DESCRIPTION

THERAPEUTIC AGENT FOR PRURITUS

Technical Field

The present invention relates to agents for treating or preventing pruritus.

Background Art

Many cytokines are known as humoral factors involved in the growth and differentiation of various types of cells, or in the activation of differentiated mature cell functions. Cytokine-stimulated cells produce different types of cytokines, thereby forming networks of multiple cytokines in the body. Biological homeostasis is maintained by a delicate balance of the mutual regulation between cytokines in these networks. Many inflammatory diseases are thought to result from a failure of such cytokine networks. Thus, monoclonal antibody-based anti-cytokine therapy is drawing much attention. For example, anti-TNF antibodies and anti-IL-6 receptor antibodies have been demonstrated to be highly effective clinically. On the other hand, there are many examples of failure where no therapeutic effects were produced when a single cytokine, such as IL-4, was blocked alone, due to the activation of compensatory pathways in actual pathological conditions.

The present inventors succeeded in isolating a novel cytokine receptor NR10 that was highly homologous to gp130, a receptor for IL-6 signal transduction (Patent Document 1). NR10 forms a heterodimer with oncostatin M receptor (OSMR) and functions as an IL-31 receptor (Non-patent Document 1). NR-10 is also known as glm-r (Non-patent Document 2), GPL (Non-patent Document 3), IL-31RA (Non-patent Document 4), and such. It has also been reported that transgenic mice overexpressing IL-31 spontaneously develop pruritic dermatitis (Non-patent Document 4).

However, it cannot be asserted that forced cytokine expression in mice or a high concentration of blood cytokine in pathological mice are actual causes of the disease. It is totally unclear whether signal blockage by an antibody produces a therapeutic effect. For example, transgenic mice in which IL-18 is overexpressed in keratinocytes develop pruritic dermatitis. In spontaneous atopic dermatitis model mice NC/Nga, the blood concentration of IL-18 increases with advancement of the pathological conditions. From these findings, the overexpression of IL-18 was presumed to be a cause of the disease. Actually, however, the administration of a neutralizing antibody exhibited no therapeutic effect (Non-patent Document 5).

Thus, the inhibition of cytokine function does not necessarily produce a therapeutic

effect in diseases with elevated cytokine expression. It is thus difficult to predict from the expression level of a cytokine what disease the inhibition of the cytokine produces a therapeutic effect on. Therefore, it is important to identify diseases on which the inhibition of signaling of a target cytokine actually produces a therapeutic effect.

Prior art documents of the present invention are described below:

Patent Document 1: WO00/75314

Non-patent Document 1: IL-31 is associated with cutaneous lymphocyte antigen-positive skin homing T cells in patients with atopic dermatitis., J Allergy Clin Immunol. 2006 Feb;117(2):418-25.

Non-patent Document 2: A novel type I cytokine receptor is expressed on monocytes, signals proliferation, and activates STAT-3 and STAT-5.J Biol Chem 277, 16831-6, 2002

Non-patent Document 3: GPL, a novel cytokine receptor related to GP130 and leukemia inhibitory factor receptor. J Biol Chem 278, 49850-9, 2003

Non-patent Document 4: Interleukin 31, a cytokine produced by activated T cells, induces dermatitis in mice. Nat Immunol 5, 752-60, 2004

Non-patent Document 5: Administration of anti-interleukin 18 antibody fails to inhibit development of dermatitis in atopic dermatitis-model mice NC/Nga., British Journal of Dermatology 149: 39-45, 2003

Present Disclosure

[Problems to be Solved]

The present disclosure was achieved in view of the circumstances described above. An objective of the present disclosure is to provide agents for treating or preventing pruritus.

[Means for Solving the Problems]

The present inventors conducted dedicated studies to achieve the objective described above. The present inventors discovered that NR10 antagonists such as neutralizing antibodies against NR10 are useful as therapeutic or preventive agents for pruritus, thereby completing the present invention.

The present disclosure relates to agents for treating or preventing pruritus. More specifically, the present disclosure provides:

[1] a preventive or therapeutic agent for pruritus, which comprises an NR10 antagonist as an active ingredient;

[2] the preventive or therapeutic agent of [1], wherein the NR10 antagonist is an antibody having a neutralizing activity against NR10;

[3] the preventive or therapeutic agent of [2], wherein the antibody is a monoclonal antibody;

[4] the preventive or therapeutic agent of [3], wherein the antibody is a monoclonal antibody having a neutralizing activity against human NR10;
[5] the preventive or therapeutic agent of any one of [2] to [4], wherein the antibody is a recombinant antibody; and
[6] the preventive or therapeutic agent of any one of [2] to [5], wherein the antibody is a chimeric, humanized, or human antibody.

In a first aspect, there is provided a preventive or therapeutic agent for pruritus, which comprises an anti-NR10 antibody having a neutralizing activity against NR10 as an active ingredient, wherein the anti-NR10 antibody recognizes domain 1 (amino acids at positions 21-120) in the amino acid sequence of human NR10 of SEQ ID NO: 7.

In a second aspect, there is provided the use of an anti-NR10 antagonist antibody having a neutralizing activity against NR10 in the manufacture of an agent for preventing or treating pruritus, wherein the anti-NR10 antibody recognizes domain 1 (amino acids at positions 21-120) in the amino acid sequence of human NR10 of SEQ ID NO:7.

In a third aspect, there is provided an anti-NR10 antibody having a neutralizing activity against NR10 for use in the prevention or treatment of pruritus, wherein the antibody recognizes domain 1 (amino acids at positions 21-120) in the amino acid sequence of human NR10 of SEQ ID NO: 7.

Brief Description of the Drawings

Fig. 1 is a graph showing the assessment of IL-31-induced scratching behavior. Mean ± standard error.

Fig. 2 is a graph showing the assessment of mite antigen-induced scratching behavior. Mean ± standard error.

Fig. 3 is a graph showing the time course of percent body weight changes in DSS colitis model mice.

Fig. 4 is a graph showing the time course of changes in the auricular thickness in the picryl chloride-induced acute contact dermatitis model.

Fig. 5 is a graph showing the pruritus-suppressing effect of anti-NR10 antibody H0L0 assessed using the number of scratchings as an indicator.

Mode for Carrying Out the Invention

NR10 is a protein that forms a heterodimer with oncostatin M receptor (OSMR) and functions as an IL-31 receptor. NR10 is also known as glm-r (J Biol Chem 277, 16831-6, 2002), GPL (J Biol Chem 278, 49850-9, 2003), IL-31RA (Nat Immunol 5, 752-60, 2004), and such. Thus, NR10 in the present invention also includes proteins called by these names.

NR10 in the present invention includes those derived from humans, mice, and other mammals. Preferred NR10 includes, without particular limitation, those derived from humans and mice. There are multiple known splicing variants of human-derived NR10 (WO 00/075314). Of the above-described splicing variants, NR10.1 consists of 662 amino acids and contains a transmembrane domain. NR10.2 is a soluble receptor-like protein consisting of 252 amino acids without the transmembrane domain. Meanwhile, known NR10 splicing variants that function as transmembrane receptor proteins include NR10.3 and IL-31RAv3. The human NR10 of the present invention is not particularly limited, as long as it forms a heterodimer with oncostatin M receptor (OSMR) and functions as an IL-31 receptor. Preferred NR10 includes NR10.3 (also referred to as ILRAv4 (Nat Immunol 5, 752-60, 2004)) and IL-31RAv3. NR 10.3 (IL-31RAv4) consists of 662 amino acids (WO 00/075314; Nat Immunol 5, 752-60, 2004) and IL-31RAv3 consists of 732 amino acids (GenBank Accession No: NM_139017). The amino

acid sequence of IL-31RAv4 is shown in SEQ ID NO: 6, and the amino acid sequence of IL-31RAv3 is shown in SEQ ID NO: 7. Meanwhile, mouse-derived NR10 includes proteins comprising the amino acid sequence of SEQ ID NO: 5.

In the present invention, the term NR10 antagonist refers to a substance that blocks intracellular signaling mediated by NR10 activation through binding to NR10 and thus causes loss or suppression of the biological activity of the cells. The biological activity includes, but is not limited to, for example, activities of inducing or suppressing the production of a bioactive substance (for example, chemokines, inflammatory cytokines, and such), activities of promoting or suppressing the secretion of a bioactive substance, growth activities, growth-inducing activities, survival activities, differentiation activities, differentiation-inducing activities, transcriptional activities, membrane transport activities, binding activities, proteolytic activities, phosphorylation/dephosphorylation activities, oxidation-reduction activities, transfer activities, nucleolytic activities, dehydration activities, cell death-inducing activities, and apoptosis-inducing activities.

The presence of the antagonistic activity can be determined by methods known to those skilled in the art. For example, a test compound can be contacted with NR10 expressed on cell surface in the presence of a ligand to determine whether the intracellular signal transduction that serves an indicator for NR10 activation occurs or not. This determination can be performed, for example, according to the method described in the reference "Dillon SR, *et al*., Interleukin 31, a cytokine produced by activated T cells, induces dermatitis in mice. Nat Immunol. 2004 Jul; 5(7):752-60". Compounds that inhibit the intracellular signaling responding to the ligand stimulation are considered to be NR10 antagonists.

The antagonists of the present invention may be naturally-occurring or artificial compounds. Known antagonists can be used in the present invention. It is also possible to use novel compounds that have been determined to have an antagonistic activity by the methods described above.

An embodiment of the NR10 antagonist of the present invention includes antibodies that bind to NR10. Such antibodies that bind to NR10 are not particularly limited; however, antibodies that specifically bind to NR10 are preferred. A preferred embodiment of the antibodies that bind to NR10 includes antibodies having a neutralizing activity against NR10. In the present invention, the "antibody having a neutralizing activity against NR10" refers to an antibody having an activity of suppressing a biological activity based on NR10. In the present invention, "antibodies having a neutralizing activity against NR10" may be polyclonal or monoclonal antibodies; however, in a preferred embodiment, the antibodies are monoclonal antibodies.

The antibodies of the present invention are not particularly limited as long as they bind to NR10, and include recombinant antibodies such as chimeric antibodies, humanized antibodies, and human antibodies. The chimeric antibodies contain, for example, the heavy and light chain constant regions of a human antibody, and the heavy and light chain variable regions of a non-human mammal, such as mouse. The chimeric antibodies can be produced by known methods. For example, the antibodies can be produced by cloning an antibody gene from hybridomas, inserting it into an appropriate vector, and introducing the construct into hosts (see, for example, Carl, A. K. Borrebaeck, James, W. Larrick, THERAPEUTIC MONOCLONAL ANTIBODIES, Published in the United Kingdom by MACMILLAN PUBLISHERS LTD, 1990). Specifically, cDNAs of the antibody variable regions (V regions) are synthesized from mRNA of hybridomas using reverse transcriptase. Once DNAs encoding the V regions of an antibody of interest are obtained, these are linked with DNAs encoding the constant regions (C regions) of a desired human antibody. The resulting constructs are inserted into expression vectors. Alternatively, the DNAs encoding the antibody V regions may be inserted into expression vectors comprising DNAs encoding the C regions of a human antibody. The DNAs are inserted into expression vectors so that they are expressed under the regulation of the expression regulatory regions, for example, enhancers and promoters. In the next step, host cells can be transformed with the expression vectors to allow expression of chimeric antibodies.

Humanized antibodies are also referred to as reshaped human antibodies, and they are prepared by transferring the complementarity determining regions (CDRs) of an antibody derived from a non-human mammal such as a mouse, to the CDRs of a human antibody. General genetic recombination techniques for their preparation are also known. Specifically, a DNA sequence designed such that the CDRs of the mouse antibody are linked with framework regions (FRs) of human antibody is synthesized by PCR using, as primers, several oligonucleotides that have portions overlapping the ends of both CDRs and FRs. The resulting DNA is then ligated to a DNA encoding a human antibody constant region, inserted into an expression vector, and introduced into a host to produce the antibody (see European Patent Application Publication No. EP 239400 and International Patent Application Publication No. WO 96/02576) FRs to be linked via CDRs are selected so that the CDRs form a favorable antigen-binding site. If needed, amino acids in the framework regions of antibody variable region may be substituted so that the CDRs of the reshaped human antibody form a proper antigen-binding site (Sato, K. *et al.*, Cancer Res. (1993) 53, 851-856).

Methods for obtaining human antibodies are also known. For example, desired human antibodies with antigen-binding activity can be obtained by sensitizing human lymphocytes with antigens of interest or cells expressing antigens of interest *in vitro*; and fusing the sensitized lymphocytes with human myeloma cells such as U266 (see Japanese Patent Application Kokoku

Publication No. (JP-B) H01-59878 (examined, approved Japanese patent application published for opposition)). Alternatively, the desired human antibody can also be obtained by immunizing a transgenic animal having an entire repertoire of human antibody genes with a desired antigen (see International Patent Application Publication Nos. WO 93/12227, WO 92/03918, WO 94/02602, WO 94/25585, WO 96/34096, and WO 96/33735).

Furthermore, techniques to obtain human antibodies by panning with a human antibody phage library are known. For example, the variable region of a human antibody is expressed as a single chain antibody (scFv) on the surface of a phage, using a phage display method, and phages that bind to the antigen can be selected. By analyzing the genes of selected phages, the DNA sequences encoding the variable regions of human antibodies that bind to the antigen can be determined. If the DNA sequences of scFvs that bind to the antigen are identified, appropriate expression vectors comprising these sequences can be constructed to obtain human antibodies. Such methods are well known. Reference can be made to WO 92/01047, WO 92/20791, WO 93/06213, WO 93/11236, WO 93/19172, WO 95/01438, WO 95/15388, and such.

The amino acid sequence of heavy or light chain variable region may have a substitution, deletion, addition, and/or insertion of one or more amino acids in the amino acid sequence of the heavy or light chain variable region of an antibody whose neutralizing activity against NR10 has been confirmed. Methods well known to those skilled in the art to prepare the amino acid sequence of the heavy or light chain variable region of an antibody having a neutralizing activity against NR10 in which one or more amino acids are substituted, deleted, added, and/or inserted in the amino acid sequence of the heavy or light chain variable region, include methods for introducing mutations into proteins. For example, those skilled in the art can prepare mutants functionally equivalent to the heavy or light chain variable region of the antibody having a neutralizing activity against NR10 by introducing appropriate mutations into the amino acid sequence of the heavy or light chain variable region of the antibody having a neutralizing activity against NR10 using site-directed mutagenesis (Hashimoto-Gotoh, T, Mizuno, T, Ogasahara, Y, and Nakagawa, M. (1995) An oligodeoxyribonucleotide-directed dual amber method for site-directed mutagenesis. Gene 152, 271-275, Zoller, MJ, and Smith, M.(1983) Oligonucleotide-directed mutagenesis of DNA fragments cloned into M13 vectors. Methods Enzymol. 100, 468-500, Kramer, W, Drutsa, V, Jansen, HW, Kramer, B, Pflugfelder, M, and Fritz, HJ (1984) The gapped duplex DNA approach to oligonucleotide-directed mutation construction. Nucleic Acids Res. 12, 9441-9456、Kramer W, and Fritz HJ (1987) Oligonucleotide-directed construction of mutations via gapped duplex DNA Methods. Enzymol. 154, 350-367, Kunkel, TA (1985) Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc Natl Acad Sci USA. 82, 488-492) or the like. Thus, the heavy or light chain variable regions of antibodies that contain one or more amino acid mutations in the heavy or light chain variable

regions and have a neutralizing activity against NR10 are also included in the heavy or light chain variable regions of the present invention.

When an amino acid residue is altered, the amino acid is preferably mutated for a different amino acid(s) that conserves the properties of the amino acid side-chain. Examples of amino acid side chain properties are: hydrophobic amino acids (A, I, L, M, F, P, W, Y, and V), hydrophilic amino acids (R, D, N, C, E, Q, G, H, K, S, and T), amino acids containing aliphatic side chains (G, A, V, L, I, and P), amino acids containing hydroxyl group-containing side chains (S, T, and Y), amino acids containing sulfur-containing side chains (C and M), amino acids containing carboxylic acid- and amide-containing side chains (D, N, E, and Q), amino acids containing basic side chains (R, K, and H), and amino acids containing aromatic side chains (H, F, Y, and W) (amino acids are represented by one-letter codes in parentheses). Amino acid substitutions within each group are called conservative substitutions. It is already known that a polypeptide containing a modified amino acid sequence in which one or more amino acid residues in a given amino acid sequence are deleted, added, and/or substituted with other amino acids can retain the original biological activity (Mark, D. F. *et al.*, Proc. Natl. Acad. Sci. USA; (1984) 81:5662-6; Zoller, M. J. and Smith, M., Nucleic Acids Res. (1982) 10:6487-500; Wang, A. *et al.*, Science (1984) 224:1431-3; Dalbadie-McFarland, G. *et al.*, Proc. Natl. Acad. Sci. USA (1982) 79:6409-13). Such mutants have an amino acid identity of at least 70%, more preferably at least 75%, even more preferably at least 80%, still more preferably at least 85%, yet more preferably at least 90%, and most preferably at least 95%, with the heavy or light chain variable regions before the amino acid mutations. Herein, sequence identity is defined as the percentage of residues identical to those in the original amino acid sequence of the heavy chain variable region or light chain variable region, determined after the sequences are aligned and gaps are appropriately introduced to maximize the sequence identity as necessary. The identity of amino acid sequences can be determined by the method described above.

Alternatively, the amino acid sequences of heavy or light chain variable regions that have a substitution, deletion, addition, and/or insertion of one or more amino acids in the amino acid sequence of the heavy or light chain variable regions and retain the neutralizing activity against NR10 can be obtained from nucleic acids that hybridize under stringent conditions to nucleic acid composed of the nucleotide sequence encoding the amino acid sequence of the heavy or light chain variable regions. Stringent hybridization conditions to isolate a nucleic acid that hybridizes under stringent conditions to a nucleic acid that includes the nucleotide sequence encoding the amino acid sequence of the heavy or light chain variable regions include, for example, the conditions of 6M urea, 0.4% SDS, 0.5x SSC, and 37°C, or hybridization conditions with stringencies equivalent thereto. With more stringent conditions, for example, the conditions of 6M urea, 0.4% SDS, 0.1x SSC, and 42°C, isolation of nucleic acids with a

much higher homology can be expected. The sequences of the isolated nucleic acids can be determined by the known methods described below. The overall nucleotide sequence homology of the isolated nucleic acid is at least 50% or higher sequence identity, preferably 70% or higher, more preferably 90% or higher (for example, 95%, 96%, 97%, 98%, 99%, or higher).

Nucleic acids that hybridize under stringent conditions to a nucleic acid composed of the nucleotide sequence encoding the amino acid sequence of the heavy or light chain variable regions can also be isolated using, instead of the above-described methods using hybridization techniques, gene amplification methods such as polymerase chain reaction (PCR) using primers synthesized based on the information of nucleotide sequence encoding the amino acid sequence of the heavy or light chain variable regions.

Specifically, the identity of one nucleotide sequence or amino acid sequence to another can be determined using the algorithm BLAST, by Karlin and Altschul (Proc. Natl. Acad. Sci. USA (1993) 90, 5873-7). Programs such as BLASTN and BLASTX were developed based on this algorithm (Altschul *et al.*, J. Mol. Biol. (1990) 215, 403-10). To analyze nucleotide sequences according to BLASTN based on BLAST, the parameters are set, for example, as score= 100 and wordlength= 12. On the other hand, parameters used for the analysis of amino acid sequences by BLASTX based on BLAST include, for example, score= 50 and wordlength= 3. Default parameters for each program are used when using the BLAST and Gapped BLAST programs. Specific techniques for such analyses are known in the art (see the website of the National Center for Biotechnology Information (NCBI), Basic Local Alignment Search Tool (BLAST); http://www.ncbi.nlm.nih.gov).

Alternatively, the antibodies of the present invention may be low-molecular-weight antibodies. The low-molecular-weight antibodies of the present invention include antibody fragments lacking some portions of a whole antibody (for example, whole IgG), and are not particularly limited as long as they retain an NR10-neutralizing activity. The low-molecular-weight antibodies of the present invention are not particularly limited, as long as they contain a portion of whole antibodies. The low-molecular-weight antibodies preferably contain a heavy chain variable region (VH) or light chain variable region (VL). Particularly preferred low-molecular-weight antibodies contain both VH and VL. In addition, preferred examples of the low-molecular-weight antibodies of the present invention include low-molecular-weight antibodies containing CDRs of an antibody. The CDRs contained in the low-molecular-weight antibodies may include some or all of the six CDRs of an antibody.

The low-molecular-weight antibodies of the present invention preferably have a smaller molecular weight than whole antibodies. However, the low-molecular-weight antibodies may form multimers, for example, dimers, trimers, or tetramers, and thus their molecular weights can be greater than those of whole antibodies.

An example of the low-molecular-weight antibodies of the present invention includes scFv antibodies. ScFv antibodies are single-chain polypeptides produced by linking a heavy chain variable region ([VH]) and a light chain variable region ([VL]) via a linker or such (Huston, J. S. *et al.*, Proc. Natl. Acad. Sci. U.S.A. (1988) 85, 5879-5883; Pluckthun "The Pharmacology of Monoclonal Antibodies" Vol. 113, eds., Resenburg and Moore, Springer Verlag, New York, pp. 269-315, (1994)). The order of the heavy chain and light chain variable regions to be linked together is not particularly limited, and they may be arranged in any order. Examples of the arrangement are listed below.

[VH] linker [VL]

[VL] linker [VH]

The amino acid sequence of the heavy chain variable region or light chain variable region may contain a substitution, deletion, addition, and/or insertion. Furthermore, the heavy chain variable region and light chain variable region may also lack some portions or be added with other polypeptides, as long as they have antigen binding activity when linked together. Alternatively, the variable regions may be chimerized or humanized.

In the present invention, linkers which bind the variable regions of the antibody include arbitrary peptide linkers that can be introduced using genetic engineering, or synthetic linkers such as those disclosed in Protein Engineering, 9(3), 299-305, 1996.

The preferred linkers in the present invention are peptide linkers. The lengths of the peptide linkers are not particularly limited and those skilled in the art can appropriately select the lengths depending on the purpose. Typical lengths are one to 100 amino acids, preferably 3 to 50 amino acids, more preferably 5 to 30 amino acids, and particularly preferably 12 to 18 amino acids (for example, 15 amino acids).

Amino acid sequences of such peptide linkers include, for example:

Ser;
Gly-Ser;
Gly-Gly-Ser;
Ser-Gly-Gly;
Gly-Gly-Gly-Ser (SEQ ID NO: 8);
Ser-Gly-Gly-Gly (SEQ ID NO: 9);
Gly-Gly-Gly-Gly-Ser (SEQ ID NO: 10);
Ser-Gly-Gly-Gly-Gly (SEQ ID NO: 11);
Gly-Gly-Gly-Gly-Gly-Ser (SEQ ID NO: 12);
Ser-Gly-Gly-Gly-Gly-Gly (SEQ ID NO: 13);
Gly-Gly-Gly-Gly-Gly-Gly-Ser (SEQ ID NO: 14);
Ser-Gly-Gly-Gly-Gly-Gly-Gly (SEQ ID NO: 15);

(Gly-Gly-Gly-Gly-Ser (SEQ ID NO: 10))n; and
(Ser-Gly-Gly-Gly-Gly (SEQ ID NO: 11))n,
where n is an integer of 1 or larger.

Synthetic chemical linkers (chemical crosslinking agents) include crosslinking agents that are routinely used to crosslink peptides, for example, N-hydroxy succinimide (NHS), disuccinimidyl suberate (DSS), bis(sulfosuccinimidyl) suberate (BS^3), dithiobis(succinimidyl propionate) (DSP), dithiobis(sulfosuccinimidyl propionate) (DTSSP), ethylene glycol bis(succinimidyl succinate) (EGS), ethylene glycol bis(sulfosuccinimidyl succinate) (sulfo-EGS), disuccinimidyl tartrate (DST), disulfosuccinimidyl tartrate (sulfo-DST), bis[2-(succinimidoxycarbonyloxy)ethyl] sulfone (BSOCOES), and bis[2-(sulfosuccinimidoxycarbonyloxy)ethyl] sulfone (sulfo-BSOCOES). These crosslinking agents are commercially available.

The antibodies of the present invention include antibodies in which two or more amino acid residues have been added to the amino acid sequence of an antibody of the present invention. Further, fusion proteins which result from a fusion between one of the above antibodies and a second peptide or protein is included in the present invention. The fusion proteins can be prepared by ligating a polynucleotide encoding an antibody of the present invention and a polynucleotide encoding a second peptide or polypeptide in frame, inserting this into an expression vector, and expressing the fusion construct in a host. Some techniques known to those skilled in the art are available for this purpose. The partner peptide or polypeptide to be fused with an antibody of the present invention may be a known peptide, for example, FLAG (Hopp, T. P. *et al.*, BioTechnology 6, 1204-1210 (1988)), 6x His consisting of six His (histidine) residues, 10x His, influenza hemagglutinin (HA), human c-myc fragment, VSV-GP fragment, p18HIV fragment, T7-tag, HSV-tag, E-tag, SV40 T antigen fragment, lck tag, α-tubulin fragment, B-tag, and Protein C fragment. Other partner polypeptides to be fused with the antibodies of the present invention include, for example, GST (glutathione-S-transferase), HA (influenza hemagglutinin), immunoglobulin constant region, β-galactosidase, and MBP (maltose-binding protein). A polynucleotide encoding one of these commercially available peptides or polypeptides can be fused with a polynucleotide encoding an antibody of the present invention. The fusion polypeptide can be prepared by expressing the fusion construct.

Furthermore, the antibodies of the present invention may be conjugated antibodies which are linked to any of various molecules including polyethylene glycol (PEG), hyaluronic acid, radioactive substances, fluorescent substances, luminescent substances, enzymes, and toxins. Such conjugated antibodies can be obtained by chemically modifying the obtained antibodies. Methods for modifying antibodies have been established in this field (for example, US 5057313 and US 5156840). The "antibodies" of the present invention also include such

conjugated antibodies.

In addition, a preferred embodiment of the anti-NR10 antibodies in the present invention includes, without particular limitation, antibodies that recognize domain 1. In the present invention, domain 1 refers to the region of amino acids at positions 21 to 120 (LPAKP to LENIA) in the amino acid sequence of human NR10 of SEQ ID NO: 7, where the amino acid numbering is based on the sequence including the signal peptide.

The antibodies of the present invention may differ in amino acid sequence, molecular weight, isoelectric point, presence/absence of sugar chains, and conformation depending on the cell or host producing the antibody or the purification method as described below. However, a resulting antibody is included in the present invention as long as it has a function as an NR10 antagonist. For example, when an antibody of the present invention is expressed in prokaryotic cells, for example *E. coli*, a methionine residue is added to the N terminus of the original antibody amino acid sequence. Such antibodies are included in the present invention.

Monoclonal antibodies having a neutralizing activity against NR10 can be obtained, for example, by the following procedure: anti-NR10 monoclonal antibodies are prepared by using as an antigen NR10 or a fragment thereof that is derived from a mammal such as human or mouse by known methods, and then antibodies having a neutralizing activity against NR10 are selected from the thus obtained anti-NR10 monoclonal antibodies. Specifically, a desired antigen or cells expressing the desired antigen are used as a sensitizing antigen for immunization according to conventional immunization methods. Anti-NR10 monoclonal antibodies can be prepared by fusing the obtained immune cells with known parental cells using conventional cell fusion methods, and screening them for monoclonal antibody-producing cells (hybridomas) by conventional screening methods. Animals to be immunized include, for example, mammals such as mice, rats, rabbits, sheep, monkeys, goats, donkeys, cows, horses, and pigs. The antigen can be prepared using the known NR10 gene sequence according to known methods, for example, by methods using baculovirus (for example, WO 98/46777).

Hybridomas can be prepared, for example, according to the method of Milstein *et al.* (Kohler, G. and Milstein, C., Methods Enzymol. (1981) 73: 3-46) or such. When the immunogenicity of an antigen is low, immunization may be performed after linking the antigen with a macromolecule having immunogenicity, such as albumin.

Embodiments of the antibodies of the present invention that have a neutralizing activity against NR10 include monoclonal antibodies that have a neutralizing activity against human NR10. Antigens used to prepare monoclonal antibodies that have a neutralizing activity against human NR10 are not particularly limited, as long as they enable preparation of antibodies that have a neutralizing activity against human NR10. For example, it is known that there are a number of variants of human NR10, and any variant may be used as an immunogen as long as it

enables preparation of antibodies that have a neutralizing activity against human NR10. Alternatively, under the same condition, a peptide fragment of NR10 or a protein in which artificial mutations have been introduced into the natural NR10 sequence may be used as an immunogen. Human NR10.3 is one of preferred immunogens in preparing antibodies that have an activity of binding and/or neutralizing NR10 in the present invention.

Furthermore, the neutralizing activity of antibody against NR10 can be measured, for example, by observing the effect of suppressing the growth of the IL-31-dependent cell line as described in the Referential Examples.

Meanwhile, monoclonal antibodies can also be obtained by DNA immunization. DNA immunization is a method in which a vector DNA constructed such that the gene encoding an antigen protein can be expressed in an animal to be immunized is administered to the animal, and the immunogen is expressed within the body of the animal to provide immunostimulation. As compared to common immunization methods based on the administration of protein antigens, the DNA immunization is expected to be advantageous in that:

- it enables immunostimulation while retaining the structure of a membrane protein; and
- the immunogen does not need to be purified.

On the other hand, it is difficult to combine DNA immunization with an immunostimulating means such as an adjuvant.

In order to obtain a monoclonal antibody by DNA immunization, first, DNA encoding NR10 is administered to an animal to be immunized. The DNA encoding NR10 can be synthesized by known methods such as PCR. The resulting DNA is inserted into an appropriate expression vector, and administered to the animal to be immunized. Expression vectors that can be used include commercially available expression vectors such as pcDNA3.1. The vector can be administered to the living body by conventional methods. For example, DNA immunization can be carried out by introducing gold particles coated with the expression vector into cells by gene gun. Booster using NR10-expressing cells after DNA immunization is a preferred method to yield a monoclonal antibody.

Once the mammal is immunized as described above and the serum level of a desired antibody is confirmed to be increased, immune cells are collected from the mammal and subjected to cell fusion. Preferred immune cells are spleen cells in particular.

Mammalian myeloma cells are used for fusion with the above immune cells. It is preferred that myeloma cells have appropriate selection markers for screening. The selection marker refers to a phenotype that allows (or does not allow) survival under particular culture conditions. Known selection markers include hypoxanthine-guanine phosphoribosyltransferase deficiency (hereinafter abbreviated as "HGPRT deficiency") and thymidine kinase deficiency (hereinafter abbreviated as "TK deficiency"). HGPRT- or TK-deficient cells exhibit

hypoxanthine-aminopterin-thymidine sensitivity (hereinafter abbreviated as "HAT sensitivity"). In HAT selection medium, HAT-sensitive cells cannot synthesize DNA and thus will die. However, when fused with normal cells, they can continue to synthesize DNA via the salvage pathway of the normal cells and thus can grow even in HAT selection medium.

HGPRT- or TK-deficient cells can be selected using a medium containing 6-thioguanine, 8-azaguanine (hereinafter abbreviated as "8AG"), or 5'-bromodeoxyuridine. While normal cells are killed due to incorporation of these pyrimidine analogs into DNA, cells lacking these enzymes can survive in the selection medium because they cannot incorporate these pyrimidine analogs. Another selection marker called G418 resistance confers resistance to 2-deoxystreptamine antibiotics (gentamicin analogs) due to the neomycin resistance gene. Various myeloma cells suitable for cell fusion are known.

Cell fusion between immune cells and myeloma cells can be essentially carried out according to known methods, for example, the method by Kohler and Milstein (Kohler. G. and Milstein, C., Methods Enzymol. (1981) 73, 3-46).

More specifically, cell fusion can be carried out, for example, in a common culture medium in the presence of a cell fusion-promoting agent. The fusion-promoting agent includes, for example, polyethylene glycol (PEG) and Sendai virus (HVJ). If required, an auxiliary agent such as dimethyl sulfoxide may also be added to improve fusion efficiency.

The immune cells and myeloma cells may be used at an arbitrarily determined ratio. For example, the ratio of immune cells to myeloma cells is preferably from 1 to 10. Culture media to be used for cell fusion include, for example, media that are suitable for the cell growth of myeloma cell line, such as RPMI 1640 and MEM, and other common culture media used for this type of cell culture. In addition, the culture media may also be supplemented with serum supplement such as fetal calf serum (FCS).

Predetermined amounts of immune cells and myeloma cells are mixed well in the culture medium, and then mixed with a PEG solution pre-heated to 37°C to produce fused cells (hybridomas). In the cell fusion method, for example, PEG with mean molecular weight of about 1,000-6,000 can be added to the cells typically at a concentration of 30% to 60% (w/v). Then, successive addition of the appropriate culture medium listed above and removal of supernatant by centrifugation are repeated to eliminate the cell fusion agent and such, which are unfavorable to the growth of hybridomas.

The resulting hybridomas can be screened using a selection medium according to the selection marker possessed by myeloma cells used in the cell fusion. For example, HGPRT- or TK-deficient cells can be screened by culturing them in a HAT medium (a medium containing hypoxanthine, aminopterin, and thymidine). Specifically, when HAT-sensitive myeloma cells are used in cell fusion, cells successfully fused with normal cells can be selectively grown in the

HAT medium. The cell culture using the above HAT medium is continued for a sufficient period of time to allow all cells except the desired hybridomas (non-fused cells) to die. Specifically, in general, the desired hybridomas can be selected by culturing the cells for several days to several weeks. Then, screening and single cloning of hybridomas that produce an antibody of interest can be carried out by performing ordinary limiting dilution methods. Alternatively, antibodies that recognize NR10 can be prepared by the method described in WO 03/104453.

Screening and single cloning of an antibody of interest can be suitably carried out by known screening methods based on antigen-antibody reaction. For example, an antigen is bound to a carrier such as beads made of polystyrene or such and commercially available 96-well microtiter plates, and then reacted with the culture supernatant of hybridoma. Next, the carrier is washed and then reacted with an enzyme-labeled secondary antibody or such. When the culture supernatant contains an antibody of interest reactive to the sensitizing antigen, the secondary antibody binds to the carrier via this antibody. Finally, the secondary antibody bound to the carrier is detected to determine whether the culture supernatant contains the antibody of interest. Hybridomas producing a desired antibody capable of binding to the antigen can be cloned by the limiting dilution method or such. Not only the antigen used for immunization but also an NR10 protein substantially equivalent thereto can be preferably used as an antigen for this purpose. For example, a cell line expressing NR10, the extracellular domain of NR10, or an oligopeptide composed of a partial amino acid sequence constituting the domain may be used as the antigen.

In addition to the above-described method for preparing hybridomas through immunization of a nonhuman animal with an antigen, antibodies of interest can also be obtained by sensitizing human lymphocytes with an antigen. Specifically, first, human lymphocytes are sensitized with an NR10 protein *in vitro*. Then, the sensitized lymphocytes are fused with an appropriate fusion partner. For example, human-derived myeloma cells with the ability to divide permanently can be used as the fusion partner (see Japanese Patent Application Kokoku Publication No. (JP-B) H1-59878 (examined, approved Japanese patent application published for opposition)). Antibodies obtained by this method are human antibodies having an activity of binding to the NR10 protein.

The antibodies obtained by the above-described method can be produced by methods known to those skilled in the art. For example, based on the sequence of the NR10-recognizing antibody, the antibody can be produced by genetic recombination techniques known to those skilled in the art. Specifically, a polynucleotide encoding an antibody can be constructed based on the sequence of the NR10-recognizing antibody, inserted into an expression vector, and then expressed in appropriate host cells (see for example, Co, M. S. *et al.*, J. Immunol. (1994) 152,

2968-2976; Better, M. and Horwitz, A. H., Methods Enzymol. (1989) 178, 476-496; Pluckthun, A. and Skerra, A., Methods Enzymol. (1989) 178, 497-515; Lamoyi, E., Methods Enzymol. (1986) 121, 652-663; Rousseaux, J. *et al.*, Methods Enzymol. (1986) 121, 663-669; Bird, R. E. and Walker, B. W., Trends Biotechnol. (1991) 9, 132-137).

The vectors include M13 vectors, pUC vectors, pBR322, pBluescript, and pCR-Script. Alternatively, when aiming to subclone and excise cDNA, the vectors include, for example, pGEM-T, pDIRECT, and pT7, in addition to the vectors described above. Expression vectors are particularly useful when using vectors for producing the antibodies of the present invention. For example, when aiming for expression in *E. coli* such as JM109, DH5α, HB101, and XL1-Blue, the expression vectors not only have the above-described characteristics that allow vector amplification in *E. coli*, but must also carry a promoter that allows efficient expression in *E. coli*, for example, lacZ promoter (Ward *et al.*, Nature (1989) 341, 544-546; FASEB J. (1992) 6, 2422-2427), araB promoter (Better *et al.*, Science (1988) 240, 1041-1043), T7 promoter or such. Such vectors include pGEX-5X-1 (Pharmacia), "QIAexpress system" (Qiagen), pEGFP, or pET (in this case, the host is preferably BL21 that expresses T7 RNA polymerase) in addition to the vectors described above.

The vectors may contain signal sequences for antibody secretion. As a signal sequence for antibody secretion, a pelB signal sequence (Lei, S. P. *et al* J. Bacteriol. (1987) 169, 4379) may be used when a protein is secreted into the *E. coli* periplasm. The vector can be introduced into host cells by calcium chloride or electroporation methods, for example.

In addition to vectors for *E. coli,* the vectors for producing the antibodies of the present invention include mammalian expression vectors (for example, pcDNA3 (Invitrogen), pEF-BOS (Nucleic Acids. Res. 1990, 18(17), p5322), pEF, and pCDM8), insect cell-derived expression vectors (for example, the "Bac-to-BAC baculovirus expression system" (Gibco-BRL) and pBacPAK8), plant-derived expression vectors (for example, pMH1 and pMH2), animal virus-derived expression vectors (for example, pHSV, pMV, and pAdexLcw), retroviral expression vectors (for example, pZIPneo), yeast expression vectors (for example, "Pichia Expression Kit" (Invitrogen), pNV11, and SP-Q01), and *Bacillus subtilis* expression vectors (for example, pPL608 and pKTH50), for example.

When aiming for expression in animal cells such as CHO, COS, and NIH3T3 cells, the vectors must have a promoter essential for expression in cells, for example, SV40 promoter (Mulligan *et al.*, Nature (1979) 277, 108), MMLV-LTR promoter, EF1α promoter (Mizushima *et al.*, Nucleic Acids Res. (1990) 18, 5322), and CMV promoter, and more preferably they have a gene for selecting transformed cells (for example, a drug resistance gene that allows evaluation using an agent (neomycin, G418, or such). Vectors with such characteristics include pMAM, pDR2, pBK-RSV, pBK-CMV, pOPRSV, and pOP13, for example.

In addition, the following method can be used for stable gene expression and gene amplification in cells: CHO cells deficient in a nucleic acid synthesis pathway are introduced with a vector (for example, pSV2-dhfr (Molecular Cloning 2nd edition, Cold Spring Harbor Laboratory Press, 1989)) that carries a DHFR gene which compensates for the deficiency, and the vector is amplified using methotrexate (MTX). Alternatively, the following method can be used for transient gene expression: COS cells with a gene expressing SV40 T antigen on their chromosome are transformed with a vector (pcD and such) with an SV40 replication origin. Replication origins derived from polyoma virus, adenovirus, bovine papilloma virus (BPV), and such can also be used. To amplify gene copy number in host cells, the expression vectors may further carry selection markers such as aminoglycoside transferase (APH) gene, thymidine kinase (TK) gene, *E. coli* xanthine-guanine phosphoribosyltransferase (Ecogpt) gene, and dihydrofolate reductase (dhfr) gene.

The antibodies of the present invention obtained by the methods described above can be isolated from inside host cells or from outside the cells (the medium, or such), and purified to homogeneity. The antibodies can be isolated and purified by methods routinely used for isolating and purifying antibodies, and the type of method is not limited. For example, the antibodies can be isolated and purified by appropriately selecting and combining column chromatography, filtration, ultrafiltration, salting out, solvent precipitation, solvent extraction, distillation, immunoprecipitation, SDS-polyacrylamide gel electrophoresis, isoelectrofocusing, dialysis, recrystallization, and such.

The chromatographies include, for example, affinity chromatography, ion exchange chromatography, hydrophobic chromatography, gel filtration, reverse phase chromatography, and adsorption chromatography (Strategies for Protein Purification and Characterization: A Laboratory Course Manual. Ed Daniel R. Marshak *et al.*, Cold Spring Harbor Laboratory Press, 1996). The chromatographic methods described above can be conducted using liquid chromatography, for example, HPLC and FPLC. Columns that can be used for affinity chromatography include protein A columns and protein G columns. Columns using protein A include, for example, Hyper D, POROS, and Sepharose FF (GE Amersham Biosciences). The present invention includes antibodies that are highly purified using these purification methods.

The NR10-binding activity of the antibodies can be determined by methods known to those skilled in the art. Methods for determining the antigen-binding activity of an antibody include, for example, ELISA (enzyme-linked immunosorbent assay), EIA (enzyme immunoassay), RIA (radioimmunoassay), and fluorescent antibody method. For example, when enzyme immunoassay is used, antibody-containing samples, such as purified antibodies and culture supernatants of antibody-producing cells, are added to antigen-coated plates. A secondary antibody labeled with an enzyme, such as alkaline phosphatase, is added and the

plates are incubated. After washing, an enzyme substrate, such as p-nitrophenyl phosphate, is added, and the absorbance is measured to evaluate the antigen-binding activity.

Moreover, the neutralizing activity of an antibody against NR10 can be determined, for example, by the method described in the Reference Examples, in which the growth inhibition effect on the IL-31-depenedent cell line is observed.

The NR10 antagonists or antibodies having a neutralizing activity against NR10 in the present invention can be used as preventive or therapeutic agents for pruritus. The present inventors administered antibodies having a neutralizing activity against mouse NR10 to pruritus model animals and demonstrated that the antibodies produced a marked therapeutic effect. Furthermore, non-antibody NR10 antagonists are also expected to have a therapeutic effect against pruritus as observed in the Examples.

The present inventors discovered that antagonistic antibodies against NR10 had a therapeutic effect on pruritus. On the other hand, it was revealed that in the acute contact dermatitis model and the DSS acute colitis model, anti-NR10 antagonistic antibodies had no therapeutic effect against these diseases themselves.

In the present invention, the treatment of pruritus is distinguished from the treatment of diseases and symptoms that cause pruritus (for example, the diseases described below, such as atopic dermatitis and type C hepatitis). Therefore, the therapeutic or preventive agents of the present invention for pruritus target pruritus itself, and they are not for treating or preventing diseases and symptoms that cause pruritus. The therapeutic or preventive agents of the present invention are administered to patients in need of treatment or prevention of pruritus for the purpose of treating or preventing pruritus, but not for the purpose of treating or preventing diseases and symptoms that cause pruritus.

Pruritus to be treated by the present invention is not particularly limited, and may be any type of pruritus. Specific examples of pruritus to be treated by the present invention include, for example, pruritus in scabies, pediculation, bug bites and stings, urticaria, atopic dermatitis, contact dermatitis, lichen planus, miliaria, dermatitis herpetiformis, xeroderma, biliary obstruction, primary biliary cirrhosis (PBC), infectious hepatitis such as type C hepatitis, urinemia, chronic renal failure, renal dialysis, lymphoma, leukemia, polycythemia rubra vera, pregnancy, ingestion of drugs (barbiturate, salicylate, etc.), hyperthyroidism, diabetes mellitus, and organ cancers.

The preventive or therapeutic agents of the present invention for pruritus comprise as an active ingredient the above-described NR10 antagonist or antibody having a neutralizing activity against NR10. The phrase "comprise an NR10 antagonist as an active ingredient" means containing an NR10 antagonist as at least one active ingredient, and does not limit the content of the NR10 antagonist. The therapeutic or preventive agents of the present invention for pruritus

may contain other ingredients that promote the prevention or treatment of pruritus, in combination with the NR10 antagonist.

Pruritus for which the therapeutic or preventive agents of the present invention are used is not particularly limited, and may be developed by any cause; however, preferred pruritus is pruritus involving IL-31. Such pruritus involving IL-31 includes pruritus caused by IL-31 and pruritus with high expression of IL-31.

The NR10 antagonists of the present invention may be prepared as formulations according to standard methods (see, for example, Remington's Pharmaceutical Science, latest edition, Mark Publishing Company, Easton, USA). Further, they may contain pharmaceutically acceptable carriers and/or additives if necessary. For example, they may contain surfactants (for example, PEG and Tween), excipients, antioxidants (for example, ascorbic acid), coloring agents, flavoring agents, preservatives, stabilizers, buffering agents (for example, phosphoric acid, citric acid, and other organic acids), chelating agents (for example, EDTA), suspending agents, isotonizing agents, binders, disintegrators, lubricants, fluidity promoters, and corrigents. However, without limitation to these, the agents for preventing or treating inflammatory diseases of the present invention may contain other commonly used carriers. Such carriers specifically include light anhydrous silicic acid, lactose, crystalline cellulose, mannitol, starch, carmelose calcium, carmelose sodium, hydroxypropylcellulose, hydroxypropylmethylcellulose, polyvinylacetaldiethylaminoacetate, polyvinylpyrrolidone, gelatin, medium chain fatty acid triglyceride, polyoxyethylene hydrogenated castor oil 60, sucrose, carboxymethylcellulose, corn starch, and inorganic salt. The agents may also contain other low-molecular-weight polypeptides, proteins such as serum albumin, gelatin, and immunoglobulin, and amino acids such as glycine, glutamine, asparagine, arginine, and lysine. When the NR10 antagonist is prepared as an aqueous solution for injection, the NR10 antagonist may be dissolved in an isotonic solution containing, for example, physiological saline, dextrose, or other adjuvants. The adjuvants may include, for example, D-sorbitol, D-mannose, D-mannitol, and sodium chloride. In addition, appropriate solubilizing agents, for example, alcohols (for example, ethanol), polyalcohols (for example, propylene glycols and PEGs), and non-ionic detergents (polysorbate 80 and HCO-50) may be used concomitantly.

If necessary, NR10 antagonists may be encapsulated in microcapsules (microcapsules made of hydroxymethylcellulose, gelatin, polymethylmethacrylate, and the like), and made into components of colloidal drug delivery systems (liposomes, albumin microspheres, microemulsions, nano-particles, and nano-capsules) (for example, see "Remington's Pharmaceutical Science 16th edition" &, Oslo Ed. (1980)). Moreover, methods for making sustained-release drugs are known, and these can be applied for NR10 antagonists (Langer *et al.*, J. Biomed. Mater. Res. (1981) 15, 167-277; Langer, Chem. Tech. (1982) 12, 98-105; US Patent

No. 3,773,919; European Patent Application (EP) No. 58,481; Sidman *et al.*, Biopolymers (1983) 22, 547-56; EP 133,988).

The agents for preventing or treating pruritus of the present invention can be administered either orally or parenterally, but are preferably administered parenterally. Specifically, the agents are administered to patients by injection or percutaneous administration. Injections include, for example, intravenous injections, intramuscular injections, and subcutaneous injections, for systemic or local administration. The agents may be given to sites where inflammation is to be suppressed, or areas surrounding the sites by local infusion, intramuscular injection in particular. The administration methods can be properly selected according to the patient's age and condition. The single-administration dose can be selected, for example, from within the range of 0.0001 to 100 mg of the active ingredient per kg body weight. Alternatively, for example, when the agents are administered to human patients, the dose of the active ingredient can be selected from within the range of 0.001 to 1,000 mg/kg body weight. The single-administration dose preferably contains, for example, about 0.01 to 50 mg/kg body weight of an NR10 antagonist. However, the dose of an agent for preventing or treating pruritus of the present invention is not limited to these examples.

The present invention also provides therapeutic agents for pruritus, which comprise an IL-31 antagonist as an active ingredient. Such IL-31 antagonists are not particularly limited, as long as they are substances that inhibit the biological activity of IL-31 by binding to IL-31. Preferred IL-31 antagonists include, for example, anti-IL-31 antibodies (for example, WO 2006/088955, WO 2006/88956, and WO 2006/122079). Preparation, alteration, modification, production, purification, administration, formulation, and such of anti-IL-31 antibodies can be performed according to the above description regarding the anti-NR10 antibodies.

All prior art documents cited in the present specification are incorporated herein by reference.

Examples

Hereinbelow, the present invention is specifically described using Examples, but it is not to be construed as being limited thereto.

[Example 1]

Assessment of IL-31-induced scratching behavior

Ten μg of mouse IL-31 (in house) was intravenously administered to 9-week-old normal female BALB/c mice (Charles River Laboratories Japan). The scratching behavior was monitored and analyzed for 12 hours immediately after administration using a scratching counting system (MicroAct; NeuroScience Inc.). As a result, the IL-31-administered group

(n=8) showed a significant increase in the number of scratchings with a peak at about five hours after the administration, as compared to the group administered with a vehicle (PBS containing 0.5 % BALB/c mouse serum) (n=8). This IL-31-induced scratching behavior was completely suppressed by intravenous administration of 350 mg/kg BM095, an anti-mouse NR10 neutralizing antibody, prior to IL-31 administration (n=8) (Fig. 1). This result demonstrates that the anti-NR10 neutralizing antibody has an effect of suppressing IL-31-induced pruritus.

Effect of anti-NR10 neutralizing antibody in mite antigen-induced dermatitis model

Five μg of *Dermatophagoides pteronyssinus* (Dp) crude extract (Cosmo Bio LSL) was administered as a mite antigen intradermally to 9-week-old SPF female NC/Nga Tnd Crlj mice (Charles River Laboratories Japan) on the ventral side of their ears three times a week for three weeks to induce dermatitis (Int Arch Allergy Immunol 2004;133:55-63). In the solvent control group for Dp, 5 μl of normal saline (Otsuka Pharmaceutical Co.) was administered on the same schedule (n=7). In this pathological model, the anti-mouse NR10 neutralizing antibody BM-095 was intravenously administered at 20 mg/kg on days 0, 3, 7, 10, 14, 17, and 21 (n=8). For a vehicle control group, 200 mmol/L NaCl/20 mmol/L sodium acetate buffer (pH 5.5) was intravenously administered on the same schedule (n=8).

The pruritus was assessed by counting the number of scratchings during the measuring time of 12 hours on day 21 using a scratching counting system (MicroAct; NeuroScience Inc.). The result showed that the number of scratchings was significantly increased in the vehicle control group as compared to the solvent control group for Dp ($p<0.005$). In the BM095-administered group, the number of scratchings was significantly reduced as compared to the vehicle control group ($p<0.05$) (Fig. 2).

This result demonstrates that the anti-NR10 neutralizing antibody has a suppressing effect against pruritus.

[Example 2] Suppressing effect of H0L0 against IL-31-induced pruritus in cynomolgus monkeys

The effect of anti-human NR10 antibody H0L0 (heavy chain amino acid sequence/SEQ ID NO: 17; light chain amino acid sequence/SEQ ID NO: 18) on pruritus induced by intravenously administering cynomolgus IL-31 to 4- to 5-year-old cynomolgus monkeys was examined. PBS (vehicle) or H0L0 was intravenously administered at 0.01, 0.03, 0.06, 0.3, and 0.6 mg/kg. Twenty-four hours after intravenous administration, 1 g/kg of cynomolgus IL-31 was intravenously administered, and then the behavior was recorded with a video camera for two hours. The number of scratchings was counted while replaying the recorded video, assuming three or more consecutive scratching actions as a scratching. The result showed that H0L0 reduced the number of cynomolgus IL-31-induced scratchings in a dose-dependent manner (Fig.

5). This result demonstrates that the anti-NR10 antibody H0L0 has a suppressing effect against pruritus.

[Referential Example 1] Establishment of NR10- and OSMR-expressing Ba/F3 cell lines

The human NR10 cDNA (WO 00/75314 SEQ ID NO: 1/SEQ ID NO: 16) was inserted into the expression vector pCOS1 (Biochem. Biophys. Res. Commun. 228, p838-45, 1996), and the resulting vector was named pCosNR10.3. An oncostatin M receptor cDNA (OSMR, GenBank accession No. NM003999) was isolated by PCR from a human placental library, and the expression vector pCos1-hOSMR was constructed in the same manner. 10 μg each of the vectors were simultaneously introduced into mouse IL-3-dependent pro-B cell-derived cell line Ba/F3 by electroporation (BioRad Gene Pulser, 960 μF, 0.33 kV). After introduction, human IL-31 was added, and the cells were cultured to obtain a cell line that proliferates in an IL-31-dependent manner. In the same manner, a mouse IL-31-dependent cell line was also produced from Ba/F3 cells expressing the mouse NR10 and mouse OSMR genes.

Both cell lines exhibited an ED50 of several ng/ml and well proliferated. The human IL-31-dependent cell line did not respond to mouse IL-31, and was suppressed by addition of human NR10 protein (extracellular domain). Meanwhile, the mouse IL-31-dependent cell line did not respond to human IL-31, and was not suppressed by addition of mouse NR10 protein (extracellular domain).

[Referential Example 2] Preparation of NR10 protein (extracellular domain)

The human NR10 cDNA was used as a template to amplify only the extracellular domain by PCR. The amplified region was then attached to a FLAG tag sequence at the C terminus and inserted to the expression vector pCXND3 (WO2005/005636) (pCXND3-NR10-flag). Ten μg of the linearized vector was introduced into Chinese hamster ovary cell line DG44 by electroporation (BioRad Gene PulserII, 25 μF, 1.5 kV). A cell line showing high level expression was obtained. The supernatant of the cell line cultured on a large scale was purified using anti-FLAG antibody column (Sigma) and gel filtration to obtain a purified sample, which was subjected to the experiments described below. Mouse NR10 (extracellular domain) in which a FLAG tag sequence has been added at the C terminus was also produced in the same manner.

[Referential Example 3] Isolation of scFv having anti-mouse NR10-neutralizing activity and preparation of chimeric IgG BM095

Candidate clones were screened from a human antibody phage library by the panning method using biotinylated mouse NR10 protein (extracellular domain). Secretory scFvs were

purified from these clones and added to the IL-31-dependent Ba/F3 cell growth assay system described in Referential Example 1. As a result, a clone exhibiting a strong growth-suppressing activity, BM095, was successfully obtained.

The sequences of human H chain variable region (VH) and light chain variable region (VL) of BM095 were linked to mouse IgG2a constant region (after CH1) and λ chain constant region, respectively, by PCR to construct an expression vector. This VH amino acid sequence is shown in SEQ ID NO: 1, and the nucleotide sequence encoding the amino acid sequence is shown in SEQ ID NO: 2. The VL amino acid sequence is shown in SEQ ID NO: 3, and the nucleotide sequence encoding the amino acid sequence is shown in SEQ ID NO: 4. The respective linearized expression vectors were simultaneously introduced into DG44 cells, and a cell line expressing the chimeric IgG at a high level was selected. A purified sample was obtained from the supernatant of a large-scale culture of this cell line by Protein A (rProtein A Sepharose Fast Flow, GE Amersham Biosciences) column chromatography and cation exchange (SP-TOYOPEARL 650M, TOSOH) column chromatography. Then, ActiClean Etox (Sterogen) resin was used to reduce pyrogens below the detection limit.

[Referential Example 4] Efficacy of BM095 on dextran sulfate sodium (DSS)-induced colitis

The DSS-induced colitis model (J Immunol 2003; 171:5507-5513), which has been reported as a pathological model for inflammatory bowel disease (IBD), was prepared to examine the effect of BM-095, an anti-mouse NR10 neutralizing antibody. An aqueous solution of 5% (w/v) dextran sulfate sodium salt (Wako Pure Chemical Industries) was prepared using distilled water sterilized by filtration with 0.22-μm filter (Millipore). Six-week-old male Balb/cAnN Crj mice (Charles River Laboratories Japan) were allowed to freely consume the solution from water bottles for seven days. The body weights were measured, and the percent change in the body weight relative to that on the first day of DSS administration was used to assess the drug efficacy.

In order to test whether the pathological condition is improved in this model by neutralization of IL-31 signaling, the anti-mouse NR10 neutralizing antibody BM095 was intravenously administered at 10 mg/kg on the day before DSS administration, and the weight loss was assessed (n=10). To the vehicle control group, the vehicle (a mixture of acetate buffer (20 mmol/L sodium acetate, 20 mmol/L sodium chloride) and phosphate-buffered saline (PBS; GIBCO) at a volume ratio of 1 : 5) was intravenously administered on the day before DSS administration (vehicle group; n=10). Furthermore, percent body weight changes of a Balb/cAnN Crj mouse of the same age and sex as those in the DSS administration group were also monitored (n=1) to evaluate percent body weight changes of normal mouse.

The time course of body weight changes is shown in Fig. 3. DSS administration

resulted in a decrease in the percent body weight in the vehicle group. On the other hand, the BM095-administered group showed similar body weight changes to the vehicle group; however, after four and five days of the DSS administration, a significant decrease of the percent body weight was observed in the BM095 group as compared to the vehicle group. From these results, BM095 administration had no therapeutic effect on colitis in this model.

Although IL-31RA expression was reported to be enhanced in this model (WO 2004/003140), the above experimental results revealed that neutralizing antibodies against the molecule had no therapeutic effect against colitis in this model.

[Referential Example 5] Efficacy of BM095 on picryl chloride-induced acute contact dermatitis model

Dermatitis resulting from delayed hypersensitivity reaction sensitized/induced by picryl chloride application, which has been reported as an acute contact dermatitis model (Clin Immunol 2003; 108: 257-262), was created to assess the effect of BM-095, an anti-mouse NR10 neutralizing antibody. Eight-week-old female Balb/cAnN Crj mice (Charles River Laboratories Japan) were sensitized by applying 50 μL of 7% picryl chloride (nacalai tesque, Inc.) solution (ethanol : acetone = 3:1, v/v) onto the abdominal skin. After five days, contact dermatitis was elicited by applying 20 μL of 1% picryl chloride solution (acetone : olive = 1:4, v/v) onto the skin of right auricle (induction). For a control for assessing the influence of the solvent on the auricular thickness, 20 μl of the solvent (acetone : olive = 1:4, v/v) was applied onto the skin of left auricle of the same mice (positive control; n = 6). The thicknesses of right and left auricles were measured with a dial thickness gauge (OZAKI MFG. CO., LTD.) immediately before induction and 24, 48, and 72 hours after induction. The change in the auricular thickness relative to the thickness immediately before induction was used to assess the drug efficacy.

To assess the establishment of the pathological condition, a group in which ethanol-acetone mixed solution (3:1, v/v) without picryl chloride was applied onto the abdominal skin at the time of sensitization, and after five days 20 μL of 1% picryl chloride solution was applied onto the skin of right auricle and 20 μl of the solvent (acetone : olive =1:4, v/v) was applied onto the skin of left auricle, was studied as a control group (negative control group; n=6).

To assess the effect of administration of anti-NR10 antibody on the pathological condition in this model, a group in which acute contact dermatitis was elicited by the method used for the above positive control group and 10 mg/kg of BM095 was intravenously administered on the day before sensitization and the day before induction (BM095 group, n=6), and a group in which the vehicle (a mixture of acetate buffer (20 mmol/L sodium acetate, 20 mmol/L sodium chloride) and phosphate-buffered saline (PBS; GIBCO) at a volume ratio of 1:5) was administered at the same timing (vehicle group, n=5), were studied.

The time course of auricular thickness changes up to 72 hours after induction is shown in Fig. 4. Auricles were significantly thickened in the positive control group at all time points of 24, 48, and 72 hours after induction, as compared to the negative control group, showing the establishment of the pathological condition. In the meantime, the BM-095 group showed similar time course of auricular thickness changes to the vehicle group, and thus no significant suppression was observed.

These results revealed that BM095 administration had no therapeutic effect against acute contact dermatitis observed in this model.

Industrial Applicability

NR10 antagonists, such as neutralizing antibodies against NR10, provided by the present invention are useful as therapeutic or preventive agents for pruritus.

CLAIMS

1. A preventive or therapeutic agent for pruritus, which comprises an anti-NR10 antibody having a neutralizing activity against NR10 as an active ingredient, wherein the anti-NR10 antibody recognizes domain 1 (amino acids at positions 21-120) in the amino acid sequence of human NR10 of SEQ ID NO: 7.

2. The preventive or therapeutic agent of claim 1, wherein the antibody is a monoclonal antibody.

3. The preventive or therapeutic agent of claim 1 or 2, wherein the antibody is a recombinant antibody.

4. The preventive or therapeutic agent of any one of claims 1 to 3, wherein the antibody is a chimeric, humanized, or human antibody.

5. Use of an anti-NR10 antagonist antibody having a neutralizing activity against NR10 in the manufacture of an agent for preventing or treating pruritus, wherein the anti-NR10 antibody recognizes domain 1 (amino acids at positions 21-120) in the amino acid sequence of human NR10 of SEQ ID NO:7.

6. The use of claim 5, wherein the antibody is a monoclonal antibody.

7. The use of claim 5 or 6, wherein the antibody is a recombinant antibody.

8. The use of any one of claims 5 to 7, wherein the antibody is a chimeric, humanized, or human antibody.

9. An anti-NR10 antibody having a neutralizing activity against NR10 for use in the prevention or treatment of pruritus, wherein the antibody recognizes domain 1 (amino acids at positions 21-120) in the amino acid sequence of human NR10 of SEQ ID NO: 7.

10. The preventive or therapeutic agent of claim 1, wherein the anti-NR10 antibody is H0L0 (heavy chain amino acid sequence/SEQ ID NO:17; light chain amino acid sequence/SEQ ID NO:18).

11. The preventive or therapeutic agent of claim 1, wherein the anti-NR10 antibody comprises the heavy or light chain variable region of H0L0 with one or more amino acid substitution, deletion, addition, and/or insertion, and has a neutralizing activity against NR10 equivalent to H0L0.

12. The preventive or therapeutic agent of claim 1, wherein the anti-NR10 antibody comprises a heavy or light chain variable region having an amino acid identity of at least 90% with the heavy or light chain variable region of H0L0, and has a neutralizing activity against NR10 equivalent to H0L0.

13. The preventive or therapeutic agent of claim 1, wherein the pruritis is selected from the group consisting of pruritis in scabies, pediculation, bug bites and stings, urticaria, atopic dermatitis, contact dermatitis, lichen planus, miliaria, dermatitis herpetiformis, xeroderma, biliary obstruction, primary biliary cirrhosis (PBC), infectious hepatitis such as type C hepatitis, urinemia, chronic renal failure, renal dialysis, lymphoma, leukemia, polycythemia rubra vera, pregnancy, ingestion of drugs (barbiturate, salicylate, etc.), hyperthyroidism, diabetes mellitus, and organ cancers.

14. The use of claim 5, wherein the anti-NR10 antibody is H0L0 (heavy chain amino acid sequence/SEQ ID NO:17; light chain amino acid sequence/SEQ ID NO:18).

15. The use of claim 5, wherein the anti-NR10 antibody comprises a heavy or light chain variable region of H0L0 with one or more amino acid substitution, deletion, addition, and/or insertion, and has a neutralizing activity against NR10 equivalent to H0L0.

16. The use of claim 5, wherein the anti-NR10 antibody comprises a heavy or light chain variable region having an amino acid identity of at least 90% with the heavy or light chain variable region of H0L0, and has a neutralizing activity against NR10 equivalent to H0L0.

17. The use of claim 5, wherein the pruritis is selected from the group consisting of pruritis in scabies, pediculation, bug bites and stings, urticaria, atopic dermatitis, contact dermatitis, lichen planus, miliaria, dermatitis herpetiformis, xeroderma, biliary obstruction, primary biliary cirrhosis (PBC), infectious hepatitis such as type C hepatitis, urinemia, chronic renal failure, renal dialysis, lymphoma, leukemia, polycythemia rubra vera, pregnancy, ingestion of drugs (barbiturate, salicylate, etc.), hyperthyroidism, diabetes mellitus, and organ cancers.

ABSTRACT

The present inventors isolated clone BM095 from a human antibody phage library, which had a strong growth inhibitory activity in the IL-31-dependent Ba/F3 cell growth assay system. When administered to pruritus model mice, the anti-mouse NR10 neutralizing antibody exhibited a marked symptom-suppressing effect. Thus, it was revealed that anti-NR10 neutralizing antibodies are useful as therapeutic agents for pruritus.

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<150> JP 2007-315144
<151> 2007-12-05

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Val Glu Cys Pro Pro Cys Pro Ala Pro Pro Val Ala Gly Pro Ser Val

225 230 235 240

Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr
245 250 255

Pro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu
260 265 270

Val Gln Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys
275 280 285

Thr Lys Pro Arg Glu Glu Gln Phe Asn Ser Thr Phe Arg Val Val Ser
290 295 300

Val Leu Thr Val Val His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys
305 310 315 320

Cys Lys Val Ser Asn Lys Gly Leu Pro Ala Pro Ile Glu Lys Thr Ile
325 330 335

Ser Lys Thr Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro
340 345 350

Pro Ser Arg Glu Glu Met Thr Lys Asn Gln Val Ser Leu Thr Cys Leu
355 360 365

Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn
370 375 380

Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Met Leu Asp Ser
385 390 395 400

Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg
405 410 415

Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu
420 425 430

His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys
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<220>
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Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
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Asp Arg Val Thr Ile Thr Cys Arg Thr Ser Glu Asn Ile Tyr Ser Phe
            20                  25                  30

Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
        35                  40                  45

Tyr Asn Ala Lys Thr Leu Ala Lys Gly Val Pro Ser Arg Phe Ser Gly
    50                  55                  60

Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
65                  70                  75                  80

Glu Asp Phe Ala Thr Tyr Tyr Cys Gln His His Tyr Glu Ser Pro Leu
                85                  90                  95

Thr Phe Gly Gly Gly Thr Lys Val Glu Ile Lys Arg Thr Val Ala Ala
            100                 105                 110

Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly
        115                 120                 125

Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala
    130                 135                 140

Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln
145                 150                 155                 160

Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser
                165                 170                 175
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Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr
180 185 190

Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser
195 200 205

Phe Asn Arg Gly Glu Cys
210