarding the transformants in wells in which production of the anti-hIL-5R α CDR-grafted antibody was observed in culture supernatants, the MTX concentration was increased to 100 nM and then to 200 nm, and a transformant capable of growing n the RPMI1640 FBS(10) medium containing 0.5 mg/ml of G418 and 200 nM MTX and of producing the anti-hIL-5R α CDR-grafted antibody in a large amount was finally obtained in the same manner as described above. The obtained transformant was made into a single cell (cloning) by limiting dilution twice. The obtained anti-hIL-5R α CDR-grafted antibody-producing transformed cell clone No. 3 has been deposited on Apr. 5, 1999, as FERM BP-6690 in

National Institute of Bioscience and Human Technology, Agency of industrial Science and Technology (Higashi 1-1-3, Tsukuba, Ibaraki, Japan).

(2) Production of Producer Cell Using CHO/dhfr$^-$ Cell

After introducing 4 μg of the anti-hIL-5R α CDR-grafted antibody expression vector, pKANTEX1259HV3LV0, described in WO 97/10354 into 1.6×10^6 cells of CHO/dhfr$^-$ by electroporation (*Cytotechnology*, 3, 133 (1990)), the cells were suspended in 10 ml of IMDM-FBS(10) and dispensed in 200 μl/well into a 96 well culture plate (manufactured by Iwaki Glass). Twenty-four hours after culturing at 37° C. in a 5% CO_2 incubator, G418 was added to give a concentration of 0.5 mg/ml, followed by culturing for 1 to 2 weeks. The culture supernatant was recovered from respective well in which colonies of transformants showing G418 resistance were formed and growth of colonies was observed, and the antigen binding activity of the anti-hIL-5R α CDR-grafted antibody in the supernatant was measured by the ELISA shown in the item 2 of Example 3.

Regarding the transformants in wells in which production of the anti-hIL-5R α CDR-grafted antibody was observed in culture supernatants, in order to increase amount of the antibody production using a DHFR gene amplification system, each of the transformants was suspended in an IMDM-dFBS (10) medium containing 0.5 mg/ml of G418 and 10 nM MTX to give a density of 1 to 2×10^5 cells/ml, and the suspension was dispensed in 0.5 ml into wells of a 24 well plate (manufactured by Iwaki Class). Transformants showing 10 nM MTX resistance were induced by culturing at 37-C for 1 to 2 weeks in a 5% CO_2 incubator. Regarding the transformants in wells in which their growth was observed, the MTX concentration was increased to 100 nM and then to 500 nM, and a transformant capable of growing in the IMDM-dFBS(10) medium containing 0.5 mg/ml of G418 and 500 nM MTX and of producing the anti-hIL-5R α CDR-grafted antibody in a large amount was finally obtained in the same manner as described above. The obtained transformant was made into a single cell (cloning) by limiting dilution twice.

(3) Production of Producer Cell Using Mouse Myeloma NS0 Cell

An anti-hIL-BR α CDR-grafted antibody expression vector was prepared in accordance with the method of Yarranton et al. (*BIO/TECHNOLOGY*, 10, 169 (1992)) and using the antibody H chain and L chain cDNA on the anti-hIL-5R α CDR-grafted antibody expression vector, pKANTEX1259HV3LV0, described in WO 97/10354, and NS0 cell was transformed to obtain a transformant capable of producing the anti-hIL-5R a CDR-grafted antibody in a large amount. The obtained transformant was made into a single cell (cloning) by limiting dilution twice.

2. Measurement of Binding Activity of Antibody to hIL-5R α (ELISA)

The binding activity of the antibody to hIL-5R α was measured as described below.

A solution was prepared by diluting the anti-hIL-5R cc mouse antibody, KM1257, described in WO 97/10354 with PBS to give a concentration of 10 μg/ml, and 50 μl of the resulting solution was dispensed into each well of a 96 well plate for ELISA (manufactured by Greiner), followed by reaction at 4° C. for 20 hours. After the reaction, 1% BSA-PBS was dispensed in 100 μl/well, and then the reaction was carried out at room temperature for 1 hour for blocking remaining active groups. After discarding 1% BSA-PBS, a solution prepared by diluting the soluble hIL-5R α described in WO 97/10354 with 1% BSA-PBS to give a concentration of 0.5 μg/ml was dispensed in 50 μl/well, followed by reaction at 4° C. for 20 hours. After the reaction, each well was washed with Tween-PBS, culture supernatants of transformants or diluted solutions of a purified human CDR-grafted antibodies were dispensed in 50 μg/well to carry out the reaction at room temperature for 2 hours. After the reaction, each well was washed with Tween-PBS, a peroxidase-labeled goat anti-human IgG (H & L) antibody solution (manufactured by American Qualex) diluted 3,000 times with 1% BSA-PBS was dispensed in 50 μl/well as a secondary antibody solution, followed by reaction at room temperature for 1 hour. After the reaction and subsequent washing with Tween-PBS, ABTS substrate solution (a solution prepared by dissolving 0.55 g of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) ammonium salt in 1 liter of 01 M citrate buffer (pH 4.2) and adding 1 μl/ml of hydrogen peroxide to the solution just before use) was dispensed in 50 μl/well for color development, and then the absorbance at OD415 was measured.

3. Purification of Anti-hIL-5R α CDR-Grafted Antibody

(1) Culturing of YB2/0 Cell-Derived Producer Cell and Purification of Antibody

The anti-hIL-5R α CDR-grafted antibody-producing transformed cell clone obtained in the above item 1(1) of Example 3 was suspended in the GIT medium containing 0.5 mg/ml of G418 and 200 nM MTX to give a density of 3×10^5 cells/ml and dispensed in 200 ml into 175 mm^2 flasks (manufactured by Greiner). Eight days after culturing at 37° C. in a 5% CO_2 incubator, the culture supernatant was recovered. The anti-hIL-5R α CDR-grafted antibody was purified from the culture supernatant using ion exchange chromatography and a gel filtration method. The purified anti-hIL-5R α CDR-grafted antibody was named YB2/0-hIL-5RCDR antibody.

(2) Culturing of CHO/dhfr$^-$ Cell-Derived Producer Cell and Purification of Antibody

The anti-hIL-5R α CDR-grafted antibody-producing transformed cell clone obtained in the above item 1(2) of Example 3 was suspended in the EX-CELL302 medium containing 3 mM L-Gln, 0.5% CDLC and 0.3% PF68 to give a density of 3×10^5 cells/ml and cultured using a 4.0 liter capacity spinner bottle (manufactured by Iwaki Glass) under agitating at a rate of 100 rpm. Ten days after culturing at 37° C. in a temperature-controlling room, the culture supernatant was recovered. The anti-hIL-5R α CDR-grafted antibody was purified from the culture supernatant using ion exchange chromatography and a gel filtration method. The purified anti-hIL-5R α CDR-grafted antibody was named CHO/d-hIL-5RCDR antibody.

(3) Culturing of NS0 Cell-Derived Producer Cell and Purification of Antibody

The anti-hIL-5R α CDR-grafted antibody-producing transformed cell clone obtained in the above item 1(3) of Example 3 was cultured in accordance with the method of Yarranton et al. (*BIO/TECHNOLOGY*, 10, 169 (1992)) and then a culture supernatant was recovered. The anti-hIL-5R α CDR-grafted antibody was purified from the culture supernatant using ion exchange chromatography and the gel filtration method. The purified anti-hIL-5R α CDR-grafted antibody was named NS0-hIL-5RCDR antibody.

4. Analysis of Purified Anti-hIL-5R α CDR-Grafted Antibodies

In accordance with a known method (*Nature*, 227, 680, (1970)), 4 μg of each of the three anti-hIL-5R α CDR-grafted antibodies produced by and purified from respective animal cells, obtained in the above item 3 of Example 3, was subjected to SDS-PAGE to analyze the molecular weight and purification degree. The results are shown in FIG. 4. As shown in FIG. 4, a single band of about 150 Kd in molecular weight

was found under non-reducing conditions, and two bands of about 50 Kd and about 25 Kd under reducing conditions, in each of the purified anti-hIL-5R α CDR-grafted antibodies. These molecular weights almost coincided with the molecular weights deduced from the cDNA nucleotide sequences of H chain and L chain of the anti-body (H chain: about 49 Kd, L chain: about 23 Kd, whole molecule; about 144 Kd), and also coincided with the reports stating that the IgG antibody has a molecular weight of about 150 Kd under non-reducing conditions and is degraded into H chains having a molecular weight of about 50 Kd and L chains having a molecular weight of about 25 Kd under reducing conditions due to cutting of the disulfide bond (hereinafter referred to as "S—S bond") in the molecule (*Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory*, Chapter 14, 1998; *Monoclonal Antibodies: Principles and Practice*, Academic Press Limited, 1996), so that it was confirmed that each anti-hIL-5R α CDR-grafted antibody was expressed and purified as an antibody molecule having the true structure.

Example 4

Activity Evaluation of Anti-hIL-5R α CDR-Grafted Antibody:

1. Binding Activity of Anti-hIL-5R α CDR-Grafted Antibody to hIL-5R α (ELISA)

The activity of the three purified anti-hIL-5R α CDR-grafted antibodies obtained in the above item 2 of Example 3 to bind to hIL-5R α was measured by the ELISA shown in the item 2 of Example 3. FIG. 5 shows a result of the examination of the binding activity measured by changing concentration of the anti-hIL-5R α CDR-grafted antibody to be added. As shown in FIG. 5, the three anti-hIL-5R α CDR-grafted antibodies showed almost the same binding activity to hIL-5R α. This result shows that the antigen binding activities of these antibodies are constant independently of the antibody producing animal cells and their culturing methods, similar to the result of the item 1 of Example 2.

2. In Vitro Cytotoxic Activity (ADCC Activity) of Anti-hIL-5R α CDR-Grafted Antibody

In order to evaluate in vitro cytotoxic activity of the three purified anti-hIL-5R α CDR-grafted antibodies obtained in the above item 3 of Example 3, the ADCC activity was measured in accordance with the following method.

(1) Preparation of Target Cell Solution

A mouse T cell line CTLL-2(h5R) expressing the hIL-5R α chain and β chain described in WO 97/10354 was cultured using the RPMI1640-FBS(10) medium to prepare a 1×10^6 cells/0.5 ml suspension, and the cells were radioisotope-labeled by reacting them with 3.7 MBq equivalents of a radioactive substance $Na_2{}^{51}CrO_4$ at 37° C. for 1.5 hours. After the reaction, the cells were washed three times through their suspension in the RPMI1640-FBS(10) medium and centrifugation, re-suspended in the medium and then allowed to stand at 4° C. for 30 minutes in ice for spontaneous dissolution of the radioactive substance. After centrifugation, the precipitate was adjusted to 2×10^5 cells/ml by adding 5 ml of the RPMI1640-FBS(10) medium and used as the target cell solution.

(2) Preparation of Effector Cell Solution

From a healthy person, 50 ml of vein blood was collected and gently mixed with 0.5 ml of heparin sodium (manufactured by Takeda Pharmaceutical). The mixture was centrifuged to separate a mononuclear cell layer using Polymorphprep (manufactured by Nycomed Pharma AS) and in accordance with the manufacture's instructions. After washing with the RPMI1640-FBS(10) medium by centrifugation three times, the resulting cells were re-suspended to give a density of 9×10^6 cells/ml using the medium and used as the effector cell solution.

(3) Measurement of ADCC Activity

Into each well of a 96 well U-shaped bottom plate (manufactured by Falcon), 50 μl of the target cell solution prepared in the above (1) (1×10^4 cells/well) was dispensed. Next, 100 μl of the effector cell solution prepared in the above (2) was dispensed (9×10^5 cells/well, the ratio of effector cells to target cells becomes 90:1). Subsequently, each of the anti-hIL-5R α CDR-grafted antibodies was added to give a final concentration from 0.001 to 0.1 μg/ml, followed by reaction at 37° C. for 4 hours. After the reaction, the plate was centrifuged, and the amount of ^{51}Cr in the supernatant was measured using a γ-counter. The amount of spontaneously released ^{51}Cr was calculated by the same operation using only the medium instead of the effector cell solution and the antibody solution and measuring the amount of ^{51}Cr in the supernatant. The amount of total released ^{51}Cr was calculated by the same operation using only the medium instead of the antibody solution and adding 1 N hydrochloric acid instead of the effector cell solution, and measuring the amount of ^{51}Cr in the supernatant.

The ADCC activity was calculated from the following equation.

$$ADCC\ activity\ (\%) = \frac{{}^{51}Cr\ in\ sample\ supernatant - spontaneously\ released\ {}^{51}Cr}{total\ released\ {}^{51}Cr - spontaneously\ released\ {}^{51}Cr} \times 100$$

The results are shown in FIG. 6. As shown in FIG. 6, among the three anti-hIL-5R α CDR-grafted antibodies, the YB2/0-hIL-5RCDR antibody showed the highest ADCC activity, followed by the CHO/d-hIL-5RCDR antibody and the NS0-hIL-5RCDR antibody in this order. Similar to the result of the item 2 of Example 2, the above results show that the ADCC activity of antibodies greatly varies depending on the animal cells to be used in their production. In addition, since the antibodies produced by the YB2/0 cell showed the highest ADCC activity in both cases of the two humanized antibodies, it was revealed that an antibody having high ADCC activity can be produced by the use of the YB2/0 cell,

3. In Vivo Activity Evaluation of Anti-hIL-5R α CDR-Grafted Antibody

In order to evaluate in vivo activity of the three purified anti-hIL-5R α CDR-grafted antibodies obtained in the above item 3 of Example 3, the inhibition activity in an hIL-5-induced eosinophilia increasing model of *Macaca faseicularis* was examined in accordance with the following method.

The hIL-5 (preparation method is described in WO 97/10354) was administered to *Macaca faseicularis* under the dorsal skin at a dose of 1 μg/kg, starting on the first day and once a day for a total of 14 times. Each anti-hIL-5R α CDR-grafted antibody was intravenously administered at a dose of 0.3 mg/kg one hour before the hIL-5 administration on the day zero. An antibody-non-added group was used as the control. In the antibody-administered groups, three animals of *Macaca faseicularis* were used in each group (No. 301, No. 302, No. 303, No. 401, No. 402, No. 403, No. 501, No. 502 and No. 503), and two animals (No. 101 and No. 102) were used in the antibody-non-added group Starting 7 days before commencement of the administration and until 42 days after

the administration, about 1 ml of blood was periodically collected from a saphena or a femoral vein, and the number of eosinophils in 1 μl of peripheral blood was measured. The results are shown in FIG. 7. As shown in FIG. 7, increase in the blood eosinophil was completely inhibited in the group to which the YB2/0-hIL-5RCDR antibody was administered. On the other hand, complete inhibition activity was found in one animal in the group to which the CHO/d-hIL-5RCDR antibody was administered, but the inhibition activity was not sufficient in two animals. In the group to which NS0-hIL-5RCDR antibody was administered, complete inhibition activity was not found and its effect was not sufficient. The above results show that the in vivo activity of antibodies greatly varies depending on the animal cells to be used in their production. In addition, since a positive correlation was found between the degree of the in vivo activity of the anti-hIL-5R α CDR-grafted antibody and the degree of its ADCC activity described in the item 2 of Example 4, it was indicated that the degree of ADCC activity is markedly important for its activity expression.

Based on the above results, it is expected that an antibody having high ADCC activity is useful also in the clinical field for various diseases in human.

Example 5

Analysis of ADCC Activity-Increasing Sugar Chain:

1. Preparation of 2-aminopyridine-Labeled Sugar Chain (PA-Treated Sugar Chain)

The humanized antibody of the present invention was acid-hydrolyzed with hydrochloric acid to remove sialic acid. After hydrochloric acid was completely removed, the sugar chain was cut off from the protein by hydrazinolysis (*Method of Enzymology*, 83, 263, 1982). Hydrazine was removed, and N-acetylation was carried out by adding an ammonium acetate aqueous solution and acetic anhydride. After freeze-drying, fluorescence labeling with 2-aminopyridine was carried out (*J. Biochem.*, 95, 197 (1984)). The fluorescence-labeled sugar chain (PA-treated sugar chain) was separated as an impurity using Superdex Peptide HR 10/30 Column (manufactured by Pharmacia). The sugar chain fraction was dried using a centrifugal concentrator and used as a purified PA-treated sugar chain.

2. Reverse Phase HPLC Analysis of PA-Treated Sugar Chain of Purified Anti-hIL-5R α CDR-Grafted Antibody

Using respective anti-hIL-5R α CDR-grafted antibody PA-treated sugar chains prepared in the above item 1 of Example 5, reverse phase HPLC analysis was carried out by CLC-ODS column (manufactured by Shimadzu). An excess amount of α-L-fucosidase (derived from bovine kidney, manufactured by SIGMA) was added to the PA-treated sugar chain for digestion (37° C., 15 hours), and then the products were analyzed by reverse phase HPLC (FIG. 8). Using PA-treated sugar chain standards manufactured by Takara Shuzo it was confirmed that the asparagine-linked sugar chain is eluted during a period of from 30 minutes to 80 minutes. The ratio of sugar chains whose reverse phase HPLC elution positions were shifted (sugar chains eluted during a period from 48 minutes to 78 minutes) by the α-L-fucosidase digestion was calculated. The results are shown in Table 1.

TABLE 1

Antibody-producing cell	α-1,6-Fucose-linked sugar chain (%)
YB2/0	47
NS0	73

About 47% of the anti-hIL-5RCDR-grafted antibody produced by the YB2/0 cell and about 73% of the anti-hIL-5RCDR-grafted antibody produced by the NS0 cell were sugar chains having α-1,6-fucose. Thus, sugar chains having no α-1,6-fucose were more frequent in the antibody produced by the YB2/0 cell in comparison with the antibody produced by the NS0 cell.

3. Analysis of Monosaccharide Composition of Purified Anti-hIL-5R α CDR-Grafted Antibody

Sugar chains of anti-hIL-5R α CDR-grafted antibodies produced by the YB2/0 cell, NS0 cell and CHO/d cell were hydrolyzed into monosaccharides by acid hydrolysis with trifluoroacetic acid, and monosaccharide composition analysis was carried out using BioLC (manufactured by Dionex).

Among N-glycoside-linked sugar chains, there are 3 mannose units in one sugar chain in the complex type N-glycoside-linked sugar chain. A relative ratio of each monosaccharide obtained by calculating the number of mannose as 3 is shown in Table 2.

TABLE 2

Antibody-producer cell	Fuc	GlcNAc	Gal	Man	ADCC activity (%)*
YB2/0	0.60	4.98	0.30	3.00	42.27
NS0	1.06	3.94	0.66	3.00	16.22
CHO/dhFr⁻	0.85	3.59	0.49	3.00	25.73
	0.91	3.80	0.27	3.00	

*Antibody concentration: 0.01 μg/ml

Since the relative ratios of fucose were in an order of YB2/0<CHO/d<NS0, the sugar chain produced in the antibody produced by YB2/0 cell showed the lowest fucose content as also shown in the present results.

Example 6

Sugar Chain Analysis of Antibody Produced by CHO/dhfr⁻ Cell:

PA-treated sugar chains were prepared from purified anti-hIL-5R α CDR-grafted antibody produced by CHO/dhfr⁻ cell, and reverse phase HPLC analysis was carried out using CLC-ODS column (manufactured by Shimadzu) (FIG. 9). In FIG. 9, an elution time from 35 to 45 minutes corresponded to sugar chains having no fucose and an elution time from 45 to 60 minutes corresponded to sugar chains having fucose. Similar to the case of the antibody produced by mouse myeloma NS0 cell, the anti-hIL-5R α CDR-grafted antibody produced by CHO/dhfr⁻ cell had less fucose-free sugar chain content than the antibody produced by rat myeloma YB2/0 cell.

Example 7

Separation of High ADCC Activity Antibody:

The anti-hIL-5R α CDR-grafted antibody produced by rat myeloma YB2/0 cell was separated using a lectin column which binds to sugar chains having fucose. HPLC was carried

out using LC-6A manufactured by Shimadzu at a flow rate of 1 ml/min and at room temperature as the column temperature. After equilibration with 50 mM Tris-sulfate buffer (pH 7.3), the purified anti-hIL-5R α CDR-grafted antibody was injected and then eluted by a linear density gradient (60 minutes) of 0.2 M α-methylmannoside (manufactured by Nakalai Tesque). The anti-hIL-5R α CDR-grafted antibody was separated into non-adsorbed fraction and adsorbed fraction. When the non-adsorbed fraction and a portion of the adsorbed fraction were sampled and their binding activity to hIL-5R α was measured, they showed similar binding activity (FIG. **10**, upper graph). When the ADCC activity was measured, the non-adsorbed fraction showed higher ADCC activity than that of the portion of adsorbed fraction (FIG. **10**, lower graph). In addition, PA-treated sugar chains were prepared from the non-adsorbed fraction and a portion of the adsorbed fraction, and reverse HPLC analysis was carried out using CLC-ODS column (manufactured by Shimadzu) (FIG. **11**). The non-adsorbed fraction was an antibody mainly having fucose-free sugar chains, and the portion of adsorbed fraction was an antibody mainly having fucose-containing sugar chains.

Example 8

Determination of Transcription Product of α1,6-fucosyltransferase (FUT8) Gene in Host Cell Line:

(1) Preparation of Single-Stranded cDNA Derived from Various Cell Lines

Chinese hamster ovary-derived CHO/DG44 cell was suspended in the IMDM medium (manufactured by Life Technologies) supplemented with 10% FBS (manufactured by Life Technologies) and 1× concentration of MT supplement (manufactured by Life Technologies) and inoculated into a T75 flask for adhesion cell culture (manufactured by Greiner) at a density of 2×10^5 cells/ml. Also, the rat myeloma-derived YB2/0 cell was suspended in the RPMI1640 medium (manufactured by Life Technologies) supplemented with 10% FBS (manufactured by Life Technologies) and 4 mM glutamine (manufactured by Life Technologies) and inoculated into a T75 flask for suspension cell culture (manufactured by Greiner) at a density of 2×10^5 cells/ml. These cells were cultured at 37° C. in a 5% CO_2 incubator, and 1×10^7 cells of each host cell were recovered on the 1st, 2nd, 3rd, 4th and 5th day to extract total RNA using RNAeasy (manufactured by QUIAGEN).

The total RNA was dissolved in 45 μl of sterile water, mixed with 0.5 U/μl of RQ1 RNase-Free DNase (manufactured by Promega) and 5 μl of attached 10× DNase buffer and 0.5 μl of RNasin Ribonuclease inhibitor (manufactured by Promega), followed by reaction at 37° C. for 30 minutes. After the reactions the total RNA was again purified using RNAeasy (manufactured by QUIAGEN) and dissolved in 50 μl of sterile water.

According to SUPERSCRIPT™ Preamplification System for First Strand cDNA Synthesis (manufactured by Life Technology), 3 μg of the obtained total RNA each was subjected to a reverse transcription reaction in a 20 μl system using oligo (dT) as a primer to thereby synthesize cDNA. A solution of 1× concentration of the solution after the reverse transcription reaction was used for cloning of FUT8 and β-actin derived from each host cell, and a solution after the reverse transcription reaction further diluted 50 times with water was used for the determination of the transcription quantity of each gene using the competitive PCR, and each of the solutions was stored at −80° C. until use.

(2) Preparation of Respective cDNA Partial Fragments of Chinese Hamster FUT8 and Rat FUT8

Respective cDNA partial fragments of Chinese hamster FUT8 and of rat FUT8 were obtained as described below. First, primers (shown in SEQ ID NO:1 and SEQ ID NO:2) specific for nucleotide sequences common in a human FUT8 cDNA (*Journal of Biochemistry*, 121, 626 (1997)) and a swine FUT8 cDNA (*Journal of Biological Chemistry*, 271, 27810 (1996)) were designed.

Next, using a DNA polymerase ExTaq (manufactured by Takara Shuzo), 25 μl of a reaction solution constituted by ExTaq buffer (manufactured by Takara Shuzo), 0.2 mM dNTPs, 0.5 μM of each of the above specific primers (SEQ ID NO:1 and SEQ ID NO:2), and 1 μl of each of the cDNA derived from CHO cell and the cDNA derived from YB2/0 cell, each obtained on the 2nd day of culturing in (1), was prepared, and polymerase chain reaction (PCR) was carried out. The PCR was carried out under conditions in which, after heating at 94° C. for 1 minute, a cycle consisting of reactions at 94° C. for 30 seconds, 55° C. for 30 seconds and 72° C. for 2 minutes is repeated 30 cycles and then the reaction solution is heated at 72° C. for 10 minutes. Each specific amplified fragment of 979 bp obtained by the PCR was connected to a plasmid pCR2.1 using TOPO TA Cloning Kit (manufactured by Invitrogen) to obtain a plasmid containing respective cDNA partial fragment of Chinese hamster FUT8 or rat FUT8 (CHFT8-pCR2.1 or YBFT8-pCR2.1).

The nucleotide sequence of each cDNA obtained was determined using DNA Sequencer 377 (manufactured by Parkin Elmer) and BigDye Terminator Cycle Sequencing FS Ready Reaction Kit (manufactured by Parkin Elmer) to confirm that the obtained cDNAs encode open reading frame (ORF) partial sequences of Chinese hamster FUT8 and rat PUTS (shown in SEQ ID NOs:3 and 4).

(3) Preparation of Chinese Hamster β-Actin and Rat β-Actin cDNA

Since it is considered that the β-actin gene is constantly transcribed in each cell and its transcription quantity is almost the same among cells, transcription quantity of the β-actin gene is determined as a standard of the efficiency of synthesis reaction of cDNA derived from respective cells.

Chinese hamster β-actin and rat β-actin were obtained by the following method. First, a forward primer (shown in SEQ ID NO:5) specific for a common sequence containing a translation initiation codon and reverse primers (shown in SEQ ID NO:6 and SEQ ID NO:7) specific for the respective sequence containing a translation termination codon were designed from a Chinese hamster β-actin genomic sequence (GenBank, U20114) and a rat β-actin genomic sequence (*Nucleic Acid Research*, 11, 1759 (1983)).

Next, using a DNA polymerase, KOD (manufactured by TOYOBO), 25 μl of a reaction solution constituted by KOD buffer #1 (manufactured by TOYOBO), 0.2 mM dNTPs, 1 mM $MgCl_2$, 0.4 μM of each of the above gene specific primers (SEQ ID NO:5 and SEQ ID NO 6, or SEQ ID NO:5 and SEQ ID NO:7), 5% DMSO, and 1 μl of each of the cDNA derived from CHO cell and the cDNA derived from YB2/0 cell, each obtained on the 2nd day of culturing in (1), was prepared, and polymerase chain reaction (PCR) was carried out. The PCR was carried out under a condition in which, after heating at 94° C. for 4 minutes, a cycle consisting of reactions at 98° C. for 15 seconds, 65° C. for 2 seconds and 74° C. for 30 seconds is repeated 25 cycles. The 5'-terminal of each specific amplified fragment of 1,128 bp obtained by the PCR was phosphorylated using MEGALABEL (manufactured by Takara Shuzo) and then digested with a restriction enzyme, EcoRV, and the resulting fragment (2.9 Kb) was connected to pBlue-

script II (KS(+) (manufactured by Stratagene) using Ligation High (manufactured by TOYOBO) to obtain a plasmid containing an ORF full length of respective cDNA of Chinese hamster β-actin or rat β-actin (CHAc-pBS or YBAc-pBS).

The nucleotide sequence of the respective cDNA obtained was determined using DNA Sequencer 377 (manufactured by Parkin Elmer) and BigDye Terminator Cycle Sequencing FS Ready Reaction Kit (manufactured by Parkin Elmer) to confirm that they respectively encode ORF full length sequences of cDNA of Chinese hamster β-actin and rat β-actin.

(4) Preparation of Standard and Internal Sequence Control

In order to measure the quantity of mRNA transcription from the FUT8 gene in producer cells, a calibration curve was firstly prepared.

As the FUT8 standard to be used in the calibration curve, plasmids, CHFT8-pCR2.1 and YBFT8-pCR2.1, obtained in (2) by inserting respective cDNA partial fragments of Chinese hamster FUT8 or rat FUT8 into pCR2.1 were digested with a restriction enzyme, EcoRI, and the resulting DNA fragments were used after making them into linear chains.

As the internal control to be used in the FUT8 determination, among CHFT8 pCR2-1 and YBFT8-pCR2.1, CHFT8d-pCR2.1 and YBFT8d-pCR2.1 obtained by deleting 203 bp between ScaI-HindIII of internal nucleotide sequences of Chinese hamster FUT8 or rat FUT8 were digested with the restriction enzyme, EcoRI, and the resulting DNA fragments were used after making them into linear chains.

As the standard of the quantity of mRNA transcribed from the β-actin gene in producer cells, plasmids CHAc-pBS and YBAc-pBS obtained in (3) by integrating the ORF full length of respective cDNAs of Chinese hamster β-actin and rat β-actin into pBluescript II KS(+) were respectively digested, the former with HindIII and PstI and the latter with HindIII and KpnI, and the resulting DNA fragments were used by making them into linear chains.

As the internal control for the determination of β-actin, among CHAc-pBS and YBAc-pBS, CHAcd-pBS and YBAcd-pBS obtained by deleting 180 bp between DraIII-DraIII of internal nucleotide sequences of Chinese hamster β-actin and rat β-actin were digested, the former with HindIII and PstI and the latter with HindIII and KpnI, and the resulting DNA fragments were used after making them into linear chains.

(5) Determination of Transcription Quantity by Competitive RT-PCR

First, a primer set (shown in SEQ ID NOs:8 and 9) common sequence-specific for internal sequences of ORF partial sequences of Chinese hamster FUT8 and rat FUT8 obtained in (2) was designed.

Next, PCR was carried out using a DNA polymerase ExTaq (manufactured by Takara Shuzo) in 20 μl in total volume of a reaction solution constituted by ExTaq buffer (manufactured by Takara Shuzo), 0.2 mM dNTPs, 0.5 μM of each of the above gene specific primers (SEQ ID NO:8 and SEQ ID NO:9), 5% DMSO, and 5 μl of a 50 times diluted solution of each of the cDNAs derived from respective host cell lines obtained in (1) and 5 μl (10 fg) of the plasmid for internal control. The PCR was carried out by heating at 94° C. for 3 minutes and then repeating 30 cycles using reactions at 94° C. for 1 minute, 60° C. for 1 minute and 72° C. for 1 minute as one cycle.

The β-actin transcription product was determined as described below. Primer sets gene-specific for internal sequences of the ORF full lengths of Chinese hamster β-actin and rat β-actin obtained in (3) (the former are shown in SEQ ID NO:10 and SEQ ID NO:11, and the latter in SEQ ID NO:12 and SEQ ID NO:13) were respectively designed.

Next, PCR was carried out using a DNA polymerase ExTaq (manufactured by Takara Shuzo) in 20 μl in total volume of a reaction solution constituted by ExTaq buffer (manufactured by Takara Shuzo), 0.2 mM dNTPs, 0.5 μM of the above gene specific primers (SEQ ID NO:10 and SEQ ID NO:11, or SEQ ID NO:12 and SEQ in NO:13), 5% DMSO, and 5 μl of a 50 times diluted solution of each of the cDNAs derived from respective host cell lines obtained in (1) and 5 μl (1 pg) of the plasmid for internal control. The PCR was carried out by heating at 94° C. for 3 minutes and then repeating 17 cycles using reactions at 94° C. for 30 seconds, 65° C. for 1 minute and 72° C. for 2 minutes as one cycle.

TABLE 3

Target gene	*Primer set	Size (bp) of PCR amplified product: Target	Competitor
FUT8	F: 5'-GTCCATGGTGATCCTGCAGTGTGG-3' (SEQ ID NO:8) R: 5'-CACCAATGATATCTCCAGGTTCC-3' (SEQ ID NO:9)	638	431
β-Actin (Chinese hamster)	F: 5'-GATATCGCTGCGCTCGTTGTCGAC-3' (SEQ ID NO:10) R: 5'-CAGGAAGGAAGGCTGGAAAAGAGC-3' (SEQ ID NO:11)	789	609
β-Actin (rat)	F: 5'-GATATCGCTGCGCTCGTCGTCGAC-3' (SEQ ID NO:12) R: 5'-CAGGAAGGAAGGCTGGAAGAGAGC-3' (SEQ ID NO:13)	789	609

*F: forward primer, R: reverse primer

Determinative PCR was carried out using the primer sets shown in Table 3. As a result, the DNA fragment having the size shown in the target column of Table 3 was amplified from the respective gene transcription product and the corresponding standard, and the DNA fragment having the size shown in the competitor column of Table 3 was amplified from the corresponding internal control.

After 7 μl of the solution after PCR was subjected to 1.75% agarose gel electrophoresis, the gel was stained with SYBR Green I Nucleic Acid Gel Stain (manufactured by Molecular Probes). The quantity of the amplified DNA fragments was measured by calculating luminescence strength of each of the amplified DNA fragments using FluorImager SI (manufactured by Molecular Dynamics).

Furthermore, PCR was carried out by changing the amount of the standard plasmid prepared in (4) to 0.1 fg, 1 fg, 5 fg, 10 fg, 50 fg, 100 fg and 500 fg, instead of the cell-derived cDNA, and the amount of amplified products was determined. A calibration curve was prepared by plotting the measured values against the amounts of standard plasmid.

Using this calibration curve, the amount of cDNA of the gene of interest in each cell was calculated from the amount of the amplified product when the total cDNA derived from each cell was used as the template, and the amount was defined as the mRNA transcription quantity in each cell.

Amounts of the FUT8 transcription product in each host cell line in using rat FUT8 sequences as the standard and internal control are shown in FIG. **12**. The CHO cell line showed a transcription quantity 10 times or more higher than that of the YB2/0 cell line throughout the culturing period. This tendency was found also when Chinese hamster FUT8 sequences were used as the standard and internal control.

Also, the FUT8 transcription quantity is shown in Table 4 as a relative value to the amount of β-actin transcription product.

TABLE 4

	Culture days				
Cell line	Day 1	Day 2	Day 3	Day 4	Day 5
CHO	2.0	0.90	0.57	0.52	0.54
YB2/0	0.07	0.13	0.13	0.05	0.02

While the FUT8 transcription quantity of the YB2/0 cell line was about 0.1% β-actin, that of the CHO cell line was from 0.5 to 2%.

Based on the above results, it was shown that the amount of the FUT8 transcription product in the YB2/0 cell line was significantly smaller than that in the CHO cell line.

INDUSTRIAL APPLICABILITY

The present invention relates to a sugar chain which controls the activity of an immunologically functional molecule, such as an antibody, a protein, a peptide or the like, as well as an antibody, a protein or a peptide having the sugar chain. The present invention further relates to methods for the production of the sugar chain and an antibody, a protein or a peptide having the sugar chain, as well as a diagnostic agent, a preventive agent and a therapeutic agent which contain these products as an active ingredient.

SEQUENCE LISTING

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ccgatacaca gaaaattcac ttcggggcgt gatcctggat atacactttc tctcccaggc 660

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We claim:

1. An antibody composition comprising antibody molecules, wherein 100% of the antibody molecules comprising an Fc region comprising complex N-glycoside-linked sugar chains bound to the Fc region through N-acetylglucosamines of the reducing terminal of the sugar chains do not contain sugar chains with a fucose bound to the N-acetylglucosamines, and wherein said antibody molecules bind to an interleukin-5 receptor protein.

2. The antibody composition according to claim 1, wherein the antibody molecules are selected from the group consisting of (a), (b) and (c);
(a) human antibodies;
(b) humanized antibodies;
(c) antibody fragments.

3. The antibody composition according to claim 1, wherein the antibody molecules belong to an IgG class.

* * * * *

Exhibit D

Copy of maintenance fee reports for the 7,718,175 patent

United States Patent and Trademark Office

Office of the Commissioner for Patents

Maintenance Fee Statement

FEE ADDRESS	CUSTOMER #	ENTITY STATUS	STATEMENT GENERATED
CPA GLOBAL LIMITED 2318 MILL ROAD 12TH FLOOR ALEXANDRIA, US 22314	197	UNDISCOUNTED	01/09/2018 09:48:41

Invention

METHOD OF MODULATING THE ACTIVITY OF FUNCTIONAL IMMUNE MOLECULES TO INTERLEUKIN-5 RECEPTOR PROTEIN

7718175	11686379	03/15/2007	05/18/2010

Payment Details

PAYMENT DATE	DATE POSTED	TRANSACTION ID	ATTORNEY DOCKET #	TOTAL PAYMENT
11/07/2017	11/07/2017	110717INTMTFEE00008788504623		$3,600.00

Fee Code	Description		Fee Amount
1552	MAINTENANCE FEE DUE AT 7.5 YEARS	110717INTMTFEE00008788	$3,600.00

According to the records of the United States Patent and Trademark Office (USPTO), the maintenance fee and any necessary surcharge have been timely paid for the patent listed above. The payment shown above is subject to actual collection. If the payment is refused or charged back by a financial institution, the payment will be void and the maintenance fee and any necessary surcharge unpaid

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METHOD OF MODULATING THE ACTIVITY OF FUNCTIONAL IMMUNE MOLECULES TO INTERLEUKIN-5 RECEPTOR PROTEIN

PATENT #	APPLICATION #	FILING DATE	ISSUE DATE
7718175	11686379	03/15/2007	05/18/2010

Payment Window Status

Approved for use through 7/31/2018. OMB 0651-0016.

Maintenance fee has already been paid.

Fee information is available at uspto.gov

WINDOW	STATUS	FEES
7.5 Year	Closed	Paid

Window	First Day to Pay	Surcharge Starts	Last Day to Pay	Status	Fees
3.5 Year	05/18/2013	11/19/2013	05/19/2014	Closed	Paid
7.5 Year	05/18/2017	11/21/2017	05/18/2018	Closed	Paid
11.5 Year	05/18/2021	11/19/2021	05/18/2022	Not Open	Not Due

Patent Holder Information

Customer #	197
Entity Status	UNDISCOUNTED
Phone Number	(703) 739-2234
Address	CPA GLOBAL LIMITED 2318 Mill Road 12th Floor ALEXANDRIA, VA 22314 UNITED STATES

Exhibit E

Power of Attorney and Statement documents

PTO/SB/81A (12-08)
Approved for use through 11/30/2011. OMB 0651-0035
U.S. Patent and Trademark Office; U.S DEPARTMENT OF COMMERCE

PATENT - POWER OF ATTORNEY OR REVOCATION OF POWER OF ATTORNEY WITH A NEW POWER OF ATTORNEY AND CHANGE OF CORRESPONDENCE ADDRESS

Patent Number	7,718,175
Issue Date	05-18-2010
First Named Inventor	Nobuo Hanai
Title	Method of modulating the activity of functional immune molecules to interleukin-5 receptor protein
Attorney Docket Number	Q104522

I hereby revoke all previous powers of attorney given in the above-identified patent.

☐ A Power of Attorney is submitted herewith.

OR

☒ I hereby appoint Practitioner(s) associated with the following Customer Number as my/our attorney(s) or agent(s) with respect to the patent identified above, and to transact all business in the United States Patent and Trademark Office connected therewith: 1131

OR

☐ I hereby appoint Practitioner(s) named below as my/our attorney(s) or agent(s) with respect to the patent identified above, and to transact all business in the United States Patent and Trademark Office connected therewith:

Practitioner(s) Name	Registration Number

Please recognize or change the correspondence address for the above-identified patent to:

☒ The address associated with the above-mentioned Customer Number.

OR

☐ The address associated with Customer Number:

OR

☐ Firm or Individual Name					
Address					
City		State		Zip	
Country					
Telephone		Email			

I am the:

☐ Inventor, having ownership of the patent.

OR

☒ Patent owner,
Statement under 37 CFR 3 73(b) (Form PTO/SB/96) submitted herewith or filed on _____.

SIGNATURE of Inventor or Patent Owner

Signature	Kenya Shitara	Date	January 5, 2018.
Name	Kenya Shitara	Telephone	+81-3-5205-7200
Title and Company	Executive Officer and Director, Legal and Intellectual Property Department, Kyowa Hakko Kirin Co., LTD.		

NOTE: Signatures of all the inventors or patent owners of the entire interest or their representative(s) are required. Submit multiple forms if more than one signature is required, see below*

☐ *Total of _____ forms are submitted.

Privacy Act Statement

The **Privacy Act of 1974 (P.L. 93-579)** requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (*i.e.*, GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

PTO/SB/96 (07-09)
Approved for use through 07/31/2012. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

STATEMENT UNDER 37 CFR 3.73(b)

Applicant/Patent Owner: KYOWA HAKKO KIRIN CO., LTD.

Application No./Patent No.: 7,718,175 Filed/Issue Date: 05-18-2010

Titled: METHOD OF MODULATING THE ACTIVITY OF FUNCTIONAL IMMUNE MOLECULES TO INTERLEUKIN-5 RECEPTOR PROTEIN

KYOWA HAKKO KIRIN CO., LTD. , a corporation

(Name of Assignee) (Type of Assignee, e.g., corporation, partnership, university, government agency, etc.

states that it is:

1. ☒ the assignee of the entire right, title, and interest in;

2. ☐ an assignee of less than the entire right, title, and interest in (The extent (by percentage) of its ownership interest is _____ %); or

3. ☐ the assignee of an undivided interest in the entirety of (a complete assignment from one of the joint inventors was made)

the patent application/patent identified above, by virtue of either:

A. ☐ An assignment from the inventor(s) of the patent application/patent identified above. The assignment was recorded in the United States Patent and Trademark Office at Reel _____, Frame _____, or for which a copy therefore is attached.

OR

B. ☒ A chain of title from the inventor(s), of the patent application/patent identified above, to the current assignee as follows:

1. From: Hanai, N; Nakamura, K; Yamasaki, M; Uchida, K; Shinkawa, T; Imahegpu, S; Kanda, Y; Hosaka, F; Yamane, N; Anazawa, H To: KYOWA HAKKO KOGYO CO., LTD.

The document was recorded in the United States Patent and Trademark Office at

Reel 012301, Frame 0276, or for which a copy thereof is attached.

2. From: KYOWA HAKKO KOGYO CO., LTD. To: KYOWA HAKKO KIRIN CO., LTD.

The document was recorded in the United States Patent and Trademark Office at

Reel 022542, Frame 0823, or for which a copy thereof is attached.

3. From: _____ To: _____

The document was recorded in the United States Patent and Trademark Office at

Reel _____, Frame _____, or for which a copy thereof is attached.

☐ Additional documents in the chain of title are listed on a supplemental sheet(s).

☒ As required by 37 CFR 3.73(b)(1)(i), the documentary evidence of the chain of title from the original owner to the assignee was, or concurrently is being, submitted for recordation pursuant to 37 CFR 3.11.

[NOTE: A separate copy (*i.e.*, a true copy of the original assignment document(s)) must be submitted to Assignment Division in accordance with 37 CFR Part 3, to record the assignment in the records of the USPTO. See MPEP 302.08]

The undersigned (whose title is supplied below) is authorized to act on behalf of the assignee.

Kenya Shitara
Signature

January 5, 2018.
Date

Kenya Shitara
Printed or Typed Name

Executive Officer and Director, Legal and Intellectual Property Department, Kyowa Hakko Kirin Co., LTD.
Title

Privacy Act Statement

The **Privacy Act of 1974 (P.L. 93-579)** requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (*i.e.*, GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Exhibit F

Letter from AstraZeneca AB authorizing Kyowa Hakko Kirin Co., Ltd to rely on any and all activities before the FDA necessary to extend the term of the '175 patent

Letter from MedImmune, LLC authorizing Kyowa Hakko Kirin Co., Ltd to rely on any and all activities before the FDA necessary to extend the term of the '175 patent

Letter from BioWa, Inc. authorizing Kyowa Hakko Kirin Co., Ltd to rely on any and all activities before the FDA necessary to extend the term of the '175 patent

AstraZeneca

December 19, 2017

AstraZeneca AB
SE-151 85 Södertälje, Sweden
T: +46 8 553 260 00
F: +46 8 553 290 00
astrazeneca.com

Commissioner of Patents
U.S. Patent and Trademark Office 600
Dulany Street (Madison Building)
Alexandra, VA 22314

Commissioner of Food and Drugs
U.S. Food and Drug Administration
10903 New Hampshire Avenue
Silver Spring, MD 20993

Re.: Patent Term Extension – FASENRA™ (benralizumab)
Biologics License Application No. 761070

Dear Commissioners:

AstraZeneca AB ("AstraZeneca") hereby authorizes Kyowa Hakko Kirin Co., Ltd. to rely upon the activities of AstraZeneca before the U.S. Food and Drug Administration during the regulatory review period for FASENRA™ (benralizumab) in making its application for extension of patent terms of U.S. Patent Nos. 7,179,464, 7,718,175 and 8,101,185. Kyowa Hakko Kirin Co., Ltd. is the assignee of the entire right, title and interest in and to U.S. Patent Nos. 7,179,464, 7,718,175 and 8,101,185. AstraZeneca further grants the Commissioner for Patents and the Secretary for Health and Human Services and Commissioner of Food and Drugs the right to refer to the above-approved Biologics License Application (BLA) in determining the eligibility of Kyowa Hakko Kirin Co., Ltd. for such extension.

BioWa, Inc. began the clinical investigation of benralizumab under IND No. 100,237. Effective as of March 7, 2007, BioWa, Inc. transferred ownership of the investigational new drug exemption to MedImmune, Inc. (now MedImmune, LLC). Subsequently, AstraZeneca, through its U.S. agent, AstraZeneca Pharmaceuticals LP, became the original marketing authorization applicant for FASENRA™ (benralizumab), submitted on November 16, 2016. AstraZeneca is the current holder of BLA No. 761070 for FASENRA™ (benralizumab), which was approved by the U.S. Food and Drug Administration on November 14, 2017.

Sincerely,

ASTRAZENECA AB

By: _____

Name: Jan-Olof Jacke

Title: President AstraZeneca AB

Date: 19 Dec 2017

MedImmune

January 3, 2018

Commissioner of Patents
U.S. Patent and Trademark Office
600 Dulany Street (Madison Building)
Alexandra, VA 22314

Commissioner of Food and Drugs
U.S. Food and Drug Administration
10903 New Hampshire Avenue
Silver Spring, MD 20993

Re.: Patent Term Extension - FASENRA™ (benralizumab)
Investigational New Drug No. 100,237
Biologics License Application No. 761070

Dear Commissioners:

MedImmune, LLC ("MedImmune") hereby authorizes Kyowa Hakko Kirin Co., Ltd. to rely upon the activities of MedImmune before the U.S. Food and Drug Administration during the regulatory review period for FASENRA™ (benralizumab) in making its application for extension of patent terms of U.S. Patent Nos. 7,179,464, 7,718,175 and 8,101,185. Kyowa Hakko Kirin Co., Ltd. is the assignee of the entire right, title and interest in and to U.S. Patent Nos. 7,179,464, 7,718,175 and 8,101,185. MedImmune further grants the Commissioner for Patents and the Secretary for Health and Human Services and Commissioner of Food and Drugs the right to refer to the above-referenced Investigational New Drug application (IND), in relation to Biologics License Application (BLA) 761070, in determining the eligibility of Kyowa Hakko Kirin Co., Ltd. for such extension.

BioWa, Inc. began the clinical investigation of benralizumab under IND No. 100,237. Effective as of March 7, 2007, BioWa, Inc. transferred ownership of the investigational new drug exemption to MedImmune, Inc. (now MedImmune, LLC). Subsequently, AstraZeneca AB, through its U.S. agent, AstraZeneca Pharmaceuticals LP, became the original marketing authorization applicant for FASENRA™ (benralizumab), submitted on November 16, 2016. AstraZeneca AB is the current holder of BLA No. 761070 for FASENRA™ (benralizumab), which was approved by the U.S. Food and Drug Administration on November 14, 2017.

Sincerely,

MEDIMMUNE, LLC.
By: Patrick Scott Ahern
Name: PATRICK SCOTT AHERN
Title: VP, IP and MedImmune LLC Corporate Secretary
Date: JANUARY 3, 2018

One MedImmune Way · Gaithersburg, Maryland 20878 · 301-398-9000 · Fax: 301-398-9306

BioWa

a member of the Kyowa Hakko Kirin Group

January 4, 2018

Commissioner of Patents

U.S. Patent and Trademark Office 600
Dulany Street (Madison Building)

Alexandra, VA 22314

Commissioner of Food and Drugs

U.S. Food and Drug Administration

10903 New Hampshire Avenue
Silver Spring, MD 20993

Re.: Patent Term Extension – FASENRA™ (benralizumab)
Investigational New Drug No. 100,237
Biologics License Application No. 761070

Dear Commissioners:

BioWa, Inc. ("BioWa") hereby authorizes Kyowa Hakko Kirin Co., Ltd. to rely upon the activities of BioWa before the U.S. Food and Drug Administration during the regulatory review period for FASENRA™ (benralizumab) in making its application for extension of patent term of U.S. Patent Nos. 7,179,464, 7,718,175 and 8,101,185. Kyowa Hakko Kirin Co., Ltd. is the assignee of the entire right, title and interest in and to U.S. Patent Nos. 7,179,464, 7,718,175 and 8,101,185. BioWa further grants the Commissioner for Patents and the Secretary for Health and Human Services and Commissioner of Food and Drugs the right to refer to the above-referenced Investigational New Drug application (IND), in relation to Biologics License Application (BLA) 761070, in determining the eligibility of Kyowa Hakko Kirin Co., Ltd. for such extension.

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BioWa

a member of the Kyowa Hakko Kirin Group

AstraZeneca AB is the current holder of BLA No. 761070 for FASENRA™ (benralizumab), which was approved by the U.S. Food and Drug Administration on November 14, 2017.

Sincerely,

BIOWA, INC.

By: _____

Name: Takeshi Masuda

Title: President and CEO

Date: January 5, 2018

Exhibit G

Certificate of Conversion – MedImmune, LLC

Delaware

The First State

I, HARRIET SMITH WINDSOR, SECRETARY OF STATE OF THE STATE OF DELAWARE DO HEREBY CERTIFY THAT THE ATTACHED IS A TRUE AND CORRECT COPY OF THE CERTIFICATE OF CONVERSION OF A DELAWARE CORPORATION UNDER THE NAME OF "MEDIMMUNE, INC." TO A DELAWARE LIMITED LIABILITY COMPANY, CHANGING ITS NAME FROM "MEDIMMUNE, INC." TO "MEDIMMUNE, LLC", FILED IN THIS OFFICE ON THE TWENTY-FIFTH DAY OF MARCH, A.D. 2008, AT 9:31 O'CLOCK P.M.

AND I DO HEREBY FURTHER CERTIFY THAT THE EFFECTIVE DATE OF THE AFORESAID CERTIFICATE OF CONVERSION IS THE FIRST DAY OF APRIL, A.D. 2008, AT 12:01 O'CLOCK A.M.

Harriet Smith Windsor

Harriet Smith Windsor, Secretary of State

2130616 8100V

080355665

AUTHENTICATION: 6478252

DATE: 03-26-08

You may verify this certificate online at corp.delaware.gov/authver.shtml

State of Delaware
Secretary of State
Division of Corporations
Delivered 09:31 PM 03/25/2008
FILED 09:31 PM 03/25/2008
SRV 080355665 - 2130616 FILE

CERTIFICATE OF CONVERSION

CONVERTING

MEDIMMUNE, INC.
(A Delaware Corporation)

TO

MEDIMMUNE, LLC
(A Delaware Limited Liability Company)

MedImmune, LLC, the continuing Delaware limited liability company (the "**Company**"), following the conversion of MedImmune, Inc. (the "**Converting Corporation**") to the Company, hereby certifies that:

1. Name of Converting Corporation. The name of the Converting Corporation immediately prior to the filing of this Certificate of Conversion was "MedImmune, Inc."

2. Date and Jurisdiction of Organization of Converting Corporation. The date on which, and the jurisdiction where, the Converting Corporation was organized are as follows:

Date	Jurisdiction
June 29, 1987	Delaware

3. Name of Converted Limited Liability Company. The name of the Delaware limited liability company to which the Converting Corporation has been converted and the name set forth in the Certificate of Formation of the Company filed in accordance with Section 18-214(b) of the Delaware Limited Liability Company Act is "MedImmune, LLC."

4. Approval of Conversion. The conversion of the Converting Corporation to the Company has been approved in accordance with the provisions of Section 266 of the Delaware General Corporation Law and Section 18-214 of the Delaware Limited Liability Company Act.

5. Effective Time. This Certificate shall be effective as of 12:01 a.m. on April 1, 2008 after its filing in the Office of the Secretary of the State of the State of Delaware.

{signature page follows}

IN WITNESS WHEREOF, the undersigned have duly executed this Certificate of Conversion as of March 25, 2008.

MedImmune, LLC

By: /s/William C. Bertrand
Name: William C. Bertrand, Jr.
Title: Senior Vice President, General Counsel and Corporate Secretary

Delaware

The First State

I, HARRIET SMITH WINDSOR, SECRETARY OF STATE OF THE STATE OF DELAWARE DO HEREBY CERTIFY THAT THE ATTACHED IS A TRUE AND CORRECT COPY OF CERTIFICATE OF FORMATION OF "MEDIMMUNE, LLC" FILED IN THIS OFFICE ON THE TWENTY-FIFTH DAY OF MARCH, A.D. 2008, AT 9:31 O'CLOCK P.M.

AND I DO HEREBY FURTHER CERTIFY THAT THE EFFECTIVE DATE OF THE AFORESAID CERTIFICATE OF FORMATION IS THE FIRST DAY OF APRIL, A.D. 2008, AT 12:01 O'CLOCK A.M.

Harriet Smith Windsor

Harriet Smith Windsor, Secretary of State

2130616 8100V

080355665

AUTHENTICATION: 6478252

DATE: 03-26-08

You may verify this certificate online at corp.delaware.gov/authver.shtml

State of Delaware
Secretary of State
Division of Corporations
Delivered 09:31 PM 03/25/2008
FILED 09:31 PM 03/25/2008
SRV 080355665 - 2130616 FILE

CERTIFICATE OF FORMATION
OF
MEDIMMUNE, LLC

This Certificate of Formation is being executed as of March 25, 2008 for the purpose of forming a limited liability company pursuant to the Delaware Limited Liability Company Act, 6 Del. C. §§ 18-101 et seq. (the "**Delaware LLC Act**").

The undersigned, being duly authorized to execute and file this Certificate of Formation, does hereby certify as follows:

1. Name. The name of the limited liability company is MedImmune, LLC (the "**Company**").

2. Registered Office and Registered Agent. The Company's registered office in the State of Delaware is located at Corporation Trust Center, 1209 Orange Street, Wilmington, New Castle County, Delaware 19801. The registered agent of the Company for service of process at such address is The Corporation Trust Company.

3. Conversion. The Company has been converted to a Delaware limited liability company pursuant to Section 18-214 of the Delaware LLC Act.

4. Effective Time. This Certificate shall be effective as of 12:01 a.m. on April 1, 2008 after its filing in the Office of the Secretary of the State of the State of Delaware.

IN WITNESS WHEREOF, the undersigned has duly executed this Certificate of Formation as of the day and year first above written.

/s/William C. Bertrand
Name: William C. Bertrand, Jr.
Title: Senior Vice President, General Counsel and Secretary

Exhibit H

Paper by Kolbeck, R., et al., *J. Allergy Clin. Immunol.*, 125(6):1344-1353 (2010)

MEDI-563, a humanized anti-IL-5 receptor α mAb with enhanced antibody-dependent cell-mediated cytotoxicity function

Roland Kolbeck, PhD,[a] Alexander Kozhich, PhD,[a] Masamichi Koike, PhD,[b] Li Peng, PhD,[c] Cecilia K. Andersson, PhD,[d] Melissa M. Damschroder, BA,[c] Jennifer L. Reed, PhD,[a*] Robert Woods, MS,[c] William W. Dall'Acqua, PhD,[c] Geoffrey L. Stephens, PhD,[a] Jonas S. Erjefalt, PhD,[d] Leif Bjermer, MD,[e] Alison A. Humbles, PhD,[a] David Gossage, MD,[f] Herren Wu, PhD,[c] Peter A. Kiener, PhD,[a**] George L. Spitalny, PhD,[b] Charles R. Mackay, PhD,[a***] Nestor A. Molfino, MD,[f] and Anthony J. Coyle, PhD[a] *Princeton, NJ, Gaithersburg and Rockville, Md, Lund, Sweden, and Darlinghurst, Australia*

Background: Peripheral blood eosinophilia and lung mucosal eosinophil infiltration are hallmarks of bronchial asthma. IL-5 is a critical cytokine for eosinophil maturation, survival, and mobilization. Attempts to target eosinophils for the treatment of asthma by means of IL-5 neutralization have only resulted in partial removal of airway eosinophils, and this warrants the development of more effective interventions to further explore the role of eosinophils in the clinical expression of asthma. Objective: We sought to develop a novel humanized anti–IL-5 receptor α (IL-5Rα) mAb with enhanced effector function (MEDI-563) that potently depletes circulating and tissue-resident eosinophils and basophils for the treatment of asthma. Methods: We used surface plasmon resonance to determine the binding affinity of MEDI-563 to FcγRIIIa. Primary human eosinophils and basophils were used to demonstrate antibody-dependent cell-mediated cytotoxicity. The binding epitope of MEDI-563 on IL-5Rα was determined by using site-directed mutagenesis. The consequences of MEDI-563 administration on peripheral blood and bone marrow eosinophil depletion was investigated in nonhuman primates. Results: MEDI-563 binds to an epitope on IL-5Rα that is in close proximity to the IL-5 binding site, and it inhibits IL-5–mediated cell proliferation. MEDI-563 potently induces antibody-dependent cell-mediated cytotoxicity of both eosinophils (half-maximal effective concentration = 0.9 pmol/L) and basophils (half-maximal effective concentration = 0.5 pmol/L) *in vitro*. In nonhuman primates MEDI-563 depletes blood eosinophils and eosinophil precursors in the bone marrow. Conclusions: MEDI-563 might provide a novel approach for the treatment of asthma through active antibody-dependent cell-mediated depletion of eosinophils and basophils rather than through passive removal of IL-5. (J Allergy Clin Immunol 2010;125:1344-53.)

Key words: Asthma, eosinophil, antibody-dependent cell-mediated cytotoxicity, FcγRIIIα, basophil, IL-5, IL-5 receptor, monoclonal antibody

Activated eosinophils are the cellular source of granule-associated basic proteins,[1] reactive oxygen species,[2] and lipid mediators,[3] which collectively can damage surrounding cells and induce airway hyperresponsiveness and mucus hypersecretion.[4,5] IL-5 is the principal cytokine mediating eosinophil mobilization, maturation, activation, and survival.[6] In human subjects IL-5 receptor (IL-5R) is expressed exclusively on eosinophil and basophil progenitors in the bone marrow (BM) and on mature eosinophils and basophils.[7-10] Indeed, neutralization of IL-5 in murine[11] and nonhuman primate[12] models of asthma resulted in reduction of eosinophil counts, which was associated with improved lung pathology. Furthermore, increased numbers of eosinophils in the airways and peripheral blood of subjects with asthma have been shown to correlate with asthma severity.[13]

These findings prompted the development of IL-5–neutralizing monoclonal antibodies (mAbs). In initial clinical trials IL-5 neutralization in subjects with mild-to-moderate asthma resulted in almost complete depletion of circulating and sputum eosinophil counts, but it did not improve lung function.[14,15] These observations have recently been corroborated in subjects with severe refractory asthma,[16,17] yet IL-5 neutralization by mepolizumab in those trials significantly reduced exacerbation frequency, improved Asthma Quality of Life Questionnaire scores, and allowed prednisone sparing, demonstrating for the first time a causal role for eosinophils in asthma exacerbations.[16,17] Interestingly, although mepolizumab significantly reduced circulating and sputum eosinophil counts, its effect on reducing mucosal eosinophilia was only partial at best and did not reach significance, even after prolonged high-dose exposure (12 months, 750 mg/mo).[16,18] Therefore more powerful means of depleting eosinophils in lung tissue are needed to further explore the contribution of eosinophils to asthma pathology.

From [a]the Department of Respiratory, Inflammation & Autoimmunity, [c]the Department of Antibody Discovery and Protein Engineering, and [f]Clinical Development, MedImmune, LLC, Gaithersburg; [b]Biowa, Inc. Princeton; [d]the Department of Experimental Medical Science, Lund University; [e]the Department of Respiratory Medicine and Allergology, Lund University Hospital; and [g]the Immunology and Inflammation Department, Garvan Institute of Medical Research, Darlinghurst.

*J. L. Reed is currently affiliated with the US Food and Drug Administration, Center for Biologics Evaluation and Research, Rockville, Md.

**P. A. Kiener is currently employed by Zyngenia, Inc, Gaithersburg, Md.

***C. R. Mackay is currently affiliated with the Immunology and Stem Cell Labs, Faculty of Medicine, Nursing and Health Sciences, School of Biomedical Sciences, Monash University, Clayton, Victoria, Australia.

Supported by MedImmune, LLC, Gaithersburg, Md, and Biowa, Inc, Princeton, NJ.

Disclosure of potential conflict of interest: R. Kolbeck, A. Kozhich, L. Peng, M. M. Damschroder, R. Woods, W. W. Dall'Acqua, G. L. Stephens, A. A. Humbles, D. Gossage, H. Wu, P. A. Kiener, N. A. Molfino, and A. J. Coyle are employed by MedImmune, LLC. J. L. Reed's husband is employed by MedImmune, LLC. C. R. Mackay is the Founder and Director of G2 Therapies. M. Koike and G.L. Spitalny are employed by Biowa, Inc. The rest of the authors have declared that they have no conflict of interest.

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Reprint requests: Roland Kolbeck, PhD, MedImmune, LLC, One MedImmune Way, Gaithersburg, MD 20878. E-mail: KolbeckR@medimmune.com.

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Abbreviations used
ADCC: Antibody-dependent cell-mediated cytotoxicity
APC: Allophycocyanin
BM: Bone marrow
BMMNC: Bone marrow mononuclear cell
EC_{50}: Half-maximal effective concentration
ECP: Eosinophil cationic protein
EDN: Eosinophil-derived neurotoxin
ELISA: Enzyme linked immunosorbent assay
FcγR: Fcγreceptor
IL-5R: IL-5 receptor
mAb: Monoclonal antibody
NK: Natural killer

Here we describe the development of MEDI-563, a novel humanized afucosylated monoclonal antibody IgG1κ mAb specific for the human IL-5Rα.[19] MEDI-563 binds to a conformationally distinct epitope within domain 1 of IL-5Rα, a region previously implicated in IL-5 binding.[20] Afucosylation of the oligosaccharide core of human IgG1 has previously been shown to result in a 5- to 50-fold higher affinity to human FcγRIIIa, the main activating Fcγ receptor (FcγR) expressed on natural killer (NK) cells, macrophages, and neutrophils.[21,22] Afucosylation enhances the interaction of MEDI-563 with FcγRIIIa and heightens antibody-dependent cell-mediated cytotoxicity (ADCC) functions by more than 1,000-fold over the parental antibody. These MEDI-563 properties might result in a more complete removal of airway eosinophils and basophils and subsequently result in greater reductions of asthma exacerbations and possibly improvements in other clinical expressions of asthma.

METHODS

Epitope mapping of MEDI-563

Extracellular IL-5Rα knockout mutants were engineered by substituting regions of full-length human IL-5Rα with corresponding segments of murine IL-5Rα and *vice versa* for knock-in mutants. Mutants were transiently expressed in HEK293F cells. HEK293F transfectants were incubated with 1 μg/mL MEDI-563 for 1 hour on ice in phosphate-buffered saline (PBS). After washing, cells were incubated with goat anti-human IgG–fluorescein isothiocyanate (Jackson ImmunoResearch Laboratories, West Grove, Pa) and then analyzed with the LSRII flow cytometer (BD Biosciences, San Jose, Calif). Expression levels of swap mutants were monitored with either goat anti-human IL-5Rα polyclonal antibody (GeneTex, San Antonio, Tex) or goat anti-mouse IL-5Rα polyclonal antibody (R&D Systems, Minneapolis, Minn).

ADCC assays

ADCC assays were performed with autologous NK cells as effector cells, as indicated. 10E4 NK cells and 10E5 bone marrow mononuclear cell (BMMNCs; approximately 1% IL-5Rα$^+$ cells) were added to each well in 96-well, flat-bottom microtiter plates. Serial dilutions of MEDI-563, parent αIL-5Rα mAb, or afucosylated hIgG1 isotype control were added. After 18 hours of incubation at 37°C, the assays were stopped. The number of IL-5Rα$^+$ cells was determined by means of flow cytometry with anti–IL-5Rα mAb KM 1257 (10 μg/mL) and phycoerythrin-labeled goat anti-mouse IgG F(ab')2 (Jackson ImmunoResearch Labs). For ADCC assays with peripheral blood–derived eosinophils or basophils, 5× 10E4 NK cells and 10E4 eosinophils or basophils were coincubated in 96-well flat-bottom plates in the presence of serial dilutions of MEDI-563 or parent αIL-5Rα mAb for 22 hours. Assays were stopped by putting plates on ice and replacing the culture medium with PBS/BSA to which Annexin V Alexa 647 at 1:500 dilution was added. Cells were analyzed on a flow cytometer (LSRII, BD Biosciences) and the percentage of Annexin V–positive eosinophils/basophils was measured. Eosinophils were identified based on their granularity (high side scatter) and basophils based on FcεRIα expression. ADCC was determined by gating on Annexin V–positive target cells. The recovery of target cells was quantitative (approximately 20% of the total cell number). Supernatants were collected at the end of ADCC assays to measure eosinophil cationic protein (ECP) and eosinophil-derived neurotoxin (EDN) levels by using an enzyme linked immunosorbent assay (ELISA). Total ECP and EDN levels were determined by means of eosinophil lysis with 1% Triton X-100 (100% degranulation), and a mixture of the cytokines RANTES (13 nmol/L), eotaxin (12 nmol/L), and IL-33 (6 nmol/L) was used as a positive control.

Administration of MEDI-563 in nonhuman primates

Thirty-four female and male cynomolgus monkeys were administered vehicle (n = 10) or MEDI-563 at 0.1 mg/kg (n = 4), 1 mg/kg (n = 6), 10 mg/kg (n = 4), or 30 mg/kg (n = 10) intravenously once on days 1, 22, 43, and 64. Six monkeys each from the vehicle, 1 mg/kg, and 30 mg/kg groups and 4 monkeys each from the 0.1 mg/kg and 10 mg/kg groups were killed and necropsied on day 67 (terminal necropsy), and the remaining monkeys were killed and necropsied on day 85 (recovery necropsies). BM smears were taken on the days of the necropsy. Eosinophil and neutrophil precursors (myeloblast and promyeloblast stages) were enumerated based on morphologic appearance. Blood was drawn on days −10 and −3 (baseline) and on days 4, 25, 46, and 64 during MEDI-563 administration, and eosinophils were enumerated with an Advia 120 hematology analyzer (Siemens, Deerfield, Ill). See the Methods section in this article's Online Repository at www.jacionline.org for additional information on reagents, proteins, and antibodies; cell lines and primary cells; measurements of kinetic rates and binding constants; Affymetrix gene array analysis; CTLL-2 proliferation assay; flow cytometry; ELISA; and immunohistochemistry.

RESULTS

MEDI-563 interaction with IL-5Rα

MEDI-563 bound to recombinant human and cynomolgus monkey IL-5Rα extracellular domains with a dissociation constant of 11 and 42 pmol/L, respectively, whereas the F(ab) fragment bound with an approximately 100-fold lower affinity, as assessed by means of surface plasmon resonance (Table I). Consistent with the specific expression of IL-5Rα on human eosinophils and basophils among a large variety of hematopoietic cell types (Fig 1, *A*), MEDI-563 exclusively stained peripheral blood eosinophils and basophils from healthy subjects (Fig 1, *B*). We consistently found that eosinophils expressed about a 3-fold higher level of IL-5Rα compared with basophils, as quantified based on median fluorescence intensity (Fig 1, *B*). In addition, MEDI-563 identified a small but specific fraction (approximately 0.9%) of BMMNCs that most likely represented the eosinophil/basophil lineage precursors (Fig 1, *B*). To further characterize the binding affinity of MEDI-563 to cell-surface IL-5Rα on peripheral blood eosinophils, we used flow cytometry in the presence of increasing concentrations of mAb. To overcome the high-background staining from the enhanced MEDI-563 interaction with FcγRIII expressed on human and cynomolgus monkey eosinophils,[23,24] we used the fucosylated parent αIL-5Rα mAb, which only differs from MEDI-563 in its lower binding affinity for FcγRIII (Table II). Parent αIL-5Rα stained human and cynomolgus monkey peripheral blood eosinophils with a half-maximal effective concentration (EC_{50}) of 26 and 40 pmol/L, respectively (Fig 1, *C*), values comparable with the MEDI-563 binding affinities to the extracellular receptor domains (Table I). Both mAbs inhibited IL-5–induced proliferation of CTLL-2 cells

TABLE I. Kinetic rate/binding constants of MEDI-563 and MEDI-563 F(ab) to human and cynomolgus monkey IL-5Rα

	MEDI-563			MEDI-563 F(ab)		
	K_{on} (1/ms × 10^5) ± SEM	K_{off} (1/s × 10^{-3}) ± SEM	K_D (nmol/L) ± SEM	K_{on} (1/ms × 10^5) ± SEM	K_{off} (1/s × 10^{-3}) ± SEM	K_D (nmol/L) ± SEM
Human IL-5Rα	43.6 ± 0.5	0.048 ± 0.02	0.011 ± 0.005	15.8 ± 2.6	1.92 ± 0.01	1.26 ± 1.0
Cyno IL-5Rα	252 ± 141	0.818 ± 0.301	0.042 ± 0.035	15.7 ± 2.1	31.8 ± 1.25	20.5 ± 1.95

Cyno, Cynomolgus monkey; K_D, dissociation constant K_{off}, off rate; K_{on}, on rate; *SEM*, standard error of the mean.

transfected with recombinant human IL-5Rαβ with identical potencies (half maximal inhibitory concentration = 0.3 nmol/L; Fig 1, *D*).

Mapping the binding epitope of MEDI-563 on IL-5Rα

The lack of MEDI-563 binding to the murine IL-5Rα was exploited as a means to identify the human IL-5Rα receptor epitope recognized by MEDI-563. Extracellular human IL-5Rα domains 1 (D1), 2 (D2), and 3 (D3) were replaced with the corresponding murine IL-5Rα domain sequences to create knockout variants or *vice versa* to create knock-in variants (Fig 2, *A*). The expression levels of all variants were monitored with anti-human or anti-mouse IL-5Rα polyclonal antibodies by using flow cytometry. In each instance MEDI-563 bound only to the constructs containing human IL-5Rα D1 (Fig 2, *A*). An alignment of human, cynomolgus monkey, and murine IL-5Rα D1 amino acid sequences identified differences between the receptors, and these areas were targeted to further characterize the binding epitope of MEDI-563 (Fig 2, *B*). Only swap mutants encoding human segment B were recognized by MEDI-563, thus identifying the region containing the binding epitope (Fig 2, *C*). Further refinement of the epitope was possible by swapping the amino acids in segment B not conserved between the human and murine sequences. Substituting amino acids N40, N42, Q46, D56, and E58 in the human IL-5Rα segment B with the corresponding murine residues had no effect on the MEDI-563 binding to human IL-5Rα. However, a single amino acid change to isoleucine at position 61 (I61) was sufficient to confer the MEDI-563 binding to murine IL-5Rα. Conversely, MEDI-563 binding to human IL-5Rα was obliterated by replacing I61 with the murine lysine residue at position 61 (K61), ultimately identifying amino acid I61 from the human IL-5Rα as the critical residue for MEDI-563 binding (Fig 2, *D*). Furthermore, segment B (amino acids 40-61) in D1 (including I61) is 100% conserved between human subjects and cynomolgus monkeys, thus explaining the cross-reactivity of MEDI-563 with cynomolgus monkey IL-5Rα (Fig 2, *B*).

MEDI-563 mediates eosinophil apoptosis *in vitro* through enhanced ADCC

The absence of the monosaccharide fucose on the oligosaccharide core of the human IgG1 has previously been shown to result in an enhanced binding affinity to human FcγRIIIa and subsequently an enhanced ADCC. Indeed, when tested by means of surface plasmon resonance with soluble human FcγR domains, the binding affinity of MEDI-563 for human FcγRIIIa was increased 6-fold compared with the fucosylated parental anti–IL-5Rα mAb (parent αIL-5Rα mAb) but was similar for all other FcγRs tested (Table II). We next investigated the potency of MEDI-563 to mediate eosinophil and basophil apoptosis *in vitro*. In the presence, but not absence, of autologous NK effector cells, MEDI-563 induced eosinophil and basophil apoptosis, as assessed by means of Annexin V staining, with EC_{50} values of 0.9 and 0.5 pmol/L, respectively (Fig 3, *A*). However, when the fucosylated parental αIL-5Rα mAb was used at concentrations 1,000-fold higher than the MEDI-563 EC_{50}, it did not induce target-cell apoptosis above background levels. (Fig 3, *A*), although its binding affinity for IL-5Rα (data not shown) and its potency to inhibit IL-5–induced cell proliferation (Fig 1, *D*) were indistinguishable from those of MEDI-563. MEDI-563 also depleted human IL-5Rα$^+$ BMMNCs when cocultured with NK effector cells, whereas an irrelevant afucosylated isotype control mAb was ineffective (Fig 3, *B*). In contrast to stimulation with a mixture of cytokines (RANTES, eotaxin, and IL-33), eosinophil apoptosis induced by MEDI-563 was not associated with the release of EDN or ECP, indicating a lack of significant eosinophil degranulation (Fig 3, *C*). Taken together, these data clearly indicate an enhanced MEDI-563 ADCC potency to deplete IL-5Rα–expressing eosinophils, basophils, and BMMNCs *in vitro* as a result of fucose deficiency.

Depletion of eosinophils in BM and peripheral blood of nonhuman primates

We have shown that MEDI-563 binds to human and cynomolgus monkey IL-5Rα on eosinophils with similar potency (Fig 1, *C*) and to human and cynomolgus monkey FcγRIIIa with a 6- and 8-fold higher affinity, respectively, compared with the parent αIL-5Rα mAb (Table II). Thus investigating the mechanism and potency of MEDI-563 to deplete eosinophils in cynomolgus monkeys proved to be a useful tool in predicting its effects in human subjects. Four MEDI-563 (0.1, 1, 10, and 30 mg/kg) or vehicle intravenous doses were administered to cynomolgus monkeys once every 3 weeks for 12 weeks. Peripheral blood eosinophils and BM eosinophil precursors were measured at different time points after drug administration. Blood eosinophil counts decreased close to the limit of detection after the first administration of MEDI-563 at all dose levels investigated and remained undetectable for the rest of the study (Fig 4, *A*). Similarly, eosinophil precursors in the BM, as assessed by histologic methods, were reduced 80% or greater in all the MEDI-563 doses 3 days after the last administration (terminal necropsy) and remained undetectable until 18 days after the last dose in the 30 mg/kg group (recovery necropsy; Fig 4, *B*). The profound effect observed with MEDI-563 in the BM was specific for the eosinophil lineage because numbers of neutrophil precursors (myeloblast and promyelocyte stages) remained unchanged (Fig 4, *C*). In conclusion, the pharmacologic efficacy and acceptable safety characteristics exhibited by MEDI-563 warrant its further investigation in subjects with asthma.

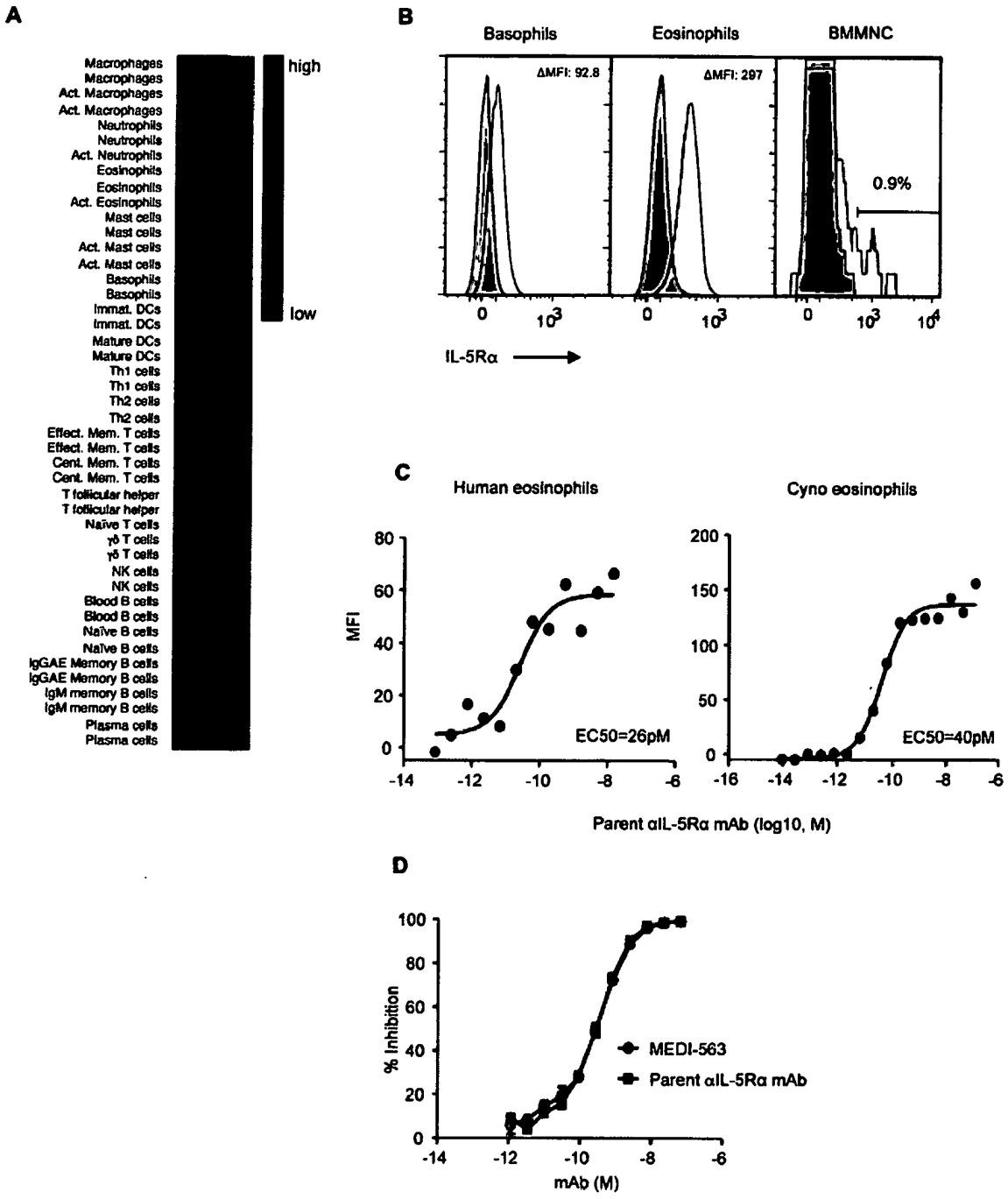


FIG 1. Interaction of MEDI-563 with IL-5Rα. **A**, *IL5RA* mRNA expression on human immune cells. **B**, Human IL-5Rα expression on basophils (n = 2), eosinophils (n = 3), and BMMNCs (n = 4) using MEDI-563 $F(ab')_2$ or an irrelevant control antibody *(shaded areas)*. **C**, Binding of parent αIL-5Rα mAb to human and cynomolgus monkey *(Cyno)* eosinophils. *MFI*, Mean fluorescence intensity. **D**, Inhibition of IL-5–induced CTLL-2 cell proliferation (± SD). Shown are representative experiments. *n*, Number of experiments performed.

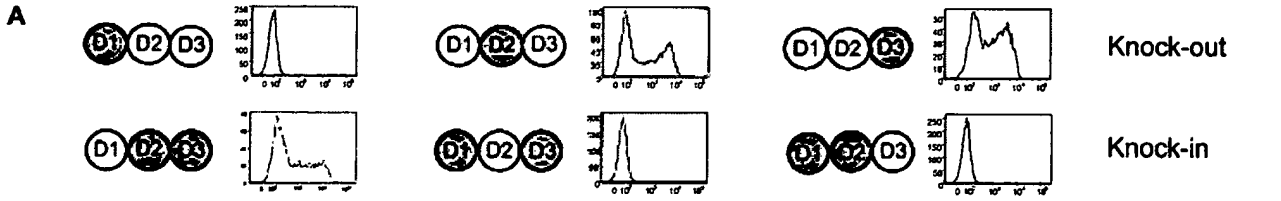
IL-5Rα expression in lung tissue of subjects with mild asthma

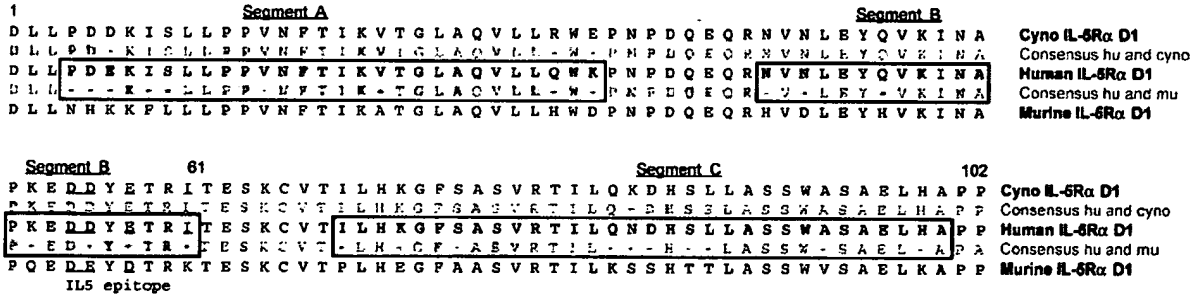
To gain better insight into the ability of MEDI-563 to bind to lung tissue–resident eosinophils, we performed immunohistochemistry studies on biopsy specimens from subjects with mild atopic asthma. Mast cells were frequently present in both bronchi (49 ± 8 cells/mm^2) and peripheral tissue (62 ± 15 cells/mm^2) along with tissue eosinophilia (bronchi, 168 ± 90 cells/mm^2; peripheral

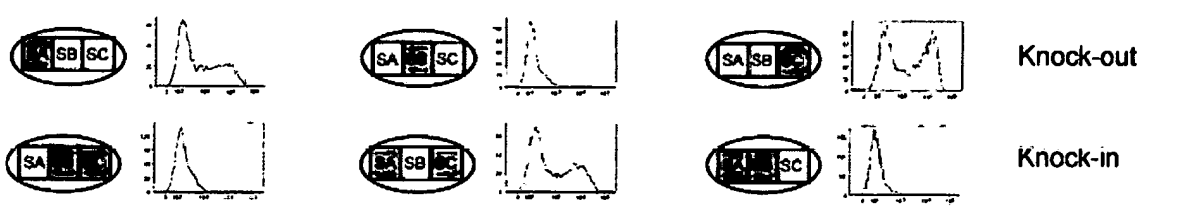
TABLE II. Binding affinities of MEDI-563 and parent αIL-5Rα mAb to human and cynomolgus monkey Fcγ receptors assessed by means of surface plasmon resonance*

	Human FcγRI, K_D (nmol/L) ± SEM	Human FcγRIIa, K_D (nmol/L) ± SEM	Human FcγRIIb, K_D (nmol/L) ± SEM	Human FcγRIIIa(V), K_D (nmol/L), ± SEM	Cyno FcγRI, K_D (nmol/L)	Cyno FcγRIIa, K_D (nmol/L)	Cyno FcγRIIb, K_D (nmol/L)	Cyno FcγRIIIa, K_D (nmol/L) ± SEM
MEDI-563	18.5± 2.5	1,280 ± 10	4,580 ± 1,150	45.5 ± 0.5	1	3,170	2,070	23 ± 3
Parent αIL-5Rα mAb	18	1,170	3,890	275.5 ± 0.5	1	2,970	1,720	195.5 ± 10.5

Cyno, Cynomolgus monkeys; K_D, dissociation constant; *SEM*, standard error of the mean.
*Note the 6- and 8-fold higher affinity of MEDI-563 for human and cynomolgus monkey FcγRIIIa, respectively, without affecting the affinity for other Fcγ receptors.







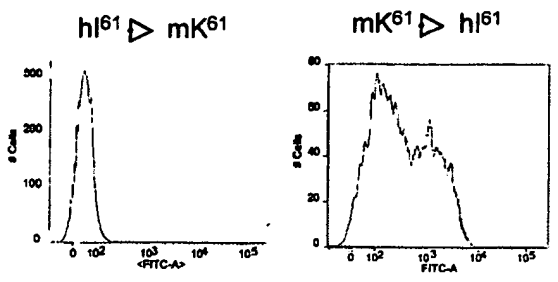


FIG 2. Identification of the MEDI-563 binding epitope to human IL-5Rα. **A**, MEDI-563 binding to extracellular chimeric human IL-5Rα *(light green)* and murine IL-5Rα *(dark green)* variants. *D*, Domain. **B**, Sequence alignment of murine, human, and cynomolgus monkey *(Cyno)* IL-5Rα D1 (differences are shown as *dashes*; segments A *[SA]*, B *[SB]*, and C *[SC]* are boxed). **C**, MEDI-563 binding to IL-5Rα segment variants. **D**, Isoleucin 61 (I61) of human IL-5Rα is the critical residue for MEDI-563 binding. I61 is shown in *red* in Fig 2, *B*.

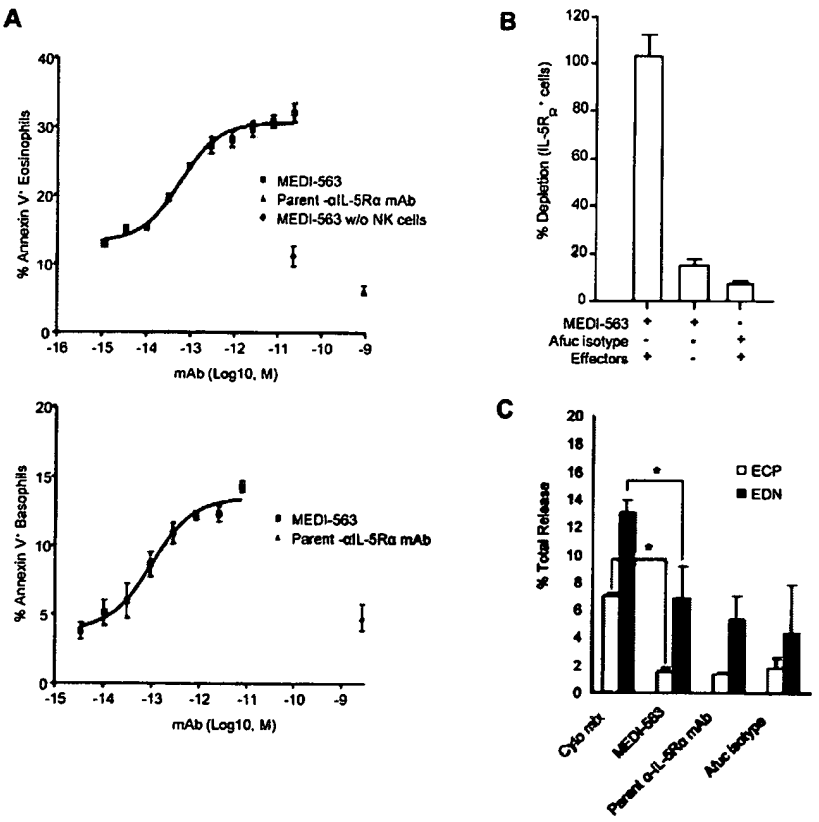


FIG 3. Enhanced ADCC of MEDI-563. **A**, MEDI-563–mediated ADCC of human eosinophils (EC_{50} = 0.9 pmol/L, n = 5) and basophils (EC_{50} = 0.5 pmol/L, n = 3). **B**, Depletion of human IL-5Rα$^+$ BMMNCs by MEDI-563 *in vitro* (n = 2). **C**, ADCC of eosinophils is not associated with ECP and EDN release (n = 2). *Afuc isotype*, Afucosylated isotype antibody control. Total ECP and EDN release induced by Triton X-100 was set to 100%. *Cyto mix*, RANTES plus eotaxin plus IL-33. Shown are representative experiments. *n*, Number of experiments performed. *Significant as determined by using the Student *t* test ($P < .05$).

tissue, 227 ± 59 cells/mm^2). In both bronchial and peripheral tissue biopsy specimens, scattered cells displayed an intense immunoreactivity with MEDI-563 (Fig 5, *A* and *B*). Generally, the staining had a characteristic surface membrane pattern (Fig 5, *A*, inset). No staining was found with isotype-matched control antibodies or in control subjects lacking primary antibodies. Furthermore, nonspecific binding of MEDI-563 to Fc receptors on eosinophils was ruled out because F(ab')$_2$ fragments of MEDI-563 produced a similarly intense immunoreactivity. Parallel identification of eosinophils and MEDI-563 staining confirmed a widespread and uniform MEDI-563–positive immunoreactivity on tissue eosinophils at all airway levels (Fig 5, *C*). Double staining for IL-5Rα and mast cell tryptase was performed to test whether MEDI-563 bound to mast cells (Fig 5, *D-F*). In short, our analysis failed to detect any colocalization, suggesting that airway mast cells do not express IL-5Rα.

In conclusion, the present study demonstrates the capacity of MEDI-563 to recognize tissue eosinophils in both central and peripheral airways of subjects with asthma. Although single intravenous administrations of MEDI-563 in an initial phase I study of subjects with mild asthma resulted in persistent peripheral blood eosinopenia lasting up to 3 months,[25] it remains to be seen whether MEDI-563 can also efficiently deplete lung tissue eosinophils.

DISCUSSION

IL-5 blockade in subjects with asthma has failed to improve parameters of lung function in response to allergen challenge despite rapid and near-complete depletion of eosinophils from peripheral blood and sputum.[14-17] However, a causal link between reduced eosinophil numbers and a reduction in exacerbation frequency and prednisone requirement has recently been demonstrated in subjects with severe refractory eosinophilic asthma.[16,17] Interestingly, results from 2 independent clinical trials demonstrated that eosinophil depletion from the airways only reached 50% to 60% compared with eosinophil counts before drug administration, even with repeated doses of the anti–IL-5 antibody mepolizumab.[16,18] Although IL-5 is considered to be essential for eosinophil differentiation and mobilization, other cytokines, including IL-3 and GM-CSF, are also important for eosinophil survival. This might provide an explanation for the eosinophil persistence during IL-5 blockade.[26,27] Therefore a strategy that depletes lung eosinophils and basophils more effectively might result in a greater reduction in exacerbation frequency and provide better asthma control.

The restricted expression of IL-5Rα on human eosinophils and basophils and their progenitors in the BM renders IL-5Rα an ideal target for specific cell depletion. Compared with IL-5–neutralization strategies, our anti–IL-5Rα mAb, MEDI-563, might

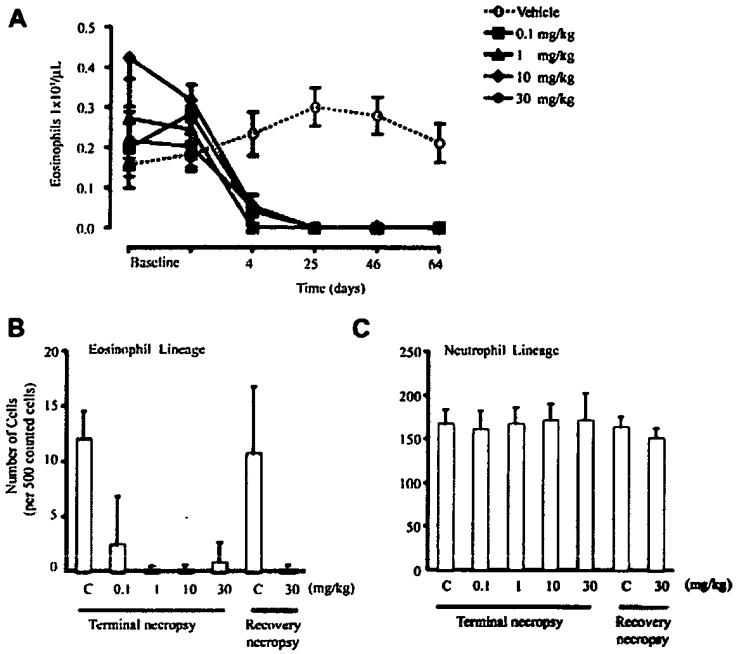


FIG 4. Eosinophil depletion in cynomolgus monkeys by MEDI-563. **A**, Peripheral blood eosinophil depletion in cynomolgus monkeys. **B** and **C**, Depletion of BM eosinophil precursors (Fig 4, *B*) and neutrophil precursors (Fig 4, *C*). Data indicate the average of 3 to 10 monkeys per group, and *error bars* represent the SD.

provide a more complete depletion of eosinophils through enhanced ADCC. MEDI-563 inhibits IL-5R signaling; however, this neutralizing effect is not central to its biological function. Afucosylation of MEDI-563 significantly increases its binding affinity to FcγRIIIa and consequently enhances its ability to engage with FcγRIIIa on effector cells, such as NK cells and macrophages. Indeed, only in the presence of NK effector cells did MEDI-563 mediate eosinophil and basophil apoptosis, with an EC_{50} of 0.9 and 0.5 pmol/L, respectively, and deplete IL-5Rα–expressing BMMNCs *in vitro*. However, even when used at concentrations 4 orders of magnitude greater than the MEDI-563 EC_{50}, the parental fucosylated αIL-5Rα mAb did not induce significant eosinophil killing, thus illustrating the importance of enhanced Fc engagement. Interestingly, although peripheral blood basophils expressed about 3-fold less IL-5Rα compared with eosinophils, the ADCC potency of MEDI-563 was indistinguishable. Therefore very low levels of cell-surface IL-5Rα expression seemed to be sufficient for the engagement of MEDI-563 in mediating potent ADCC of IL-5Rα–expressing target cells.

We have mapped the MEDI-563 binding site to an epitope that has previously been identified as a portion of the IL-5 binding site,[20] providing an explanation for its neutralizing activity. Furthermore, we have identified I61 on the human IL-5Rα D1 to be essential for MEDI-563 binding. I61 is conserved in human and cynomolgus monkey IL-5Rα, but not in murine IL-5Rα, explaining the cross-reactivity of MEDI-563 to cynomolgus monkey IL-5Rα but not to the murine homolog.

MEDI-563 bound to immobilized recombinant IL-5Rα and IL-5Rα expressed on human and cynomolgus monkey peripheral blood eosinophils with high affinity (26 and 40 pmol/L, respectively). However, the binding affinity of its F(ab) fragment to immobilized recombinant human and cynomolgus monkey IL-5Rα extracellular domains was about 100-fold lower, suggesting that avidity might enhance the binding of MEDI-563 to IL-5Rα. MEDI-563 also inhibited IL-5–induced proliferation of human IL-5Rαβ transfected CTLL-2 cells *in vitro*, although with a potency of 300-fold lower than its ADCC activity on eosinophils and basophils. It might be possible that neutralization of IL-5–mediated proliferation of transfected CTLL-2 cells requires a high degree of receptor occupancy by MEDI-563, whereas engagement of only a few receptors is sufficient to mediate potent ADCC. Eosinophils are the source of cationic granule proteins, such as major basic protein, ECP, EDN, and eosinophil peroxidase, which are stored in crystalloid granules. When released into the extracellular space, these basic proteins have been shown to be toxic to parasites and bacteria.[28,29] However, the toxicity is not only limited to foreign pathogens but also affects diverse cell types in the host. For example, major basic protein has been shown to induce airway hyperresponsiveness[5] and cytotoxicity against the airway epithelium,[30,31] whereas both ECP and EDN cause neurotoxicity.[32] Therefore ADCC-mediated killing of eosinophils might pose substantial safety risks associated with the potential release of cationic eosinophil proteins during cytolysis or accidental necrosis.[33] We measured the release of EDN and ECP in ADCC assays *in vitro* and found no indication that eosinophil apoptosis mediated by MEDI-563 was associated with increased levels of these proteins.

In nonhuman primates repeat administrations of MEDI-563 at all dose levels rapidly depleted peripheral blood eosinophils to less than the limit of detection, demonstrating the desired pharmacologic effect. Of particular note, MEDI-563 also depleted eosinophil precursors from the cynomolgus monkey BM without affecting granulocytic stem cell counts (myeloblast and promyelocyte stages). Because FcγRIIIa expression is conserved

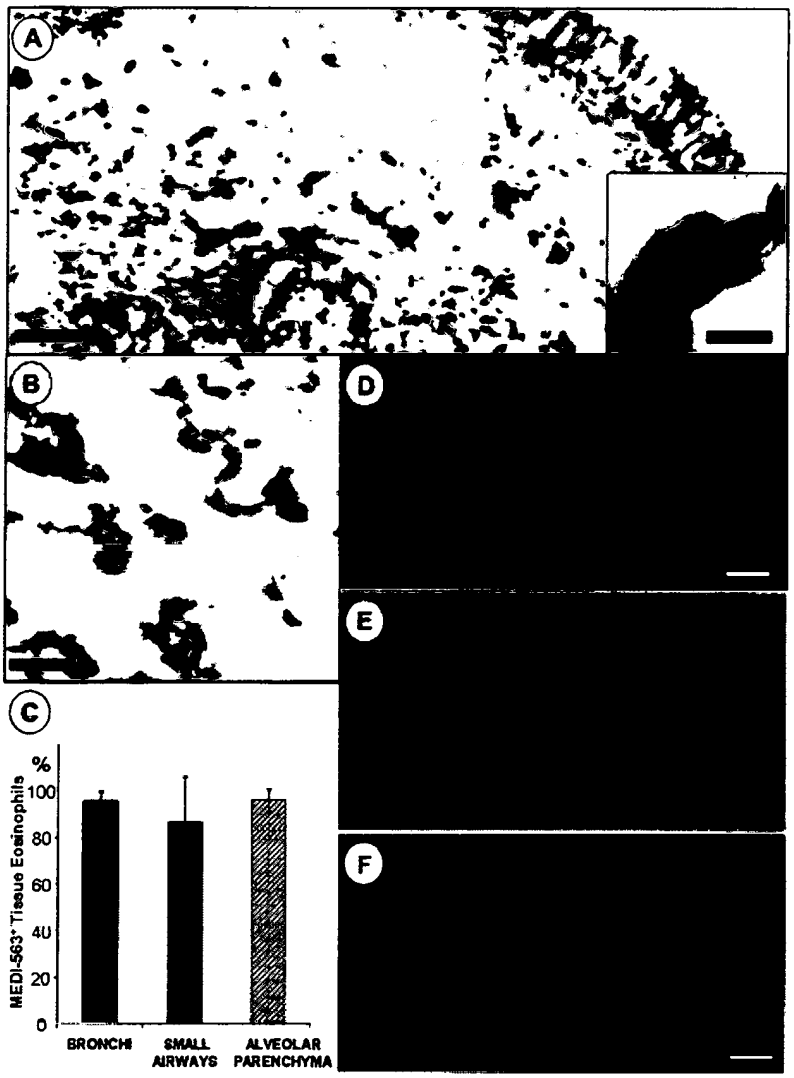


FIG 5. Immunohistochemical localization of IL-5Rα–expressing cells in lung tissue. **A** and **B**, Bright field micrographs depicting MEDI-563 immunoreactive cells *(red)* in the bronchial mucosa (Fig 5, *A*) and the alveolar parenchyma (Fig 5, *B*). Fig 5, *A, Inset*, High-power magnification. **C**, Quantification of eosinophils positive for MEDI-563 staining (± SD). **D**, Mast cell tryptase *(green)* and IL-5Rα *(red)*. **E**, Red-stained eosinophils *(E)* in Fig 5, *D*, are identified through their distinct granule morphology. **F**, Lack of IL-5Rα staining in mast cells.

between human subjects and cynomolgus monkeys,[23,34,35] the data suggest that similar mechanisms might operate in both species for the depletion of peripheral blood eosinophils and possibly that of eosinophil precursors in the BM. Indeed, administration of single intravenous MEDI-563 doses in an initial phase I study of subjects with mild asthma has demonstrated rapid, long-lasting, and reversible depletion of peripheral blood eosinophils in a dose-dependent manner.[25] Whether the depletion of eosinophil precursors in the BM of human subjects might contribute to the sustained peripheral eosinopenia induced by MEDI-563 remains to be demonstrated. In 2 case studies eosinophil and basophil deficiency have been reported to be associated with reduced immunity and recurrent infections,[36,37] raising the possibility that sustained eosinophil and basophil depletion in human subjects by MEDI-563 might result in similar safety signals. Whether this will indeed be the case is currently being addressed in clinical trials with multiple administrations of MEDI-563.

Using immunohistochemical methods, we have demonstrated that MEDI-563 prominently stained all the eosinophils from lung biopsy specimens of subjects with mild asthma, a prerequisite for the successful and swift removal of eosinophils from the lung through mechanisms involving ADCC. Active and rapid depletion of lung eosinophils will also depend on the appropriate number and localization of effector cells and sufficient levels of MEDI-563 in lung tissue. Furthermore, MEDI-563–mediated depletion of eosinophils in subjects with asthma might be influenced by the downregulation of IL-5Rα on eosinophils in the lung after an allergen challenge,[38] IL-5 binding,[39,40] and the use of corticosteroids.[41] These factors alone or in combination might affect the degree and kinetics of MEDI-563–mediated

eosinophil depletion in subjects with asthma. Currently, clinical studies are in progress to address the effectiveness of MEDI-563 in depleting lung eosinophils in subjects with eosinophilic asthma.

Basophils are associated with allergic inflammation and, similar to eosinophils, are linked to asthma severity and exacerbations. Recent *in vitro* studies have demonstrated that basophils constitutively express IL-5Rα[8] but do not require the cytokine IL-5 for survival.[42] *In vivo* basophils have been shown to play a critical role in the development of IgE-mediated chronic allergic inflammation in the skin[43] and to increase in numbers in the airways of asthmatic subjects on allergen provocation.[44] Here we present data showing that MEDI-563 potently induces basophil apoptosis *in vitro*. However, the ability of MEDI-563 to deplete basophils *in vivo* and its downstream consequences remain to be demonstrated. Also, mast cells and mast cell–derived mediators, such as biogenic amines, leukotrienes, cytokines, and chemokines, have been implicated in asthma pathogenesis. In lung biopsy specimens from subjects with mild asthma, we have not been able to detect IL-5Rα expression in mast cells, although a recent study has demonstrated IL-5Rα expression in human mast cells *in vitro*.[45] One might speculate that mast cells could express IL-5Rα *in vivo* and become transiently sensitive to MEDI-563–mediated depletion under certain circumstances. Currently, no experimental evidence exists to support such a notion.

In summary, we report the characterization of a novel anti–IL-5Rα mAb, MEDI-563, that mediates the killing of eosinophils and basophils *in vitro* and in nonhuman primates through an enhanced ADCC function. Continued investigation of MEDI-563 activity in asthmatic subjects might provide new insights into the relative contributions of eosinophils and basophils, and active depletion of these cells might provide a novel approach for the treatment of bronchial asthma.

We thank S. Phipps and S. Wilson for cloning the cynomolgus monkey IL-5Rα sequence and for providing recombinant extracellular IL-5Rα domains. We also thank Dr M. Mense, Dr J. Leininger, Dr N. Hanai, F. Okada, and other collaborators of Kyowa Hakko Kirin Co, Ltd, for helpful comments and suggestions.

Key message

- MEDI-563 is a humanized anti–IL-5Rα mAb with enhanced ADCC function that potently induces eosinophil apoptosis *in vitro* and efficiently depletes peripheral blood eosinophils and eosinophil precursors in nonhuman primates.

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METHODS

Reagents, proteins, and antibodies

Parent αIL-5Rα mAb and MEDI-563 were produced in wild-type Chinese Hamster Ovary (CHO) cells and *FUT8*-deficient CHO cells, respectively. Anti–IL-5Rα mAb KM1257 binds to an epitope on human IL-5Rα without interfering with MEDI-563 binding and *vice versa*. Antibodies were purified by means of Protein G affinity chromatography. Extracellular FcγR domains and extracellular IL-5Rα domains were constructed with C-terminal 6xHis tags, expressed in HEK293F cells, and purified by means of nickel affinity chromatography. Cytokines were purchased from R&D Systems (Minneapolis, Minn). RPMI 1640 medium containing high-glucose heat-inactivated FBS and horse serum was obtained from Invitrogen (Carlsbad, Calif).

Cell lines and primary cells

A CTLL-2 human IL-5Rαβ stably transfected cell line was received from Dr K. Takatsu (University of Tokyo). Cells were cultured in RPMI 1640 supplemented with 10% FBS, 50 μmol/L 2-mercaptoethanol, 1 ng/mL human IL-5, and 5 ng/mL murine IL-2 and cultured at 37°C and 5% CO_2 in saturating humidity. Eosinophils and basophils were isolated from heparinized peripheral blood of healthy donors. Red blood cells were removed by means of sedimentation in Hetasep (StemCell Technologies, Vancouver, British Columbia, Canada). Eosinophils or basophils were further enriched from the leukocyte fraction by means of negative selection with EasySep eosinophil or basophil enrichment kits, respectively (StemCell Technologies). The purity and viability of isolated cells were verified by means of flow cytometry. PBMCs were isolated by means of density gradient centrifugation with Histopaque-1077 (Sigma, St Louis, Mo). NK cells were isolated from PBMCs by means of negative selection with the EasySep NK enrichment kit (StemCell Technologies).

Measurements of kinetic rates and binding constants

All measurements were performed on a BIAcore 3000 instrument (BIAcore, Inc, Uppsala, Sweden). Human and cynomolgus monkey IL-5Rα was immobilized at low density onto separate flow cells on the same CM5 sensor chip by using a standard amino coupling chemistry, as outlined by the instrument's manufacturer. The final receptor domain densities were virtually identical at 585 RUs (human IL-5Rα) and 586 RUs (cynomolgus monkey IL-5Rα). A reference flow cell surface was also prepared on this sensor chip by using the identical immobilization protocol minus the protein. Serial dilutions of MEDI-563 and MEDI-563 F(ab) were prepared in HBS-EP buffer (pH 7.4) containing 0.01 mol/L HEPES, 0.15 mol/L NaCl, 3 mmol/L EDTA, and 0.005% P-20 surfactant. Samples were injected at a flow rate of 75 μL/min. Dissociation data were collected for 10 to 15 minutes, followed by a 60-second pulse of 4 mol/L $MgCl_2$ between injections to regenerate the receptor surfaces on the chip. MEDI-563 and parent αIL-5Rα were immobilized at high density onto separate flow cells on CM5 sensor chips by using a standard amino coupling chemistry, as outlined by the instrument's manufacturer, to determine the affinity (dissociation constant) for the binding of all available human and cynomolgus monkey FcγRs. The final IgG surface densities were very similar, ranging from 7,378 to 7,958 RUs. Stock solutions of FcγRs, starting at 4, 16, or 32 μmol/L were serially diluted to the desired concentrations with HBS-EP buffer. Different concentrations of FcγRs were then injected over both the IgG and reference cell surfaces at a flow rate of 5 μL/min. Binding data were collected for 50 minutes, followed by a 60-second pulse of 5 mmol/L HCl between injections to regenerate (remove bound FcγRs) from the IgG surfaces. Several buffer injections were also interspersed throughout the injection series. Select buffer injections were used along with the reference cell data to correct the raw datasets for injection artifacts, nonspecific binding interactions commonly referred to as double-referencing, or both.[E1] Fully corrected binding data were then globally fit to a 1:1 binding model (BIAevaluation 4.1 software; BIAcore, Inc) that included a term to correct for mass transport–limited binding, should it be detected. These analyses determined the kinetic rate (on or off) constants from which the apparent dissociation constant was then calculated as K_{off}/K_{on}.

Affymetrix gene array analysis

Total RNA was isolated from cells by using the RNeasy Total RNA Isolation Kit (Qiagen, Chatsworth, Calif) or TRIzol (Invitrogen Life Technologies), as per the manufacturer's instructions. cRNA was prepared and fluorescence intensities were measured on Affymetrix U133A and B arrays by using an Agilent GeneArray Laser Scanner, and absolute gene expressions were determined and scaled to 150 by using algorithms in MicroArray Analysis Suite 5.0 Software (Affymetrix). Spotfire (Somerville, Mass) was used to map gene expression patterns and produce heat maps. All of the datasets used in this study and the methods used for the purification of the relevant human leukocyte subsets have been previously described.[E2,E3]

CTLL-2 proliferation assay

CTLL-2 human IL-5Rαβ cells were washed twice with RPMI 1640 medium and cultured overnight in complete medium (no IL-2 and no IL-5). After overnight starvation, CTLL-2 human IL-5Rαβ cells were collected and adjusted to 0.2×10^6 cells/mL. Cells were plated at 50 μL per well in flat-bottom 96-well plates. IL-5 was adjusted to 1.2 ng/mL in complete medium, and 25 μL per well was added. Antibodies were adjusted to 40 μg/mL in complete medium, and serial 2-fold dilutions were prepared from these stocks. Antibody dilutions were added at 25 μL per well to the plate and incubated for 48 hours (at 5% CO_2 and 37°C). One hundred microliters of Titer Glo (Promega, Madison, Wis) assay solution was added to each well. After mixing, 150 μL per well was transferred into a new white 96-well plate (Corning, Lowell, Mass). Luminescence was read with the luminescence reader Victor 2 (PerkinElmer, Waltham, Mass).

Flow cytometry

Binding affinity of the parent αIL-5Rα mAb to eosinophils in whole blood from healthy human donors and cynomolgus monkeys was examined by means of flow cytometry. Human IgG1 was added to 100-μL whole blood serial dilutions of the parent αIL-5Rα mAb or control, and samples were vortexed briefly. After 1 hour of incubation at room temperature, samples were washed with PBS (2 × 2 mL). A 20 μL per tube 1:10 dilution of goat anti-human IgG Fcγ-specific $F(ab')_2$ allophycocyanin (APC) (Jackson ImmunoResearch Laboratories) was added and mixed well. After 1 hour at room temperature, 2 mL of FACS lysing buffer (BD Biosciences) was added. After 10 minutes, incubation samples were washed twice with PBS and analyzed on a LSRII flow cytometer. Eosinophils were identified in the granulocyte gate as cells with high autofluorescence in phycoerythrin and fluorescein isothiocyanate channels.[E4] Mean fluorescence intensity in the APC channel was recorded. Staining intensities were calculated by subtracting the isotype control mean fluorescence intensity from the parent αIL-5Rα mAb mean fluorescence intensity. Prism 5 software (Prism Software Corp, Irvine, Calif) was used to calculate binding curves and EC_{50} values.

ELISA

ECP and EDN levels in supernatants from ADCC experiments were quantified by means of ECP ELISA (MBL International, Woburn, Mass) and EDN ELISA (MBL International, Woburn, Mass), respectively.

Immunohistochemistry

A cohort consisting of 9 subjects (6 male and 3 female subjects) with mild asthma was selected. All subjects were atopic, and none were taking inhaled steroids at that time point in the study. Mean and range values for PD_{20} (methacholine) and FEV_1 percent predicted were 75.6 μg (16-145 μg) and 98.8% (88% to 124%), respectively. From each patient, multiple bronchial and transbronchial biopsy specimens were collected. Transbronchial biopsy specimens were performed under radiographic guidance. All subjects provided written informed consent to participate in the study, which was approved by the local ethics committee (Lund, Sweden). Immediately after, collected biopsy specimens were immersed in PLP fixative (2% paraformaldehyde, 0.075 mol/L

lysine, 0.037 mol/L sodium phosphate, and 0.01 mol/L periodate), subjected to fixation overnight, and thereafter rinsed in sucrose buffer before being mounted at −76°C in Tissue-Tek freeze mounting media. The usefulness of MEDI-563 for immunohistochemistry was tested through an initial screening of multiple immunohistochemical protocols. In the present study we used a protocol detecting MEDI-563 immunoreactivity in human tissues after direct labeling MEDI-563 or MEDI-563 $F(ab)_2$ with biotin or the fluorochrome Alexa-647 (Invitrogen). Briefly, 10 μmol/L cryosections were incubated with primary antibody for 2 hours at room temperature. As standard visualization, biotinylated MEDI-563 sections were incubated with AP-conjugated streptavidin, and the immunoreactivity was visualized with the New Fuchsin + Substrate-Chromogen System (K0625, Dako, Glostrup, Denmark), according to the manufacturer's directions. For visualization of the eosinophil IL-5Rα, eosinophils were identified by means of anti-ECP staining (EG-2, Pharmacia) or their characteristic granule morphology by using differential interference contrast microscopy. Mast cells were identified by antibodies directed at mast cell tryptase (clone G3; Chemicon, Temecula, Calif) and visualized by Alexa-488-labeled secondary antibodies (Molecular Probes, Eugene, Ore).

Euthanasia of cynomolgus monkeys

On the day of necropsy, animals were first sedated with ketamine and then weighed and anesthetized by using an intravenous injection of a commercial euthanasia solution containing pentobarbital and phenytoin. Euthanasia of the anesthetized animals was then performed by means of exsanguination.

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Exhibit I

Activities during IND Period

Sequence Number	Submission Date	Submission Type	Submission Description
0000	6/29/2006	original-application	Original IND
0001	2/28/2007	Clinical	Protocol Amendment
0002	3/5/2007	Other	Biowa IND transfer
0003	3/5/2007	Other	MedImmune IND Transfer
0004	4/5/2007	Quality	CMC Response to FDA Sept 20, 2006
0005	4/26/2007	Clinical	Protocol Amendment
0006	7/24/2007	Clinical	New Investigator
0007	8/16/2007	Clinical	Protocol Amendment
0008	9/27/2007	Safety	Annual Report 2007
0009	11/13/2007	Clinical	Protocol Amendment
0010	2/4/2007	Clinical	New Protocol and IB
0011	2/15/2008	Quality	DS OOS Amendment
0012	4/9/2008	Other	MedImmune name change
0013	5/7/2008	Clinical	Protocol Amendment and New Investigators
0014	6/6/2008	Quality	MEDI-563 Placebo CMC Information
0015	6/13/2008	Other	Change in Contact
0016	6/13/2008	Clinical	Protocol Amendment

Sequence Number	Submission Date	Submission Type	Submission Description
0017	7/1/2008	Quality	Reponses to FDA Questions and Comments
0018	8/22/2008	Clinical	New Investigator
0019	9/19/2008	Clinical	New Investigator
0020	9/23/2008	Safety	Annual Report 2008 - 28Jul2007 – 29Jul2008
0021	10/2/2008	Quality	Additional Processing Step of AppTec Drug Substance Amendment
0022	10/2/2008	Non-clinical	Pharmacology Toxicology Amendment
0023	10/9/2008	Clinical	New Protocol and IB
0024	11/3/2008	Non-clinical	Additional Pharmacology Toxicology Data
0025	11/14/2008	Non-clinical	Request for feedback on Tox Study Design
0026	11/14/2008	Clinical	New Investigator
0027	11/21/2008	Clinical	Protocol Amendment and New Investigator
0028	12/16/2008	Clinical	New Investigator
0029	1/15/2009	Non-clinical	Pharmacology Toxicology Information Amendment
0030	2/4/2009	Non-clinical	Pharmacology Toxicology Information Amendment
0031	2/4/2009	Non-clinical	Response to FDA Request for Info

Sequence Number	Submission Date	Submission Type	Submission Description
0032	2/6/2008	Clinical	Protocol Amendment and New Investigator
0033	2/10/2008	Clinical	Protocol Amendment
0034	4/2/2009	Clinical	IB, Protocol Amendment and New Investigators
0035	4/14/2009	Clinical	Protocol Amendment
0036	6/24/2009	Clinical	Protocol Amendment
0037	7/17/2009	Clinical	Protocol Amendment -New Investigators CP186
0038	8/6/2009	Clinical	Protocol Amendment
0039	9/1/2009	Clinical	Protocol Amendment and New Investigator
0040	9/4/2009	Clinical	Protocol Amendment
0041	9/9/2009	Quality	MedImmune Manufacturing Process and Responses to CMC Comments
0042	9/25/2009	Safety	Annual Report 2009 - 30Jul2008-29Jul2009
0043	10/9/2009	Clinical	Protocol Amendment and New Investigators
0044	10/30/2009	Clinical	New Investigator CP186 and CP166
0045	11/5/2009	Clinical	Protocol Amendment
0046	12/9/2009	Clinical	New Investigators information MI-CP186
0047	2/4/2010	Safety	Safety Report

Sequence Number	Submission Date	Submission Type	Submission Description
0048	3/22/2010	Safety	Safety Report first follow-up
0049	4/23/2010	Safety	Safety Report
0050	5/4/2010	Safety	Safety Report Second Follow-up
0051	5/19/2010	Safety	Safety Report First Follow-up
0052	6/17/2010	Non-clinical	Information Amendment-Pharmacology-Toxicology
0053	7/8/2010	Non-clinical	Information Amendment-Pharmacology-Toxicology
0054	8/11/2010	Clinical and Quality	New Protocol, IB, and CMC Amendment
0055	8/17/2010	Safety	Safety Report
0056	9/1/2010	Clinical	New Investigators - CP186
0057	9/3/2010	Safety	Safety report Follow-up
0058	9/13/2010	Safety	Safety report Follow-up
0059	9/15/2010	Safety	Safety report Follow-up
0060	9/22/2010	Safety	Annual Report 2010 - 30 July 2009 through 29 July 2010
0061	12/2/2010	Clinical	New Investigators CP220
0062	1/13/2011	Clinical	New Investigators CP221
0063	2/10/2011	Clinical	New Investigators CP222
0064	2/17/2011	Non-clinical	Information Amendment-Pharmacology-Toxicology
0065	3/2/2011	Clinical and Quality	Response to FDA Request for Information

Sequence Number	Submission Date	Submission Type	Submission Description
0066	4/29/2011	Clinical	Protocol Amendment and New Investigators
0067	5/6/2011	Clinical	New Protocol and New Investigators
0068	5/19/2011	Non-Clinical-Clinical	Information Amendment-Pharmacology-Toxicology and New Investigators
0069	7/1/2011	Non-clinical	Response to FDA Information Request-Toxicology
0070	7/12/2011	Safety	Safety Report
0071	8/4/2011	Clinical	New Investigators
0072	9/22/2011	Safety	Annual Report 30Jul2010 to 29Jul2011
0073	10/12/2011	Clinical	General correspondence
0074	10/18/2011	Clinical	CP158 and 197 CSR
0075	10/21/2011	Clinical	New Investigators and TORO
0076	10/26/2011	Other	Meeting Request
0077	11/4/2011	Clinical	IB Version 6
0078	11/22/2011	Clinical	Updated Investigators and Protocol Amendment
0079	12/22/2011	Clinical	General Correspondence-Request for Comments-Advice-Clinical
0080	12/19/2011	Clinical	New and Revised Investigators
0081	2/13/2012	Clinical	New Investigators
0082	3/29/2012	Safety	Safety Report MI-CP220

Sequence Number	Submission Date	Submission Type	Submission Description
0083	4/3/2012	Safety	Safety Report MI-CP220
0084	4/23/2012	Safety	Safety Report MI-CP220
0085	4/24/2012	Safety	Safety report Follow-up
0086	4/27/2012	Safety	Safety Report MI-CP220
0087	5/8/2012	Safety	Safety Report
0088	6/12/2012	Clinical	New Investigators
0089	6/19/2012	Safety	Safety Report MI-CP220
0090	6/21/2012	Safety	Safety report Follow-up
0091	7/18/2012	Clinical	New Investigators
0092	7/18/2012	Safety	Safety Report
0093	7/24/2012	Clinical	Protocol Amendment
0094	7/25/2012	Safety	Safety report Follow-up
0095	8/1/2012	Safety	Safety report
0096	8/2/2012	Safety	Safety Report MI-CP220
0097	8/14/2012	Safety	Safety Report MI-CP220
0098	8/17/2012	Safety	Safety Report
0099	8/22/2012	Safety	Safety report Follow-up
0100	8/23/2012	Safety	Safety report Follow-up
0101	8/24/2012	Safety	Safety Report MI-CP220
0102	9/4/2012	Safety	Safety report Follow-up
0103	9/11/2012	Safety	Safety report Follow-up

Sequence Number	Submission Date	Submission Type	Submission Description
0104	9/20/2012	Safety	Development Safety Update Report Version 1.0 - 2011-2012
0105	9/26/2012	Safety	Safety report Follow-up
0106	11/9/2012	Other	Asthma End of Phase 2 Meeting Request
0107	11/13/2012	Safety	Safety Report
0108	11/14/2012	Safety	Safety Report
0109	11/19/2012	Clinical	IB Version 7 and MI-CP166 CSR
0110	11/20/2012	Safety	Safety report Follow-up
0111	11/28/2012	Safety	Safety report Follow-up
0112	12/6/2012	Safety	Safety report Follow-up
0113	12/13/2012	Safety	Safety Report
0114	12/14/2012	Safety	Safety report Follow-up
0115	12/21/2012	Safety	Safety report Follow-up
0116	12/21/2012	Safety	Safety report Follow-up
0117	12/28/2012	Safety	Safety report Follow-up
0118	1/2/2013	Safety	Safety report Follow-up
0119	1/11/2013	Safety	Safety report Follow-up
0120	1/14/2013	Other	Asthma End of Phase 2 Meeting Package
0121	1/30/2013	Other	Errata to End of Phase 2 Meeting Package

Sequence Number	Submission Date	Submission Type	Submission Description
0122	1/31/2013	Safety	Safety report Follow-up
0123	2/5/2013	Other	Slides to support EOP2 Meeting Package
0124	2/7/2013	Non-Clinical	Validation Assay Report
0125	2/8/2013	Safety	Safety report Follow-up
0126	3/14/2013	Safety	Safety report Follow-up
0127	3/25/2013	Other	Clarification question on FDAs End of Phase 2 Meeting Minutes
0128	4/26/2013	Other	Pediatric Study Plan
0129	5/30/2013	Safety	Safety report Follow-up
0130	6/11/2013	Clinical	Protocol Amendment
0131	6/12/2013	Other	Update to SN0130 Cover Letter
0132	6/24/2013	Quality	CMC Process 3 Info
0133	7/15/2013	Clinical	Protocol Amendment
0134	8/8/2013	Clinical	Revised Pediatric Study Plan
0135	8/15/2013	Clinical	Protocol Amendment
0136	8/16/2013	Other	COPD EOP2 meeting request
0137	8/22/2013	Clinical	New Investigators
0138	9/19/2013	Clinical	New Investigators
0139	9/25/2013	Safety	DSUR 2012-2013
0140	9/20/2013	Clinical	New Investigators

Sequence Number	Submission Date	Submission Type	Submission Description
0141	10/17/2013	Other	COPD End of Phase 2 Meeting Package
0142	10/17/2013	Clinical	MI-CP186 CSR
0143	10/31/2013	Clinical	New Investigators
0144	11/8/2013	Other	Response to CMC Information Request Process 3
0145	11/15/2013	Other	COPD End of Phase 2 Meeting Correspondence - MI-CP196 SAP
0146	11/27/2013	Clinical	New Investigators
0147	12/16/2013	Other	COPD End of Phase 2 Response to Preliminary Comments
0148	1/3/2014	Clinical	New Investigators
0149	1/16/2014	Other	COPD Pediatric Study Plan Class Waiver Request
0150	1/17/2014	Other	Letter of Authorization for NIH HES Study
0151	1/29/2014	Other	Word Version of COPD initial Pediatric Study Plan
0152	2/7/2014	Clinical	New Investigators
0153	2/10/2014	Clinical	Protocol Amendment
0154	2/12/2014	Clinical	Protocol Amendment
0155	2/14/2014	Clinical	Protocol Amendment
0156	2/26/2014	Clinical	IB Version 9
0157	3/6/2014	Clinical	New Investigators

Sequence Number	Submission Date	Submission Type	Submission Description
0158	3/12/2014	Quality	Type C Meeting Request - Self Administration
0159	3/13/2014	Clinical	Response to FDA Request for AUC data 30, 100 mg
0160	3/17/2014	Quality	COPD CMC Amend 100-30mg
0161	3/19/2014	Clinical	Statistical Analysis Plan
0162	4/3/2014	Clinical	New Investigators
0163	4/25/2014	Other	Type C Briefing Package - Self Administration
0164	4/29/2014	Quality	CMC IND Amendment 10mg Dose
0165	5/1/2014	Safety	Safety Report Study 18
0166	5/2/2014	Clinical	New Investigators
0167	5/6/2014	clinical	Protocol Amendment and IB V10
0168	6/6/2014	Clinical	New Investigators
0169	6/16/2014	Clinical	Asthma Study 18,17 A1 and IB V11
0170	6/20/2014	Clinical	Statistical Analysis Plan
0171	6/25/2014	Other	Type C-clinical mtg request
0172	7/8/2014	Clinical	New Investigators
0173	7/14/2014	Clinical	Statistical Analysis Plan
0174	7/28/2014	Clinical	MI-CP196 CSR

Sequence Number	Submission Date	Submission Type	Submission Description
0175	7/29/2014	Clinical	Termination of Site on CALIMA
0176	8/6/2014	Clinical	New Investigators
0177	8/8/2014	Other	Type C Meeting Package
0178	9/4/2014	Clinical	New Investigators
0179	9/8/2014	Clinical	Protocol Amendment
0180	9/25/2014	Safety	DSUR 2013-2014
0181	9/26/2014	Safety	Safety Report Follow up - Study 18
0182	10/3/2014	Clinical	Pediatric Study Plan Amendment
0183	10/10/2014	Clinical	New Investigators
0184	10/16/2014	Safety	Safety Report
0185	10/21/2014	Safety	Safety Report
0186	10/23/2014	Clinical	Resubmission of Pediatric Study Plan Amendment
0187	10/30/2014	Clinical	Streamlined Track Changes version of PSP
0188	11/4/2014	Safety	Safety report Follow-up
0189	11/14/2014	Clinical	New Investigators
0190	11/21/2014	Clinical	Protocol Amendment and IB
0191	12/3/2014	Safety	Safety Report
0192	12/10/2014	Clinical	Statistical Analysis Plan
0193	12/16/2014	Safety	Safety Report

Sequence Number	Submission Date	Submission Type	Submission Description
0194	12/17/2014	Clinical	Statistical Analysis Plan - sample size re-estimate for study 17_18
0195	1/5/2015	Safety	Safety Report
0196	1/16/2015	Clinical	New Investigators
0197	1/21/2015	Safety	Safety Report
0198	2/3/2015	Clinical	MI-CP220 CSR
0199	1/27/2015	Safety	Safety Report
0200	2/6/2015	Safety	Safety Report
0201	2/9/2015	Safety	Safety Report
0202	2/10/2015	Safety	Safety Report
0203	2/17/2015	Safety	Safety Report
0204	2/25/2015	Clinical	Protocol Amendment
0205	2/27/2015	Clinical	New protocol
0206	3/3/2015	Safety	Safety Report
0207	3/3/2015	Safety	Safety Report
0208	3/11/2015	Clinical	New Investigators
0209	3/10/2015	Quality	CMC APFS Summative Validation Protocol
0210	3/12/2015	Clinical	Protocol Amendment Asthma and SAP for asthma study 32
0211	3/11/2015	Safety	Safety Report
0212	3/18/2015	Safety	Safety Report

Sequence Number	Submission Date	Submission Type	Submission Description
0213	3/19/2015	Safety	Safety Report
0214	3/23/2015	Safety	Safety Report
0215	3/25/2015	Safety	Safety Report
0216	3/26/2015	Safety	Safety Report
0217	3/27/2015	Clinical	New Investigators
0218	3/30/2015	Safety	Safety Report
0219	4/7/2015	Clinical	D3250C00018 CSP A2
0220	4/7/2015	Safety	Safety Report
0221	4/10/2015	Safety	Safety Report
0222	4/13/2015	Safety	Safety Report
0223	4/14/2015	Safety	Safety Report
0224	4/24/2015	Clinical	New Investigators
0225	4/21/2015	Safety	Safety Report
0226	6/10/2015	Clinical	Protocol Amendment and ICFs
0227	5/5/2015	Safety	Safety Report
0228	5/11/2015	Safety	Safety Report
0229	5/20/2015	Clinical	New Investigators
0230	5/18/2015	Safety	Safety Report
0231	5/19/2015	Safety	Safety Report
0232	5/21/2015	Safety	Safety Report
0233	5/27/2015	Safety	Safety Report
0235	6/1/2015	Safety	Safety Report

Sequence Number	Submission Date	Submission Type	Submission Description
0236	6/1/2015	Safety	Safety Report
0237	7/1/2015	Clinical	D3250C00020 CSP A1
0238	6/2/2015	Safety	Safety Report
0239	6/3/2015	Safety	Safety Report
0240	6/4/2015	Safety	Safety Report
0241	6/5/2015	Safety	Safety Report
0242	6/8/2015	Safety	Safety Report
0243	6/17/2015	Clinical	New Investigators
0244	6/15/2015	Safety	Safety Report
0245	6/22/2015	Quality	CMC Information Amendment -COPD 30mg lot resupply – Amendment
0246	6/18/2015	Safety	Safety Report
0247	7/2/2015	Clinical	Protocol Amendment
0248	6/30/2015	Safety	Safety Report
0249	6/30/2015	Safety	Safety Report
0250	7/2/2015	Safety	Safety Report
0251	7/17/2015	Clinical	New Investigators
0252	7/16/2015	Clinical	SAP-D3250C00029
0253	7/13/2015	Safety	Safety Report
0254	7/17/2015	Safety	Safety Report
0255	7/20/2015	Safety	Safety Report
0256	7/27/2015	Safety	Safety Report

Sequence Number	Submission Date	Submission Type	Submission Description
0257	7/31/2015	clinical	Protocol Amendment
0258	7/30/2015	Safety	Safety Report
0259	10/28/2015	clinical	Protocol Amendment
0260	8/3/2015	Safety	Safety Report
0261	8/5/2015	Safety	Safety Report
0262	8/14/2015	Safety	Safety Report
0263	8/24/2015	Safety	Safety Report
0264	9/1/2015	Clinical	July 1572 and CVs
0265	9/3/2015	Safety	Safety Report
0266	9/25/2015	Safety	DSUR 2014-2015
0267	9/11/2015	Safety	Safety Report
0268	9/15/2015	Safety	Safety Report
0269	2/18/2016	Safety	Safety Report
0270	9/18/2015	Safety	Safety Report
0271	9/22/2015	Safety	Safety Report
0272	9/30/2015	Clinical	New Investigators
0273	10/14/2015	Clinical	D3250C00016 CSR
0274	9/29/2015	Safety	Safety Report
0275	9/30/2015	Safety	Safety Report
0276	9/30/2015	Safety	Safcty Report
0277	10/1/2015	Safety	Safety Report
0278	10/2/2015	Safety	Safety Report

Sequence Number	Submission Date	Submission Type	Submission Description
0279	10/16/2015	Clinical	New Investigators
0280	10/8/2015	Safety	Safety Report
0281	10/21/2015	Clinical	Protocol Amendment
0282	10/13/2015	Safety	Safety Report
0283	10/21/2015	Safety	Safety Report
0284	10/20/2015	Safety	Safety Report
0285	10/20/2015	Safety	Safety Report
0286	10/21/2015	Safety	Safety Report
0287	11/3/2015	Safety	Safety Report
0288	11/5/2015	Safety	Safety Report
0289	11/17/2015	Clinical	New Investigators
0290	11/10/2015	Safety	Safety Report
0291	11/12/2015	Safety	Safety Report
0292	11/16/2015	Safety	Safety Report
0293	11/19/2015	Safety	Safety Report
0294	11/20/2015	Safety	Safety Report
0295	11/24/2015	Safety	Safety Report
0296	12/2/2015	Safety	Safety Report
0297	12/9/2015	Safety	Safety Report
0298	12/14/2015	Safety	Safety Report
0299	12/18/2015	Clinical	New Investigators
0300	12/15/2015	Safety	Safety Report

Sequence Number	Submission Date	Submission Type	Submission Description
0301	12/24/2015	Safety	Safety Report
0302	1/28/2016	Clinical	New Protocol
0303	1/19/2016	Clinical	New Investigators
0304	1/15/2016	Clinical	GREGALE SAP update
0305	1/28/2016	clinical	BORA CSP Amendment 2
0306	2/9/2016	Other	pre-BLA Type C Meeting Request Letter
0307	1/26/2016	Safety	Safety Report
0308	2/2/2016	Safety	Safety Report
0309	2/11/2016	Clinical	New Protocol
0310	2/11/2016	Clinical	Protocol Amendment resubmission
0311	2/15/2016	Clinical	New Investigators
0312	2/8/2016	Safety	Safety Report
0313	2/16/2016	Clinical	Response to FDA Question on SAP (Asthma and COPD)
0314	2/11/2016	Safety	Safety Report
0315	3/7/2016	Clinical	CSP Amendment 2 + IB Version 13.0 + MELTEMI Administrative change
0316	2/24/2016	Safety	Safety Report
0317	2/25/2016	Safety	Safety Report
0318	2/26/2016	Safety	Safety Report

Sequence Number	Submission Date	Submission Type	Submission Description
0319	2/29/2016	Other	Withdrawn Type C meeting request
0320	2/29/2016	Safety	Safety Report
0321	3/17/2016	Clinical	ALIZE Study 33 Original CSP submission
0322	3/7/2016	Safety	Safety Report
0323	3/22/2016	Clinical	New Investigators
0324	3/31/2016	Other	Request for feedback-preBLA
0325	4/6/2016	Clinical	Study 20, 32 & ADA SAP
0326	4/22/2016	Clinical	New Investigators
0327	4/11/2016	Safety	Safety Report
0328	4/12/2016	Safety	Safety Report
0329	4/19/2016	Safety	Safety Report
0330	4/25/2016	Safety	Safety Report
0331	5/3/2016	Clinical	New Investigators
0332	5/3/2016	Safety	Safety Report
0333	5/4/2016	Safety	Safety Report
0334	5/18/2016	Clinical	Investigator Data-Asthma-COPD
0335	5/19/2016	Safety	Safety Report
0336	6/8/2016	Other	Pre-BLA Type B meeting request
0337	6/30/2016	Other	Proprietary name submission

Sequence Number	Submission Date	Submission Type	Submission Description
0338	6/20/2016	Clinical	New Investigators
0339	6/16/2016	Other	Resubmission pre-BLA Type B meeting request letter
0340	9/6/2016	Clinical	New Protocol
0341	8/15/2016	Other	pre-BLA Type B meeting package
0342	7/27/2016	Clinical	New Investigators
0343	9/16/2016	Clinical	d3250c00032 (BISE) CSR
0344	10/12/2016	Clinical	Study 29 (Gregale) CSR
0345	10/12/2016	Clinical	Study 17 (Sirocco) CSR
0346	10/12/2016	Clinical	Study 18 (Calima) CSR
0347	8/24/2016	Clinical	New Investigators
0348	8/26/2016	Clinical	Summative validation protocol for AI
0349	9/22/2016	Clinical	Study 31 (GRECO) CSP submission
0350	9/23/2016	Safety	DSUR (30Jul15 to 29Jul16)
0351	9/9/2016	Other	Additional Statements to pre-BLA meeting package
0352	9/19/2016	Clinical	New Investigators
0354	9/20/2016	Other	Response to preliminary comments to pre-BLA meeting package
0355	9/27/2016	Safety	Safety Report
0356	10/14/2016	Clinical	Updated Study 32 (BISE) CSR

Sequence Number	Submission Date	Submission Type	Submission Description
0357	11/4/2016	Other	Letter of Authorization-Dr. Wechsler
0358	10/21/2016	Clinical	New Investigators
0359	10/24/2016	Clinical	Study 20 (Zonda) CSR
0360	11/10/2016	Other	Asthma Transfer of Obligations
0361	11/18/2016	Clinical	New Investigators
0362	11/22/2016	Other	Report on Scientific Misconduct
0363	11/30/2016	Other	AI sample and transcript request
0364	12/1/2016	Other	Letter of Authorization. Dr Bernstein
0365	11/29/2016	Other	Change of Contact
0366	12/2/2016	Safety	Safety Report
0367	12/9/2016	Other	Change of Contact
0368	12/20/2016	Other	Letter of Authorization. Dr Tversky
0369	12/22/2016	Clinical	New Investigators
0370	12/21/2016	Safety	Safety Report
0371	1/23/2017	Clinical	New Investigators
0372	1/20/2017	Clinical	BORA CSP Amendment 3
0373	1/31/2017	Clinical	Study 16,17,18 CSR Addendums
0374	1/31/2017	Clinical	New Investigators

Sequence Number	Submission Date	Submission Type	Submission Description
0375	2/17/2017	Clinical	New Investigators
0376	3/14/2017	Other	Meeting Request Letter Nasal Polyps
0377	3/17/2017	Clinical	New Investigators
0378	3/21/2017	Clinical	Study 17,18,20 Addenda and Errata
0379	4/6/2017	Other	Nasal Polyps pre-Phase 3 Briefing Book
0380	3/28/2017	Quality	RIR for Autoinjector Human Factors Summative Validation Protocol Review Comments
0381	4/10/2017	Safety	Safety Reporting TOO for Asthma and COPD
0382	4/17/2017	Clinical	New Investigators
0384	5/1/2017	Clinical	New Protocol
0385	4/28/2017	Clinical	Updated ICFs
0386	5/22/2017	Clinical	New Investigators
0387	6/6/2017	Other	Response to preliminary feedback from FDA
0388	6/14/2017	Clinical	Statistical Analysis Plan for Study 30,31,37,38
0389	6/19/2017	Clinical	New Investigators
0390	6/16/2017	Other	FDA Signatory List
0391	6/20/2017	Other	Letter of Authorization Dr. Bernstein resubmission
0392	7/19/2017	Clinical	New Investigators

Sequence Number	Submission Date	Submission Type	Submission Description
0393	7/27/2017	Clinical	Initial Pediatric Study Plan Nasal Polyposis
0394	9/26/2017	Clinical	COPD Human Factors Summative Validation Protocol
0395	7/26/2017	Other	Letter of Authorization Dr. Rothenberg
0396	8/18/2017	Clinical	New Investigators
0397	9/22/017	Safety	Annual DSUR 2017 (30 July 2016 to 29 July 2017)
0398	9/20/2017	Clinical	New Investigators
0399	10/20/2017	Clinical	New Investigators
0400	11/7/2017	Clinical	New Protocol
0401	11/10/2017	Clinical	Nasal Polyps agreed Initial Pediatric Study Plan

Exhibit J

Activities during BLA Period

Sequence Number	Submission Date	Submission Type	Submission Description
0000	11/16/2016	Original Application	Original BLA Submission
0001	12/09/2016	Other	Other-Change of AZ Contact for BLA
0002	01/12/2017	Other	Other-Clarification on Debarment Certification Signature
0003	02/07/2017	Other	Other-Proprietary Name Review Request
0004	02/01/2017	Other	Other-Response to Day 74 Questions
0005	03/16/2017	Clinical	4 Months Safety Update
0006	02/28/2017	Quality and Clinical	Response to Quality and Clinical Pharmacokinetic Questions Received on Feb917
0007	03/10/2017	Other	LASA Report Supporting PPN Request
0008	03/16/2017	Clinical	17, 18 and 20 Clinical Study Report Addendums and Errata
0009	03/22/2017	Clinical	Response to Clinical Questions Received on 10Mar17
0010	03/27/2017	Clinical	Population Pharmacokinetic Analysis Errata List
0011	04/04/2017	Quality	Response to Quality Questions Received on 21Mar2017 and 24Mar2017
0012	04/04/2017	Clinical	Response to Clinical Questions Received on 22March2017
0013	04/11/2017	Clinical	Response to Clinical White Blood Cells Questions Received on 24Mar2017
0014	05/09/2017	Other	Updated Financial Disclosure forms
0015	05/18/2017	Other	Proprietary Name-Fasenra-Resubmission
0016	05/25/2017	Clinical	Response to Clinical Questions Received on 16May2017
0017	06/02/2017	Clinical	Response to Safety Questions Received on 17May2017
0018	06/23/2017	Clinical	Response to Investigators and sub-Investigators Number Questions Received on 14Jun2017
0019	06/30/2017	Quality	Response to CMC Questions-14Jun2017
0020	07/10/2017	Quality	Response to CMC Questions-21Jun2017
0021	07/14/2017	Quality	Response to CMC Questions -03Jul2017

Sequence Number	Submission Date	Submission Type	Submission Description
0022	08/02/2017	Labeling	Response to Labeling Comments Received on 20July2017-
0023	08/11/2017	Labeling	Response to Labeling Requests Received on 01aug2017
0024	08/11/2017	Quality	Response to CMC Questionss-13Jul2017
0025	08/09/2017	Clinical	Response to Statistical Questions Received on 01Aug2017
0026	08/18/2017	Quality	Response to CMC Questions Received on 02Aug2017
0027	08/22/2017	Clinical	Response to Late Cycle Meeting -Adolescents Request
0028	09/01/2017	Quality	Response to CMC Questions Received on 18Aug2017
0029	09/05/2017	Labeling	Response to Label Request Received on 23Aug2017
0030	09/25/2017	Quality	Response to CMC Questions Received on 14Sept2017
0031	09/27/2017	Labeling	Response to Label Request Received on 15Sept2017
0032	10/09/2017	Quality	CMC Errata Amendment
0033	10/20/2017	Labeling	Response to Labeling Request Received on 11Oct2017
0034	10/20/2017	Other	Response to Post Marketing Requirement and Post Marketing Commitment Received on 04Oct2017
0035	10/31/2017	Labeling	Response to Labeling Request Received on 24Oct2017

Exhibit K

Tan et al., J Asthma Allergy, 9: 71-81 (2016)

Journal of Asthma and Allergy Dove

REVIEW

Benralizumab: a unique IL-5 inhibitor for severe asthma

Laren D Tan[1]
Jennifer M Bratt[2]
Dorottya Godor[3]
Samuel Louie[2]
Nicholas J Kenyon[2]

[1]Division of Pulmonary and Critical Care Medicine, Department of Internal Medicine, School of Medicine, Loma Linda University, Loma Linda, CA, USA; [2]Division of Pulmonary, Critical Care and Sleep Medicine, Department of Internal Medicine, School of Medicine, University of California Davis, Sacramento, CA, USA; [3]School of Medicine, Semmelweis University, Budapest, Hungary

Abstract: The presence of eosinophilic inflammation is a characteristic feature of chronic and acute inflammation in asthma. An estimated 5%–10% of the 300 million people worldwide who suffer from asthma have a severe form. Patients with eosinophilic airway inflammation represent approximately 40%–60% of this severe asthmatic population. This form of asthma is often uncontrolled, marked by refractoriness to standard therapy, and shows persistent airway eosinophilia despite glucocorticoid therapy. This paper reviews personalized novel therapies, more specifically benralizumab, a humanized anti-IL-5Rα antibody, while also being the first to provide an algorithm for potential candidates who may benefit from anti-IL-5Rα therapy.

Keywords: asthma, eosinophils, asthma treatments, benralizumab, IL-5, IL-5Rα, MEDI-563

Introduction

In 2007, the incremental cost due to asthma was $56 billion in the United States,[1] and worldwide the costs are much higher. Of the estimated 25–30 million asthmatics in the United States, the majority of the annual costs incurred are from the 5%–10% of patients with severe asthma. With the advent of novel biological therapies that have been most recently approved for severe asthma, health economists and society will look closely on whether these medications decrease or increase total costs. A high prevalence of severe asthmatics are seen in African-Americans, Puerto Rican Americans, Cuban Americans, women, and those of age ≥65 years. It is imperative that we comprehend the global impact of these new treatments, especially in these populations.

Current modalities of treatments have demonstrated that asthmatics will have variations in response and outcomes. The majority of asthmatics respond well to a written asthma action plan of pharmacotherapy with inhaled corticosteroids often combined with long- or short-acting β-agonist bronchodilators (LABA or SABA) and leukotriene antagonists (LTRAs). Despite aggressive therapy, it is unclear why 5%–10% of asthmatics with severe asthma do not respond to their prescribed regimen. An explanation to this conundrum may be attributed to gaps in medical knowledge and deficiencies in experience on the identification of severe asthma. Other potential barriers are clinical restraints in coordination, integration, and resources for advanced treatments such as omalizumab, bronchial thermoplasty (BT), and now a novel biologic, such as benralizumab. Several key questions need to be addressed before embracing novel biologics as a potential mainstay treatment for asthma, "Should patients be tried on omalizumab before anti-IL5 therapies?" and "If patients have an incomplete response to omalizumab, should anti-IL5 treatment be added?" In this paper, we will review

Correspondence: LD Tan
Division of Pulmonary and Critical Care Medicine, Department of Internal Medicine, School of Medicine, Loma Linda University, 11234 Anderson Street, Room 6433, Loma Linda, CA 92354, USA
Tel +1 909 558 4489
Fax +1 909 558 0581
Email latan@llu.edu

the opportunities for treating patients with severe, persistent asthma with novel biological agents, such as benralizumab, and present a framework for understanding such patients.

Better phenotyping

Most adults with severe asthma develop their disease in childhood. Corticosteroid-resistant asthmatics represent a critical subset of severe asthmatics. Although uncommon, it is estimated that corticosteroid-resistant asthma accounts for 0.01%–0.1% of all patients with asthma;[2] however, a retrospective review at National Jewish Health demonstrated that of their severe asthmatics, 25% were found to be corticosteroid resistant.[3] More recently, Woodruff et al[4] outlined a "Th-2 high and Th-2 low group". The T-helper type 2 (Th-2) lymphocytes are defined by the cytokines, namely, interleukins (ILs) IL-4, -5, and -13, all of which are important in the development and persistence of eosinophilic, allergic airway inflammation. In this 8-week study, Th-2 high subjects had an increase of 300 mL in forced expiratory volume in 1 second (FEV_1) on an average with inhaled fluticasone, and this was significantly greater than that in either the Th-2 low or the placebo (control) groups. This study was one of the first to show clear responses to therapy tailored to the specific phenotype of severe asthmatics.

To date, there is no laboratory test or biomarker that exists to readily distinguish severe asthma from less-severe asthma phenotypes and has the ability to predict a favorable response to treatment. The best predictor of adverse outcomes and excessive use of asthma control medications appears to be the baseline FEV_1.[5,6] Fraction of exhaled nitric oxide (FeNO) is increased in asthmatic patients and, although NO concentrations may vary greatly (normal <25 ppb in adults), increases and decreases in NO levels correlates well with improvement and deterioration in asthma symptoms, respectively.[7] In addition, NO does not appear to be increased in patients with chronic obstructive pulmonary disease (COPD), making the differentiation between these conditions easier. Despite FeNO and sputum eosinophils, it is still difficult to differentiate in severe asthmatics between those who respond to treatment versus those who will not.[8]

Evaluation of potential candidates

When considering advanced asthma therapies such as injections (omalizumab and benralizumab) and BT, our approach to the evaluation of a severe asthma patient would include first ascertaining that the diagnosis of severe asthma is correct, that is, we exclude other diagnoses and identify confounding comorbidities, even if this is the third or fourth evaluation (Figure 1). After reevaluation of the diagnosis of asthma, we attempt to understand the asthma phenotype by age stratification and lung function.[5] Phenotyping also includes understanding of serum total immunoglobulin E (IgE) and radioallergosorbent test panel to detect atopy and fungal sensitivities, FeNO, and peripheral blood eosinophil numbers.[9] In addition, we would consider a chest computed tomography for bronchiectasis and other structural lung changes if the diffusion capacity of the lungs for carbon monoxide is abnormal. Often, a chest radiograph will suffice. Lastly, it is important to consider fiber optic bronchoscopy with bronchoalveolar lavage and endobronchial and transbronchial biopsies. For example, if we find a positive serology for *Aspergillus* spp. or other fungus in the right clinical situation, we consider treatment for 8 months with itraconazole 200 μg twice daily.[9] If we want to truly assess the steroid responsiveness, we treat the patient with 12 days of prednisone, often in the late afternoon rather than the morning, eg, 40 mg ×3 days, 30 mg ×3 days, 20 mg ×3 days, 10 mg ×3 days, and ask him/her to return to the clinic to determine if his/her asthma is refractory to corticosteroids. A favorable response to oral corticosteroids, for example, improvement in symptoms, FeNO, and spirometry, would force us to consider leaving the patient on low-dose prednisone 1–3 mg daily in addition to high dose, inhaled steroids and LABA, as in Step 6 of the National Asthma Education and Prevention Program (NAEPP) table (Figure 1). At this point, consistent with the NAEPP guidelines, we consider additional adjunct therapies, including zileuton SR for 2–6 weeks (Step 3), which inhibits leukotriene B4 synthesis and modulates neutrophil infiltration. Also, we would consider theophylline, which is a phosphodiesterase-4-inhibitor (Step 3), but the latter is not US Food and Drug Administration (FDA) approved for asthma despite evidence of a mild bronchodilator effect and reduction in sputum eosinophils and neutrophils.[10] We tend to use montelukast only if the patient derives clear clinical benefit (Step 3). Outside the NAEPP guidelines, but consistent with more recent evidence, we strongly consider adding tiotropium and measure FEV_1 in a scheduled follow-up visit 2 weeks later at the minimum.[11] Tiotropium and LABA have an additive effect on bronchodilating the airways. At Step 5 of the guidelines, we would treat with omalizumab if IgE is elevated and radioallergosorbent test is positive for a perennial aeroallergen. We monitor such patients in clinic for anaphylaxis for 2 hours the first 3 injections (captures 75% of anaphylactic reactions) and would discontinue if there is no clinical benefit. Along with omalizumab at steps 5 and 6, we consider BT if the patient fails to improve, or we go to BT

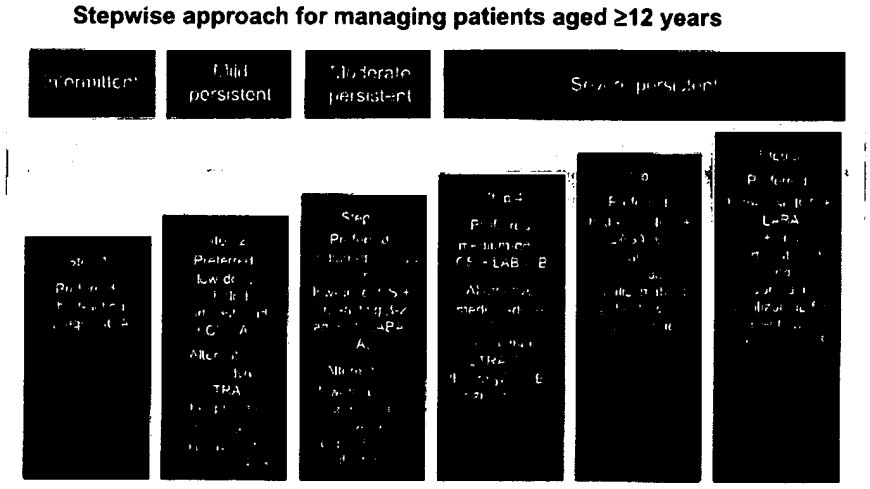

Figure 1 NAEPP stepwise approach to managing asthma with grades of recommendations.[72]
Note: Grading of Recommendations Assessment, Development and Evaluation (GRADE), (A) High quality of evidence, (B) Moderate, (C) Low, (D) Very low.
Abbreviations: ICS, inhaled corticosteroid; LABA, long-acting β-agonist bronchodilators; LTRA, leukotriene antagonist; NAEPP, National Asthma Education and Prevention Program.

immediately if the patient elects to go with BT or declines omalizumab. This is the stage of evaluation and disease where we would place benralizumab and other anti-IL-5 injection therapies, particularly in the patients with severe asthma with mild peripheral eosinophil count elevations (>300 cells/μL).

Rationale for novel biologics

There has been recognition of various asthma phenotypes and endotypes, as well as an increase in understanding of asthma pathogenesis, which allow for a targeted, personalized approach to refractory asthma.[12] Omalizumab is one of the soon-to-be many personalized approaches that will be in the armamentarium of the asthma specialist. Omalizumab is a monoclonal antibody (mAb) that binds to IgE, which has been approved for patients with refractory allergic asthma that has been shown to decrease exacerbations, inhaled corticosteroids, and improved asthma-related quality-of-life measures in refractory asthmatics.[12–14] Asthma has long been viewed as a disease by marked eosinophilia, eosinophils in airways secretions, and IgE-mediated inflammation, the pathogenesis of which is thought to be Th-2 driven. An association between eosinophilia and outcomes of asthma severity has been established in several studies,[15,16] with eosinophil numbers in induced sputum highest among severe asthmatics.[17–20] These findings support previous evidence that link airway inflammation and abnormal airway physiology indicating that reducing airway inflammation with corticosteroids improves airway function. The classic eosinophilic pathogenesis of asthma does not adequately explain the subgroups of asthma. For example, noneosinophilic (atypical Th-2 profile) asthma is more likely to have neutrophils and may be relatively corticosteroid resistant; a distinction between this subgroup is important, especially when providing a thoughtful and effective approach in treatment.

In the early 1990s, Djukanovic et al[21] were able to exhibit markers of airway inflammation and airway remodeling in bronchial lavage and bronchial biopsies in mild and moderate asthmatics. These findings, in addition to the emerging data on Th-1 and Th-2 subsets of CD4 T-cells, prompted further studies into showing that T-cells in the airways of individuals with asthma had cytokine profiles characteristic of Th-2 cells.[15] Thus, specific cytokines such as ILs have since been the primary target for biological inhibition asthma therapy, IL-2, IL-4, IL-5, IL-9, and IL-13 (Table 1).

Th-2-specific cytokines – IL-4, IL-5, IL-9, and IL-13 – have all been earlier traditionally considered to contribute to the etiology of asthma; they contribute either directly or indirectly to the accumulation of airway and peribronchial eosinophilia. Eosinophils are bone marrow-derived granulocytes that have long been recognized as the main mediator cells in allergic asthma. In addition to releasing varying cytokines and chemokines, they have been found to also

Table 1 Clinical trials with anti-interleukins

Medication	Mechanism	Targeted cytokine	Eosinophils	FEV_1	Key findings
Daclizumab	Monoclonal antibody to alpha subunit of IL-2	IL-2	Serum: ↓	↑	Decrease in β-agonist use and asthma symptoms but did not continue throughout the study.[68]
Dupilumab	Monoclonal antibody to alpha subunit of IL-4 receptor (IL-4α), inhibits both IL-4 and IL-13	IL-4/IL-13	Serum: ↓/↑	↑	Decreased asthma exacerbations with sputum eosinophilia when LABA and ICS were withdrawn.[60]
Benralizumab Reslizumab Mepolizumab	Monoclonal antibody to IL-5 or alpha-chain of IL-5 receptor (IL-5α)	IL-5/(IL-5α)	Serum: ↓ sputum	↑	Benralizumab decreased eosinophils in the airways, sputum, bone marrow, and peripheral blood.[55] Reslizumab decreased exacerbations and improved asthma symptoms.[21] Mepolizumab decreased exacerbations, oral steroid use in dependent asthmatics, and lung function in patients on high dose ICS.[53,69]
MEDI-528	Monoclonal antibody to IL-9	IL-9	Serum: ↓/↑	↓/↑*	No significant improvement in exacerbation rates or ACQ scores.[70]
Lebrikizumab	Monoclonal antibody to IL-13	IL-13	Serum: ↑ (slight increase)	↑	Improvement in FEV_1 in patients with elevated serum periostin.[71]

Note: The * indicates no significant improvement in FEV_1 with some subjects showing and increase and FEV_1 and other a decrease.
Abbreviations: ACQ, Asthma Control Questionnaire; FEV_1, forced expiratory volume in 1 second; IL, interleukin; LABA, long-acting β-agonist bronchodilators; ICS, inhaled corticosteroid.

release toxic granular proteins, all of which promote Th-2 inflammation and airway epithelial damage.[12]

It is evident that Th-2 cytokines and its resultant ILs exhibit an imperative role in triggering an inflammatory cascade seen in asthma. Unlike IL-4, IL-9, and IL-13, IL-5 is a key cytokine in eosinophil maturation, activation, survival, and proliferation. As a consequence, the selectivity of IL-5 in the differentiation, activation, recruitment, and survival of human eosinophils have prompted studies to use antibodies against human IL-5.[12,22]

Activated eosinophils are the cellular source of granules associated with basic proteins,[23] reactive oxygen species,[24] and lipid mediators,[25] which collectively can damage surrounding cells and induce airway hyperresponsiveness and mucus hypersecretion.[26,27] Increased numbers of eosinophils in the airways and peripheral blood of subjects with asthma have been shown to correlate with asthma severity.[28] Furthermore, scientific literature supports that elevated sputum eosinophil levels are associated with the cause and severity of both asthma and COPD exacerbations.[29] Severe eosinophilic asthma is identified by blood eosinophils ≥ 150 cells/μL at initiation of treatment or blood eosinophils ≥ 300 cells/μL in the past 12 months.[30] The clinical relevance of eosinophils in asthmatic patients has been confirmed in longitudinal studies demonstrating a reduction in acute exacerbations in subjects who maintained sputum eosinophil counts of < 2%–8%, depending on the specific study.[31–33] Besides reflecting the nature of asthma, eosinophilic airway inflammation also suggests decreased responsiveness to anti-inflammatory therapies, particularly with steroids. A subset of patients with refractory asthma have persistent airway eosinophilia despite chronic, high-dose, inhaled corticosteroid treatment.[34]

Eosinophils have an approximate 3-day turnover rate, with a replacement rate equivalent to the apoptotic rate. With exposure to allergen or parasitic helminth infection, IL-5 and granulocyte-macrophage colony-stimulating factor (GM-CSF) trigger eosinophil differentiation and expansion. IL-5 derived from allergen-exposed tissues act as a chemotactic agent and increase eosinophil transmigration by increasing β2 integrin-mediated adhesion to endothelial cells. As they are recruited to the affected tissues, the eosinophils are stimulated with IL-33, IL-3, IL-5, and GM-CSF, which inhibits Bid-dependent apoptotic signaling, prolonging their survival and resulting in tissue accumulation.

While present in the affected tissues, eosinophils can act as antigen-presenting cells and degranulate, resulting in the upregulation of inflammation. These granules contain cationic proteins, including eosinophil peroxidase, major basic protein, and eosinophil-associated ribonucleases (EARs), and a number of cytokines, chemokines, enzymes, and growth factors, including IL-3, IL-4, IL-5, IL-6, IL-10, IL-13, interferon gamma, tumor necrosis factor-alpha, GM-CSF, transforming growth factor-alpha, chemokine C-C motif ligand-5, C-C motif chemokine-11, and chemokine C-X-C motif ligand-5, that promote inflammation and tissue remodeling.

Accumulation of eosinophils in the parenchyma, airway wall, and lumen, characteristic of eosinophilic asthma, is because of the combined effect of increased differentiation of

bone marrow-derived progenitor lines, increased recruitment from the blood, and prolonged life span. Particularly, in obese asthmatics, eosinophils appear to be accumulating in the submucosa. This accumulation combined with reduced rates of apoptosis can lead to the accumulation of necrotic and lysis-prone eosinophils and their released granules.

Despite the use of systemic corticosteroids upon discharge, relapse rates at 12 weeks after an acute asthma exacerbation can range from anywhere between 41% and 52%.[35] Increases in airway, sputum, and blood eosinophils have been implicated in asthma exacerbations, resulting in emergency department admissions.[36,37] Emerging evidence has also been linked to eosinophilic airway inflammation and a poor response to bronchodilators.[38] Eosinophilic inflammation, which may be a consequence of IL-5 action, can be mitigated by mepolizumab, an anti-IL-5 agent.

IL-5 is involved in the maturation, differentiation, survival, and activation of eosinophils.[39,40] Basophils also express IL-5Rα[41] and have been shown to be involved in asthma allergen challenges,[42,43] cold air challenges,[44] asthma exacerbations,[45,46] and fatal asthma.[47,48] In fact, mepolizumab has been shown to reduce the number of blood and sputum eosinophils as well as the number of subsequent asthma exacerbations.[31,32,49,50]

Emerging anti-IL-5 therapies in asthma

The aforementioned findings prompted the development of IL-5-neutralizing monoclonal antibodies (mAbs). By reducing blood and sputum eosinophil counts and asthma exacerbations, anti-IL-5 antibody therapy has shown clinical benefit in severe, corticosteroid-requiring asthma associated with sputum eosinophilia. In contrast, a previous study of anti-IL-5 antibodies showed no benefit in the management of milder asthma that was not necessarily eosinophilic. Anti-IL-5 mAb therapy on airway mucosal eosinophil counts was first tested in steroid-naive subjects with mild atopic asthma, in whom intravenous mepolizumab achieved a 55% decrease in airway mucosal eosinophil counts.[51] Several trials, including the SIRIUS, MENSA, and DREAM studies, evidenced improved outcomes with intravenous and subcutaneous mepolizumab following a 12-monthly administration of the drug among patients with severe asthma and blood or sputum eosinophilia.[22,49,50,52–56] Positive outcomes were reflected by a decrease in the rate of exacerbations, blood and sputum eosinophil levels, improvement in quality of life, and a mean reduction of 50% from baseline in glucocorticoid dose.[38,49,54,55] However, IL-5 blockade in subjects with asthma has failed to improve parameters of lung function, symptom scores, and airway hyperresponsiveness despite rapid and near-complete depletion of eosinophils from blood and sputum.[55] Decrease in the number of airway and bone marrow eosinophil and basophil precursors only reached 55% and 52%, respectively.[51,55] These latter data compare unfavorably with the novel anti-IL-5 agent benralizumab that effected near total depletion. In view of the overall impact of mepolizumab on asthma, in June 2015, the FDA recommended mepolizumab (*Nucala*, GlaxoSmithKline) for add-on maintenance treatment in patients aged 18 years or above with severe eosinophilic asthma. Mepolizumab is not commercially available at present, although it is under review by regulatory agencies.

In two pivotal Phase III studies (NCT01287039 and NCT01285323), another anti-IL-5 agent, reslizumab (Cinquil, Teva), cut the frequency of clinical asthma exacerbations by at least half, that is, 50% and 60%, respectively. It proved to be effective in patients inadequately controlled by medium to high doses of inhaled corticosteroid-based therapy. It was also of benefit for asthmatics who had blood eosinophils of 400 cells/μL or higher and one or more exacerbations in the previous year. These results[22,50,56] support the use of reslizumab in patients with asthma and elevated blood eosinophil counts who are uncontrolled on inhaled corticosteroid-based therapy.[22] Reslizumab also provided significant improvement in lung function and other secondary measures of asthma control when added to an existing inhaled corticosteroid-based therapy.[50] Pending full analysis of the data, these positive results pave the way for reslizumab regulatory submissions planned for the first half of 2015. FDA ruling on the use of benralizumab in severe asthma is expected by March 2016 (Figure 2).

The mode of action of benralizumab

Eosinophils are a key target in inflammatory respiratory diseases such as asthma and in some instances of COPD. Many functions of eosinophils and basophils are driven by IL-5. Eosinophils rapidly undergo apoptosis in the absence of IL-5 or other eosinophil-active cytokines. Benralizumab, also known as MEDI-563, is a novel investigational mAb in the management of asthma and COPD. It has a unique mode of action. It binds to the α chain of the IL-5 receptor (IL-5Rα), a receptor expressed by mature eosinophils, eosinophil-lineage progenitor cells, and basophils.[40] Benralizumab is a humanized, recombinant, afucosylated IgG1κ mAb. In other words, it has been engineered without a fucose sugar residue in the CH2 region. Afucosylation enhances the interaction of benralizumab with its binding site and thus heightens antibody-dependent cell-mediated cytotoxicity (ADCC)

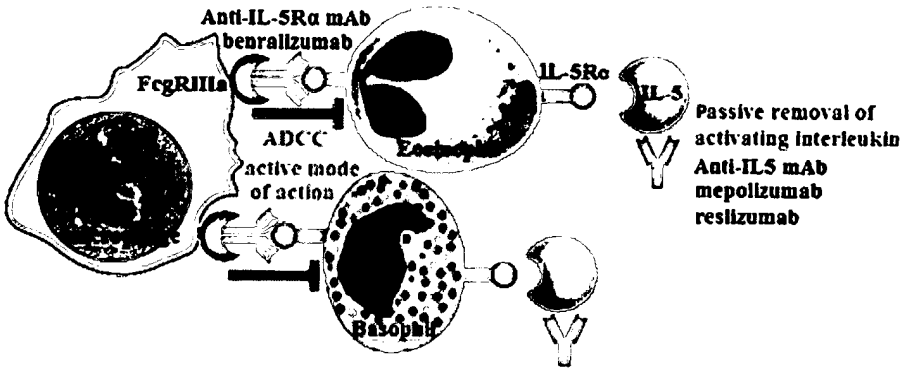

Figure 2 The mechanism of action of therapies targeting IL-5 and its receptor.
Abbreviations: IL, interleukin; ADCC, antibody-dependent cell-mediated cytotoxicity; NK, natural killer; mAB, monoclonal antibody.

functions by > 1,000-fold over the parental antibody.[31,41,57–59] Afucosylated IgG 1 mAbs have a high-binding affinity to the FcgRIIIa region, and they can overcome the inhibitory effects exhibited by serum IgG, which limit the ADCC activity of their fucosylated counterparts.[60] Other anti-IL-5 mAbs (eg, mepolizumab and reslizumab) act by neutralizing the effects of IL-5 and block the activation of eosinophils by IL-5. However, when compared to the other anti-IL-5 biologics, benralizumab targets the effector cells themselves that are circulating and lung-tissue resident tissue eosinophils and basophils. Benralizumab not only blocks all the recruitment, activation, and mobilization of eosinophils but it also allows the depletion of eosinophils in the circulation, bone marrow, and target tissues, particularly airways and lungs in asthmatics.[61] By acting on the IL-5 receptor by ADCC, benralizumab decreases blood eosinophils and basophils close to the limit of detection and reduces eosinophil precursors in the bone marrow by 80% or more.[41] Therefore, benralizumab might provide a more complete depletion of airway eosinophils and basophils through enhanced ADCC and subsequently effect in greater reductions of asthma exacerbations and possibly improvements in other clinical expressions of asthma. This makes benralizumab potentially more effective than the mAb against IL-5 itself, which allows only passive removal of IL-5.

Clinical trials, asthma, and benralizumab

The first, Phase I clinical trial on benralizumab in asthma was completed in 2008 (NCT00512486). A multicenter, double-blind, placebo-controlled trial looked into the effects of benralizumab on eosinophil counts in airway mucosal/submucosal biopsy specimens, sputum, bone marrow, and peripheral blood.[61] Two patient cohorts were administered either as a single intravenous or multiple subcutaneous doses of benralizumab. Both intravenous and subcutaneous benralizumab reduced eosinophil counts in airway mucosa, submucosa, and sputum. In a similar fashion to mepolizumab[51] and reslizumab,[50] the study with benralizumab also reported blood eosinophil count reductions of 100%. Single intravenous administrations of benralizumab resulted in marked peripheral blood eosinopenia within 24 hours of dosing, lasting up to 3 months.[57] In contrast, peripheral blood eosinophil depletion can be achieved only gradually with mepolizumab, with a peak reached at 4 weeks after dosing. Actually, intravenous mepolizumab doses only resulted in a 52% reduction in bone marrow eosinophil counts,[51] whereas benralizumab was able to suppress eosinophil counts in bone marrow and peripheral blood to undetectable levels. The greatest benefits were seen in patients with blood eosinophil levels ≥400 cells/μL, who exhibited significant improvement in annual exacerbation rates, lung function, and asthma score with benralizumab treatment. Benralizumab also demonstrated an acceptable safety profile, with only minor adverse events. The most common ones included nasopharyngitis, headache, and nausea. No adverse events were documented apart from nasopharyngitis and injection site reactions. In fact, there have been no safety concerns to date with benralizumab in Phase I, II, and III trials in asthma.[57]

A Phase II, multicenter, randomized, double-blind clinical trial completed in 2011 looked into the ability of one intravenous dose of benralizumab to reduce recurrence after acute asthma exacerbations poorly responsive to initial therapy (NCT00768079).[62] The study enrolled 110 adults with acute asthma exacerbations necessitating urgent admission into the emergency department. Participants in the study were given a single intravenous infusion of either benralizumab or placebo added to current standard

of care with bronchodilators and systemic corticosteroids. Treatment with benralizumab translated into a reduction in the rate and severity of asthma exacerbations up to 12 weeks post-ED admission. In fact, antieosinophilic therapy reduced exacerbation rates by 49% and exacerbations resulting in hospitalization by 60%. These results suggest that there is a persistent effect of a single dose of benralizumab on exacerbations lasting beyond 12 weeks, with noticeable effects up to 24 weeks. This is significant when compared to a course of systemic corticosteroids administered in an emergency setting to reduce asthma exacerbations lasting only for 3 weeks.[63] Benralizumab also produced a long-lasting reduction of blood[57] and airway[61] eosinophils to levels and duration that cannot be achieved by current standard therapy. Patients who are frequently admitted to the ED with asthma exacerbations have often been described as having incomplete adherence to post-ED controller agents. The sustained depletion of eosinophils by benralizumab is independent of compliance with standard-of-care oral and/or inhaled asthma therapy. Health care utilization was reduced in subjects treated with benralizumab who presented to the ED with an asthma exacerbation. Benralizumab had no impact on other outcomes, such as the proportion of subjects who experienced one or more subsequent exacerbations. No changes were identified in pulmonary function tests, asthma control, and self-reported quality of life. As opposed to common assumption that benralizumab would have greater effect on eosinophilic exacerbations, this study demonstrated a clinical response to benralizumab regardless of blood eosinophil count.

Encouraging Phase IIb data on benralizumab[22,56] from August 2013 revealed that in the setting of uncontrolled severe asthma and elevated baseline eosinophil counts, benralizumab can achieve a significant reduction in asthma exacerbation rate. The study recruited patients with uncontrolled asthma using medium-dose or high-dose, inhaled corticosteroids and LABA for at least 12 months before the screening. Patients were then selected on the basis of two to six exacerbations in the past year that required use of a systemic corticosteroid burst. Benralizumab reduced asthma attacks by approximately 40%–70%, depending on the dose received and baseline eosinophil numbers. In fact, the study showed that benralizumab resulted in fewer asthma exacerbations for a subgroup with higher baseline blood eosinophils. The study also met its secondary end points reflected by improvements in asthma control and lung function as measured by FEV_1 over a period of 1 year. Confirming previous literature on the ability of benralizumab to deplete blood and airway eosinophils, benralizumab decreased blood eosinophil levels after the first dose. The investigators used a baseline blood eosinophil cutoff value for the study of at least 300 cells/μL, which is a good biomarker value to identify patients with asthma who may benefit from benralizumab. In conclusion, this randomized, controlled, double-blind, dose-ranging study revealed that in adults with uncontrolled eosinophilic asthma, benralizumab at 20 mg and 100 mg doses reduces asthma exacerbations and baseline blood eosinophils of at least 300 cells/μL, and improves lung function and asthma control.

Phase II studies consistently confirm that benralizumab is able to cut exacerbation rates compared with placebo. The efficacy and safety data from benralizumab Phase II study support the launching of a Phase III Windward program for benralizumab in the management of severe asthma. The Windward program is extensive; it consists of a number of Phase III clinical trials, including CALIMA, SIROCCO, PAMPERO, ZONDA, GREGALE, and BORA. The first study is the CALIMA trial (NCT01914757); it is designed to determine whether subcutaneous benralizumab reduces the number of exacerbations in patients with uncontrolled, severe asthma receiving a double controller regimen of medium-to-high-dose inhaled corticosteroids and a LABA, with or without oral corticosteroids and additional asthma controllers. The CALIMA trial also aims to assess the impact of benralizumab on lung function, asthma symptoms, and other asthma control measures, as well as emergency room visits and hospital admissions due to asthma exacerbations. The trial design includes a personalized health care strategy in patients with eosinophila. Patients will be followed for 56 weeks, and the study is expected to be completed in March 2016.

As mentioned earlier, besides CALIMA, the Windward program includes other Phase III clinical trials as well. Asthma exacerbation rates are currently being examined for benralizumab used in conjunction with high- (SIROCCO) or medium- (PAMPERO) dose inhaled corticosteroids plus a LABA. AstraZeneca will also conduct an oral corticosteroid-reducing trial ZONDA (NCT02075255) to determine the efficacy in reducing oral steroids while on benralizumab. The GREGALE study (NCT02417961) assesses the functionality, reliability, and performance of an accessorized prefilled syringe with benralizumab administered subcutaneously in an at-home setting. Finally, the long-term safety of benralizumab will be examined in a study called BORA (NCT02258542), estimated to end in June 2018.

Clinical trials, COPD, and benralizumab

Although predominantly associated with elevated neutrophils, elevated levels of eosinophils have also been associated with the cause and severity of COPD exacerbations. Out of the 210 million people suffering from COPD globally, 10%–20% shows evidence of eosinophilic airway inflammation. By depleting blood and sputum eosinophils, benralizumab may also have a role in the management of COPD. The mode of action of benralizumab in COPD is controversial, but several possibilities exist. It has been well documented that a subset of patients with COPD may also have underlying asthma phenotype features, resulting in the diagnosis of asthma–COPD overlap syndrome (ACOS).[64] Recently, the Global Initiative for Asthma (GINA) and the Global Initiative for Chronic Obstructive Lung Disease (GOLD) issued a joint document that describes ACOS as a clinical entity that resembles features in favor of each diagnosis. Clinically if three or more features of either asthma or COPD are present, the diagnosis of ACOS should be considered. Relevant variables include age at onset, pattern and time course of symptoms, personal history or family history, variable or persistent airflow limitation, lung function between symptoms, and severe hyperinflation. Typically, asthma is a Th-2-driven cytokine pattern resulting in eosinophila, whereas neutrophilic inflammation dominates in COPD.[65–67] Although ACOS has not been extensively studied with novel biologics, the utilization of benralizumab may be beneficial, especially in those with predominant eosinophila. Currently, COPD is not characteristically a disease of eosinophila; there are a subset of patients with COPD who may benefit from novel biologics that target the eosinophilic pathway. In clearly defined patients with COPD with no evidence of asthma, 20%–30% of patients show eosinophilia (>3% sputum eosinophils) and eosinophilic bronchitis as evidenced by a sputum database analysis.[68] In fact, 18% of patients with COPD with exacerbations actually demonstrate eosinophilia.[69]

Besides asthma management, Phase II studies turned out to be promising for benralizumab in the treatment of COPD, both in terms of safety and efficacy. In 2013, 101 patients with moderate-to-severe COPD entered a trial on the basis of sputum eosinophilia of at least 3% in the previous 12 months plus at least one acute exacerbation requiring oral corticosteroids, antibiotics, or hospitalization in the past year. The primary end point of the study was to determine if benralizumab reduced acute exacerbations of COPD in patients with eosinophilia and COPD in the course of 1 year. The investigation was a randomized, double-blind, placebo-controlled, Phase IIa study (NCT01227278). Study participants received placebo or 100 mg benralizumab subcutaneously, every 4 weeks (three doses), then every 8 weeks (five doses) over 48 weeks. Benralizumab proved to be efficient in significantly improving lung function but had no effect on reducing annualized exacerbation rate in the per-protocol population. However, in the prespecified analyses, the study indicated that inpatient groups of higher baseline levels of blood eosinophils (≥200 cells/μL), benralizumab did reduce COPD exacerbations in a numerical, albeit nonsignificant way. Similar to the per-protocol population, it improved lung function and disease-specific health status. The incidence of adverse events in benralizumab-treated patients, including respiratory disorders (63%) and infections (27%), was comparable to that of placebo groups. Overall, benralizumab failed to reduce the rate of acute exacerbations of COPD compared with placebo but had a positive impact on lung function, quality of life, and COPD symptoms. The results of prespecified subgroup analysis support further investigation of benralizumab in patients with COPD and blood eosinophilia and call for careful identification of eosinophilic phenotypes in COPD (SECOPD). These findings make benralizumab the first biological agent to produce marked reduction in eosinophilic inflammation and beneficial effects in severe eosinophilic COPD.

Insights from Phase II trials prompted the design of Phase III studies for benralizumab in the treatment of COPD. In the setting of the GALATHEA and TERRANOVA trials, benralizumab is currently undergoing Phase III studies to determine if subcutaneous benralizumab reduces COPD exacerbation rate in symptomatic patients with moderate-to-very-severe COPD. Selection criteria include patients receiving standard-of-care therapies, evidence of moderate-to-very-severe COPD, two or more moderate or more than one severe COPD exacerbations requiring treatment or hospitalization in the past year, treatment with double or triple therapy throughout the year before enrollment, and a history of smoking. Health status, quality of life, pulmonary function, respiratory symptoms, rescue medication use, severity, frequency, and duration of exacerbations will be evaluated. These randomized, double-blinded, double-dummy, 56-week, placebo-controlled, multicenter trials are expected to end in the course of 2017.

Benralizumab in the treatment of hypereosinophilic syndrome

Besides the treatment of eosinophilic asthma and COPD, benralizumab seems to be a promising investigational agent in the management of a rare, chronic eosinophilic disorder called hypereosionphilic syndrome (HES). HES consists of peripheral blood eosinophilia with concomitant organ

dysfunction in the absence of parasitic, allergic, or other causes of eosinophilia. Although HES can affect any organ, eosinophilic infiltration and mediator release typically causes damage to the heart, lungs, spleen, skin, and nervous system. HES can be categorized into two broad subtypes: the myeloproliferative and lymphoproliferative variant. The myeloproliferative variant is characterized by a chromosomal defect, resulting in the *FIP1L1/PDGFRA*-associated fusion gene; it may be responsive to treatment with imatinib, a tyrosine kinase inhibitor. In contrast, the lymphoproliferative variant lacks the *FIP1L1/PDGFRA*-associated fusion gene. It is marked by a clonal population of T-cells with aberrant phenotype and is usually treated with corticosteroids.[70] Despite the high proportion of responders to corticosteroid therapy, there are also patients who become relatively refractory to steroids or develop intolerance.

Overproduction of eosinophilopoietic cytokines, such as IL-5, can be one of the underlying mechanisms that can lead to HES. Therefore, investigational agents targeting IL-5 (mepolizumab and reslizumab) or its receptor (benralizumab) can potentially be used as steroid-sparing drugs in the treatment of lymphoproliferative, *FIP1L1-PDGFRA*-negative HES. A randomized, multicenter trial of 85 patients with HES revealed that mepolizumab allows reduction in glucocorticosteroid dose and is well tolerated and effective as a long-term corticosteroid-sparing agent.[52]

An ideal alternative option in the management of HES could be the targeting of the IL-5Rα, which is conveniently restricted to eosinophils, basophils, mast cells, and their precursors. The safety and efficacy of benralizumab as an anti-IL-5Rα agent is currently being evaluated in subjects with HES (NCT02130882). Symptomatic patients with HES on stable therapy are being recruited into a Phase IIa randomized, double-blind, placebo-controlled study, ending in August 2016. The primary end point of the study is to determine the reduction in peripheral blood eosinophilia in the setting of stable background therapy at 12 weeks and 1 year. Secondary end points include the reduction of bone marrow eosinophils and mast cells, tissue eosinophilia, improvement in end organ manifestations, the frequency and severity of adverse events, pharmacokinetics, and the development of antidrug antibodies.

Conclusion

The preclinical and clinical studies with benralizumab provide compelling evidence that targeting the IL-5 pathway in eosinophilic conditions such as asthma, COPD, and HES has therapeutic potential. Through the mechanism of ADCC, eosinophils and basophils are actively and effectively depleted after administration of the mAb benralizumab. This mode of action might provide a novel treatment approach in these eosinophilic conditions. At the time of publication, benralizumab is undergoing Phase III clinical trials in both asthma and COPD and Phase II trials in the management of HES. Benralizumab proved to be effective in reducing exacerbation rate in patients with uncontrolled eosinophilic asthma.[56] In Phase II studies, benralizumab achieved mixed results in the setting of COPD by effecting a clinically significant improvement in lung function but had no impact on exacerbations.[68] In certain forms of HES, anti-IL-5 therapy effectively suppresses blood eosinophils and clinical manifestations of the disease and allows doses of corticosteroids to be tapered. These results seem promising for the potential use of benralizumab in the setting of HES.

Although the overall potential market for anti-IL-5 receptor therapy makes up just a meager 3% of the total asthma population, this group comprises 30% of patients with severe treatment-refractory disease.[71] Patients with severe asthma often fail to achieve adequate control despite heavy use of high-dose, inhaled corticosteroids and long-acting bronchodilators.

There is a strong link between severe asthma and high levels of blood, tissue, and sputum eosinophils. Still, no approved treatments exist for patients with severe asthma with predefined eosinophil levels. Benralizumab has the potential to address this pivotal medical question.

The marked heterogeneity of the pathogenesis of asthma calls for careful selection of asthmatic patients for patient-tailored therapies involving IL modulation. Biologics, such as benralizumab, will have to be offered on the basis of phenotyping patients to achieve the best outcome. It is crucial to determine patient subtypes by sputum and/or peripheral blood eosinophilia to figure out who are likely to be responders or nonresponders to targeted therapies.

This paper has also provided a novel algorithm in the evaluation of patients who may benefit from benralizumab. To date, there have been no safety concerns with benralizumab. However, there is much to be explored with regard to the long-term side effects of benralizumab (ie, infection or malignancy). Although it is still years away from potential clinical use, it is hoped that it will be approved for the management of severe, treatment-refractory asthma in the next 5 years. The development prospects for benralizumab are encouraging as a potential innovative personalized medication for exacerbation-prone patients with severe asthma, COPD, and HES inadequately controlled by current standard-of-care therapy.

Disclosure

The authors report no conflicts of interest in this work.

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Exhibit L

Evaluate™ Press Release

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Top-Line Results from the Benralizumab Phase III Programme in Severe Asthma

Source Press Release
Company Kyowa Hakko Kirin, AstraZeneca
Tags Trial Results, Presentations & Events, Phase III, Monoclonal Antibody, Respiratory
Date May 17, 2016

Tokyo, Japan, May 17th, 2016 – Kyowa Hakko Kirin Co., Ltd. (Tokyo: 4151, President and CEO: Nobuo Hanai, "Kyowa Hakko Kirin ") announced today that AstraZeneca (BK, London, CEO: Pascal Soriot) obtained the positive top-line results that benralizumab , a potential new medicine and anti-eosinophil monoclonal antibody, was well tolerated and achieved the primary end-point in two pivotal Phase III registrational trials (SIROCCO and CALIMA), demonstrating significant reductions in the annualized asthma exacerbation rate compared to placebo.

The trials evaluated the efficacy and safety of two dose regimens of benralizumab as an add-on therapy for severe, uncontrolled asthma with eosinophilic inflammation.

In SIROCCO and CALIMA, the primary analysis population included patients on high-dose inhaled corticosteroids (ICS) plus long-acting β2-agonist (LABA) with a baseline blood eosinophil count ≥ 300 cells/microliter. The safety and tolerability findings for benralizumab were generally consistent with those reported in previous studies. Patients were randomized to receive benralizumab 30 mg every 4 weeks; 30 mg every 4 weeks for the first three doses followed by 30 mg every 8 weeks; or placebo.

"We are delighted with the positive results from these pivotal Phase III studies of benralizumab in patients with uncontrolled asthma. Benralizumab is Kyowa Hakko Kirin 's second product using the POTELLIGENT ® technology that enhances the activity of antibody-dependent cell-mediated cytotoxicity (ADCC). We believe benralizumab has the potential to deliver a new therapeutic option for uncontrolled asthma patients with eosinophilic phenotype." said Yoichi Sato, Managing Executive Officer, Vice President, Head of Research and Development Division of Kyowa Hakko Kirin .

Eosinophils are the biological effector cells which drive inflammation and airways hyper -responsiveness in approximately 50% of asthma patients, leading to frequent exacerbations, impaired lung function and reduced quality of life. Benralizumab is an anti-eosinophil monoclonal antibody that depletes eosinophils via ADCC, the process by which natural killer cells are activated to target eosinophils. Benralizumab induces direct, rapid, and near complete depletion of eosinophils in the bone marrow, blood and target tissue.

Results from the SIROCCO and CALIMA trials will be presented at a future medical meeting.

The Kyowa Hakko Kirin Group companies strive to contribute to the health and well-being of people around the world by creating new value through the pursuit of advances in life sciences and technologies.

About Asthma

Asthma is a common, chronic condition in which inflammation and narrowing of the airways may cause wheezing, breathlessness, chest tightness and coughing. Asthma currently affects the health and day-to-day lifestyles of 315 million individuals worldwide, and by 2020 will likely increase in numbers to as many as 400 million people.

About SIROCCO and CALIMA

SIROCCO and CALIMA are both part of the comprehensive WINDWARD programme in asthma, the largest Phase III development programme for a biologic in respiratory disease. (A total of 2,511 patients: 1,205 in SIROCCO and 1,306 in CALIMA)

About Benralizumab

Benralizumab is an anti-eosinophil monoclonal antibody that binds to the human interleukin-5 receptor alpha subunit (IL-5Rα) expressed on the surface of eosinophils and basophils. In binding to the receptor, benralizumab leads to active, rapid and direct eosinophil depletion (apoptosis by ADCC) through natural killer cells.

Kyowa Hakko Kirin /BioWa have granted to AstraZeneca exclusive development and commercialisation rights for benralizumab in certain countries including the US and Europe and an exclusive option to commercialise benralizumab in Japan and are eligible for milestone payments and royalties related to the development and commercialisation of benralizumab in those countries. Kyowa Hakko Kirin /BioWa have all rights for benralizumab in certain countries in Asia. Kyowa Hakko Kirin retains the rights to participate in certain commercial activities alongside AstraZeneca in Japan when the option is exercised by AstraZeneca .

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