Sequence dependent aggregation

Herein is reported a method for reducing immunoglobulin aggregation in concentrated solutions by reverting one or two mutation(s) in the fourth framework region, i.e. in the J-element, of an immunoglobulin heavy chain to the hydrophilic or natural germline amino acid residue.

Background of the Invention

Due to their chemical and physical properties, such as molecular weight and domain architecture including secondary modifications, the downstream processing of immunoglobulins is very complicated. Concentrated immunoglobulin solutions are required not only for formulated drugs but also for intermediates in downstream processing (DSP) to achieve low volumes for economic handling and application storage.

Also the final formulation of the immunoglobulin requires a highly concentrated solution. For example, for subcutaneous application immunoglobulin concentrations of more than 100 mg/ml, i.e. about 150 mg/ml, are required. But at least some immunoglobulins tend to aggregate at unphysiologically high concentrations of 100 mg/ml or more.

In WO 2009/000098 a sequence based engineering and optimization of single chain antibodies is reported.

Summary of the Invention

Herein is reported a method for reducing immunoglobulin aggregation in a concentrated solution. It has been found that two or even a single reverting mutation in the fourth framework region of an immunoglobulin heavy chain can reduce the formation of aggregates allowing the provision of a highly concentrated solution of the reverted immunoglobulin variant with reduced aggregate content. Therefore, herein are reported as individual aspects a method for reducing the aggregation of an immunoglobulin in solution, a method for modifying an immunoglobulin, a method for producing an immunoglobulin, and a method for humanizing an immunoglobulin.

All the methods as reported herein comprise the following steps:

a) aligning the amino acid sequence of the fourth heavy chain framework region of an immunoglobulin with a reference amino acid sequence to achieve maximal level of amino acid sequence identity,

b) identifying aligned amino acid sequence positions with different amino acid residues in the fourth heavy chain framework region amino acid sequence of the immunoglobulin and of the reference amino acid sequence,

c) modifying the immunoglobulin amino acid sequence by substituting an or at least one amino acid residue in the fourth heavy chain framework region amino acid sequence of the immunoglobulin at a position identified in b) for the respective threonine or serine residue as present in the reference amino acid sequence, whereby at the substituted position the amino acid residue is threonine or serine in the reference amino acid sequence, and whereby the amino acid residue is not threonine and not serine in the fourth heavy chain framework region amino acid sequence of the immunoglobulin,

d) optionally providing a nucleic acid sequence encoding the modified immunoglobulin amino acid sequence.

In one embodiment only the difference of threonine residues is determined and changed. In another embodiment the reference amino acid sequence to the fourth framework region of the immunoglobulin is the amino acid sequence xxxxTTxTxSS (SEQ ID NO: 01) with x denoting any amino acid residue except threonine and serine. In a further embodiment the reference amino acid sequence is the amino acid sequence xxxxTxxTxxx (SEQ ID NO: 11) with x at positions 1, 2, 3, 4, 7 and 9 denoting any amino acid residue except threonine and serine and x at position 6 denoting threonine and x at position 10 and 11 denoting serine, whereby the difference of threonine residues at position 5 and/or 8 in the reference amino acid sequence to the amino acid sequence of the fourth framework region of the immunoglobulin heavy chain is identified in step b) and modified in step c). In one embodiment the reference amino acid sequence is a human germline amino acid sequence. In one embodiment of all aspects as reported herein the methods comprise the following steps:

a) aligning the amino acid sequence of the fourth heavy chain framework region of the immunoglobulin with the amino acid sequence WGQGTLVTVSS (SEQ ID NO: 02), or the amino acid sequence WGRGTLVTVSS (SEQ ID NO: 03), or the amino acid sequence WGQGTMVTVSS (SEQ ID NO: 04), or the amino acid sequence WGQGTTVTVSS (SEQ ID NO: 05), or the amino acid sequence WGKGTTVTVSS (SEQ ID NO: 06) to achieve maximal level of amino acid sequence identity,

b) identifying aligned amino acid positions with different amino acid residues in the fourth heavy chain framework region amino acid sequence of the immunoglobulin and the reference amino acid sequence,

c) modifying the immunoglobulin by substituting an amino acid residue in the fourth heavy chain framework region amino acid sequence at a position identified in b) for the respective threonine or serine residue present in the reference amino acid sequence, whereby the amino acid residue at the position is threonine or serine in the reference amino acid sequence and whereby the amino acid residue at the position is not threonine and not serine in the fourth heavy chain framework region amino acid sequence of the immunoglobulin,

d) optionally providing a nucleic acid sequence encoding the modified immunoglobulin amino acid sequence.

In one embodiment of the aspects as reported herein the methods comprise as first step the step of providing or determining the amino acid sequence of the fourth framework region of the immunoglobulin heavy chain. In one embodiment the reference sequence is the amino acid sequence WGQGTLVTVSS (SEQ ID NO: 02). In another embodiment the reference amino acid sequence is xxxxTxxTxxx (SEQ ID NO: 11). In one embodiment the difference of the threonine residues at position 5 or/and 8 of the reference amino acid sequence is determined. In one embodiment only the difference of threonine residues is determined and changed.

In one aspect as reported herein the method is a method for producing an immunoglobulin comprising the following steps:

a) aligning the amino acid sequence of the fourth heavy chain framework region of the immunoglobulin with the reference amino acid sequence xxxxTTxTxSS (SEQ ID NO: 01) or xxxxTxxTxxx (SEQ ID NO: 11) to achieve maximal level of amino acid sequence identity,

b) identifying aligned amino acid sequence positions with different amino acid residues at position 5 and/or 6 and/or 8 and/or 10 and/or 11 in the fourth heavy chain framework region amino acid sequence of the immunoglobulin and the reference amino acid sequence,

c) modifying the fourth heavy chain framework region amino acid sequence of the immunoglobulin by substituting an amino acid residue in the fourth heavy chain framework region amino acid sequence at a position identified in b) for the respective threonine or serine residue as in the reference amino acid sequence, whereby the amino acid residue at the position is threonine or serine in the reference amino acid sequence, and whereby the amino acid residue at the position is not threonine and not serine in the fourth heavy chain framework region amino acid sequence of the immunoglobulin,

d) cultivating a mammalian cell comprising the nucleic acid encoding the modified immunoglobulin heavy chain amino acid sequence and a nucleic acid encoding a corresponding light chain amino acid sequence for the expression of the immunoglobulin heavy and light chain,

e) recovering the immunoglobulin from the mammalian cell or the cultivation medium and thereby producing an immunoglobulin.

In one embodiment the reference amino acid sequence is the amino acid sequence WGQGTLVTVSS (SEQ ID NO: 02), or the amino acid sequence WGRGTLVTVSS (SEQ ID NO: 03), or the amino acid sequence WGQGTMVTVSS (SEQ ID NO: 04), or the amino acid sequence WGQGTTVTVSS (SEQ ID NO: 05), or the amino acid sequence WGKGTTVTVSS (SEQ ID NO: 06). In one embodiment the step of modifying the fourth heavy chain framework region amino acid sequence of the immunoglobulin is substituting an amino acid residue in the fourth heavy chain framework region amino acid sequence at a position identified in b), which is threonine in the

reference amino acid sequence and which is not threonine in the fourth heavy chain framework region amino acid sequence, for the respective threonine residue as present in the reference amino acid sequence. In another embodiment the modifying is of at least one position identified. In one embodiment the reference amino acid sequence is the amino acid sequence WGQGTLVTVSS (SEQ ID NO: 02). In one embodiment the immunoglobulin is a human or humanized immunoglobulin. In another embodiment the mammalian cell is a CHO cell or a HEK cell. In another embodiment the difference of the threonine residues at position 5 and/or 8 of the reference amino acid sequence is determined.

Another aspect as reported herein is a method for humanizing an immunoglobulin comprising the following steps:

a) aligning the amino acid sequence of the fourth heavy chain framework region of the immunoglobulin with the reference amino acid sequence xxxxTTxTxSS (SEQ ID NO: 01) or xxxxTxxTxxx (SEQ ID NO: 11) to achieve maximal level of amino acid sequence identity,

b) identifying aligned amino acid sequence positions with different amino acid residues in the fourth heavy chain framework region amino acid sequence of the immunoglobulin and the reference amino acid sequence,

c) humanizing the immunoglobulin by substituting an amino acid residue in the fourth heavy chain framework region amino acid sequence at a position identified in b) for the respective threonine or serine residue as in the reference sequence, whereby the amino acid residue at the position is threonine or serine in the reference amino acid sequence, and whereby the amino acid residue at the position is not threonine and not serine in the fourth heavy chain framework region amino acid sequence of the immunoglobulin,

d) optionally providing a nucleic acid sequence encoding the humanized immunoglobulin.

Detailed Description of the Invention

It has been found that the exchange of two or even a single threonine and/or serine amino acid residue to a small hydrophobic or non-polar amino acid residue, such as

isoleucine or alanine, in the amino acid sequence of the fourth immunoglobulin heavy chain framework region increases the tendency of the immunoglobulin to form aggregates in solution, especially in solutions with high salt concentration and/or high immunoglobulin concentration. By reverting the exchanged amino acid residues back to the naturally occurring threonine and/or serine residue(s) the tendency to form aggregates in solution, especially in concentrated solutions, is distinctly reduced.

In one aspect the method as reported herein is a method for reducing the aggregation of an immunoglobulin in solution comprising the following steps:

- determining whether one or more threonine and/or serine residues in the fourth framework region amino acid sequence of the heavy chain of the immunoglobulin have been changed by comparing the amino acid sequence with a reference amino acid sequence for the fourth framework region amino acid sequence,
- reverting the amino acid sequence of the immunoglobulin by modifying at least one of the exchanged threonine and/or serine residues back to the reference threonine and/or serine residue and thereby reducing the aggregation of an immunoglobulin in solution.

The term “aligning” stands for the process of lining up two or more amino acid sequences to achieve maximal level of amino acid sequence identity and conservation. It comprises the determination of positional homology for molecular sequences, involving the juxtaposition of amino acids or nucleotides in homologous molecules. As a result the compared sequences are presented in a form that the regions of greatest statistical similarity are shown.

“Maximal level of amino acid sequence identity" with respect to a reference amino acid sequence is defined as the percentage of amino acid residues in a candidate amino acid sequence that are identical with the amino acid residues in the reference amino acid sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining amino acid sequence identity can be achieved in various ways, for instance, using publicly available computer software such as BLAST,

BLAST-2, ALIGN or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for aligning amino acid sequences, including any algorithms needed to achieve maximal alignment over the full length of the amino acid sequences being compared. For purposes herein, however, "% amino acid sequence identity" values are generated using the sequence comparison computer program ALIGN-2. The ALIGN-2 sequence comparison computer program was authored by Genentech, Inc., and the source code has been filed with user documentation in the U.S. Copyright Office, Washington D.C., 20559, where it is registered under U.S. Copyright Registration No. TXU510087. The ALIGN-2 program is publicly available from Genentech, Inc., South San Francisco, California, or may be compiled from the source code. The ALIGN-2 program should be compiled for use on a UNIX operating system, including digital UNIX V4.0D. All sequence comparison parameters are set by the ALIGN-2 program and do not vary.

In situations where ALIGN-2 is employed for amino acid sequence comparisons, the % amino acid sequence identity of a given amino acid sequence A to, with, or against a given amino acid sequence B (which can alternatively be phrased as a given amino acid sequence A that has or comprises a certain % amino acid sequence identity to, with, or against a given amino acid sequence B) is calculated as follows:

100 times the fraction X/Y

where X is the number of amino acid residues scored as identical matches by the sequence alignment program ALIGN-2 in that program's alignment of A and B, and where Y is the total number of amino acid residues in B. It will be appreciated that where the length of amino acid sequence A is not equal to the length of amino acid sequence B, the % amino acid sequence identity of A to B will not equal the % amino acid sequence identity of B to A.

In a further aspect the method as reported herein is a method for modifying an immunoglobulin comprising the following steps:

- determining whether one or more threonine and/or serine residues in the fourth framework region amino acid sequence of the heavy chain of the immunoglobulin have been changed by comparing the amino acid

sequence with a reference amino acid sequence for the fourth framework region amino acid sequence,

- reverting the amino acid sequence of the immunoglobulin by modifying at least one of the exchanged threonine and/or serine residues back to the reference threonine and/or serine residue and thereby modifying an immunoglobulin.

In one aspect the method as reported herein is a method for humanizing an immunoglobulin comprising the following steps:

- determining by comparing the amino acid sequence of the fourth framework region of the heavy chain of a chimeric immunoglobulin, a CDR-grafted immunoglobulin, a T-cell epitope reduced or depleted immunoglobulin, or a variant thereof with a reference amino acid sequence for the fourth framework region whether one or more threonine and/or serine residues have been replaced by a different amino acid residue,

- reverting the amino acid sequence of the immunoglobulin by modifying at least one of the exchanged threonine and/or serine residues back to the threonine and/or serine residue as in the reference amino acid sequence and thereby humanizing an immunoglobulin.

The term "fourth framework region amino acid sequence" denotes the amino acid sequence of the fourth framework region of the heavy chain of an immunoglobulin. This amino acid sequence starts with the amino acid residue directly C-terminal to the complementarity determining region 3 (CDR 3) of the immunoglobulin heavy chain and ends with the last amino acid residue of the heavy chain variable domain. In one embodiment the amino acid residues of the CDR 3 of the immunoglobulin heavy chain are determined according to Kabat.

The term "chimeric immunoglobulin" denotes an immunoglobulin comprising amino acid residues derived from an immunoglobulin of a first species and amino acid residues derived from a second species not identical with the first species. If the acceptor species is human then the chimeric immunoglobulin is a "humanized immunoglobulin". For the most part, a humanized immunoglobulin is derived from

a human immunoglobulin (recipient or acceptor immunoglobulin), in which the amino acid sequence of one or more hypervariable region(s) determined according to Kabat and/or Chothia and/or other numbering systems are changed to the amino acid sequence of a hypervariable region of a non-human species (donor immunoglobulin). A humanized immunoglobulin in which the entire hypervariable regions according to Kabat and/or Chothia and/or other numbering systems of a human acceptor immunoglobulin are replaced by the corresponding amino acid residues of a non-human donor immunoglobulin are denoted as "CDR-grafted immunoglobulin". Exemplary non-human donor immunoglobulins are mouse, rat, rabbit, dog, hamster, sheep, or non-human primate immunoglobulins, having the desired specificity and affinity towards an antigen of interest (see e.g. Morrison, S.L., et al., Proc. Natl. Acad. Sci. USA 81 (1984) 6851-6855; US 5,202,238; US 5,204,244). In some instances, framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized immunoglobulins may comprise further modifications, e.g. amino acid residues that are not found in the acceptor immunoglobulin or in the donor immunoglobulin. Such modifications result in variants of such recipient or donor immunoglobulin, which are homologous but not identical to the corresponding parent sequence. A humanized immunoglobulin optionally will also comprise at least a portion of an immunoglobulin constant region, typically that of a human immunoglobulin.

In one embodiment the immunoglobulin is a chimeric immunoglobulin, or a CDR-grafted immunoglobulin, or a T-cell epitope reduced or depleted immunoglobulin, or a variant thereof. In one embodiment the immunoglobulin comprises a human heavy chain constant region or a variant thereof. In a further embodiment the human constant region is of IgG1 subclass, or of IgG4 subclass, or of IgG2 subclass, or of IgG3 subclass, or of SEQ ID NO: 07, or SEQ ID NO: 08, or is a variant thereof.

In one embodiment the constant region is modified in such a way that no Fcγ receptor (e.g. FcγRIIIa) binding and/or no C1q binding as defined below can be detected. In one embodiment the constant region is a human constant region and is either of human IgG4 subclass or is a mutated Fc part from human IgG1 subclass. In another embodiment the constant region is of human IgG1 subclass comprising the mutations L234A and L235A (positions determined according to full length,

i.e. including the variable domain, human IgG1 heavy chain amino acid sequence). While IgG4 shows reduced Fcγ receptor (FcγRIIIa) binding, immunoglobulins of other IgG subclasses show strong binding. However Pro238, Asp265, Asp270, Asn297 (loss of Fc carbohydrate), Pro329, Leu234, Leu235, Gly236, Gly237, Ile253, Ser254, Lys288, Thr307, Gln311, Asn434, or/and His435 are residues which, if altered, provide also reduced Fcγ receptor binding (Shields, R.L., et al., J. Biol. Chem. 276 (2001) 6591-6604; Lund, J., et al., FASEB J. 9 (1995) 115-119; Morgan, A., et al., Immunology 86 (1995) 319-324; EP 0 307 434). In one embodiment the constant region is in regard to Fcγ receptor binding of IgG4 subclass or of IgG1 or IgG2 subclass, with a mutation in L234, L235, and/or D265, and/or contains the PVA236 mutation. In one embodiment the mutation is S228P, L234A, L235A, L235E, and/or PVA236 (PVA236 means that the amino acid sequence ELLG (given in one letter amino acid code) from amino acid position 233 to 236 of IgG1 or EFLG of IgG4 is replaced by PVA). In a further embodiment the mutation is S228P of IgG4, and L234A and L235A of IgG1. The constant region of an immunoglobulin is directly involved in ADCC (antibody-dependent cell-mediated cytotoxicity) and CDC (complement-dependent cytotoxicity). An immunoglobulin which does not bind Fcγ receptor and/or complement factor C1q does not elicit antibody-dependent cellular cytotoxicity (ADCC) and/or complement dependent cytotoxicity (CDC).

The term “T-cell epitope depleted immunoglobulin” denotes an immunoglobulin which was modified to remove or reduce immunogenicity by removing human T-cell epitopes (peptide sequences within proteins with the capacity to bind to MHC Class II molecules). By this method interactions between amino acid side chains of the peptide and specific binding pockets with the MHC class II binding groove are identified. The identified immunogenic regions are changed to eliminate immunogenicity. Such methods are described in general in, e.g., WO 98/52976 or in WO 98/08097.

The term ”variant thereof” denotes an immunoglobulin comprising conservative sequence modifications or an altered glycosylation pattern. The term "conservative sequence modifications" denotes amino acid substitutions including the ones in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains

(e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g. glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine, tryptophan), non-polar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine), beta-branched side chains (e.g., threonine, valine, isoleucine), and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine). Another type of variant of the immunoglobulin alters the original glycosylation pattern of the immunoglobulin. By altering is meant deleting one or more glycosylation sites found in the immunoglobulin, and/or introducing one or more glycosylation sites that are not present in the immunoglobulin. Glycosylation of immunoglobulins is typically N-linked. N-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine and asparagine-X-cysteine, where X can be any amino acid, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain.

In one embodiment the reference sequence is the sequence of SEQ ID NO: 02 to 06, or the sequence of SEQ ID NO: 01, or the sequence of SEQ ID NO: 11. In another embodiment the determining and reverting is of the replaced threonine residues.

The invention is in the following exemplified with an anti-IL13Rα1 antibody and an anti-OX40L antibody. The corresponding amino acid sequences and production methods are reported in WO 2006/072564 and WO 2006/029879 (which are incorporated herein by reference). These antibodies are only used to illustrate the aspects as reported herein and not to present any limitation. The scope of the invention is set forth in the appended set of claims.

The anti-IL13Rα1 antibody is in the following denoted as parent antibody and has an amino acid sequence of the fourth heavy chain framework region of WGQGTLVIVSS (SEQ ID NO: 09). The comparison with the amino acid sequence of SEQ ID NO: 01 and SEQ ID NO: 11 is shown below.

1
1 0
xxxxTTx**T**xSS (SEQ ID NO: 01), or
xxxxTxx**T**xxx (SEQ ID NO: 11) to
WGQGTLV**I**VSS (SEQ ID NO: 09)

It can be seen that one difference between the amino acid sequences is an isoleucine residue at the 8th position of SEQ ID NO: 09 instead of the threonine residue in SEQ ID NO: 01 or SEQ ID NO: 11. Therefore, a variant antibody has been produced in which the isoleucine residue is reverted back to a threonine residue.

Whereas the parent antibody shows high levels of aggregate formation the reverted variant antibody has a clearly reduced tendency to form aggregates. In Figure 1 the rates of the size increase (nm/h) of aggregates of the parent anti-IL13Rα1 antibody at an antibody concentration of 30 mg/ml at 50 °C and varying ionic strength is shown. Also shown in Figure 1 are the rates obtained with a variant anti-IL13Rα1 antibody in which the isoleucine residue at amino acid position 115 (corresponding to the 8th position in SEQ ID NO: 01, 11 and 09) in the fourth framework region of the heavy chain amino acid sequence has been reverted to threonine as single and sole difference to the parent anti-IL13Rα1 antibody. The single reverting mutation reduces the rate of aggregate size increase at all examined ionic strengths.

In Figure 2 the analytical size exclusion chromatograms of two samples stored for 9 weeks at 40 °C in the same formulation is shown. The respective data is listed in the following Table 1.

Table 1: Analytical data of samples stored for 9 weeks at 40°C.

	relative area monomer [%]	relative area low molecular weight compounds [%]	relative area high molecular weight compounds [%]	relative increase HMWs [%]
parent anti-IL13Rα1 antibody stored at -80°C (reference value)	98.6	0.04	1.4	-
parent anti-IL13Rα1 antibody stored at 40°C for 9 weeks	93.7	4.2	2.0	48.9
variant anti-IL13Rα1 antibody stored at -80°C (reference value)	97.3	0.1	2.5	-
variant anti-IL13Rα1 antibody stored at 40°C for 9 weeks	93.2	3.9	2.9	13.4

It can be seen that the tendency to form aggregates of the reverted variant antibody is reduced by more than 50 % compared to the parent antibody.

In Figure 3 the Kyte-Doolittle plot of the heavy chain residues 1 to 130 with an analysis or averaging window of 9 residues of the parent anti-IL13Rα1 antibody is shown. In the forth framework region the plot shows the highest hydrophobicity values for the entire heavy chain. In Figure 4 the Kyte-Doolittle plot of the heavy chain of the variant anti-IL13Rα1 antibody is shown. It can be seen that the hydrophobicity values of the fourth framework region are distinctly reduced in the reverted variant antibody.

In Figure 5 the Kyte-Doolittle plot of the heavy chain residues 1 to 130 with an analysis/averaging window of 9 residues of an anti-OX40L antibody is shown. It can be seen that also in this case the highest hydrophobicity value is in the fourth heavy chain framework region. By analyzing the amino acid sequence of the fourth

heavy chain framework region an amino acid exchange can be determined as shown below.

1	0	
xxxx**T**TxTxSS		(SEQ ID NO: 01), or
xxxx**T**xxTxxx		(SEQ ID NO: 11), or
WGQG**T**LVTVSS		(SEQ ID NO: 02) to
WGQG**A**LVTVSS		(SEQ ID NO: 10).

Reverting this single amino acid change back to the naturally occurring germline threonine residue reduced the hydrophobicity of the antibody as shown in the Kyte-Doolittle plot in Figure 6.

The following examples, sequence listing and figures are provided to aid the understanding of the present invention, the true scope of which is set forth in the appended claims. It is understood that modifications can be made in the procedures set forth without departing from the spirit of the invention.

Description of the Sequence Listing

SEQ ID NO: 01 xxxxTTxTxSS – reference amino acid sequence

SEQ ID NO: 02 WGQGTLVTVSS – partial germline FR4/J-element amino acid sequence.

SEQ ID NO: 03 WGRGTLVTVSS – partial germline FR4/J-element amino acid sequence.

SEQ ID NO: 04 WGQGTMVTVSS – partial germline FR4/J-element amino acid sequence.

SEQ ID NO: 05 WGQGTTVTVSS – partial germline FR4/J-element amino acid sequence.

SEQ ID NO: 06 WGKGTTVTVSS – partial germline FR4/J-element amino acid sequence.

SEQ ID NO: 07 human IgG1 heavy chain constant region amino acid sequence.

SEQ ID NO: 08 human IgG4 heavy chain constant region amino acid sequence.

SEQ ID NO: 09 WGQGTLVIVSS – amino acid sequence of an anti-IL13Rα1 antibody.

SEQ ID NO: 10 WGQGALVTVSS – amino acid sequence of an anti-OX40L antibody.

SEQ ID NO: 11 xxxxTxxTxxx – reference amino acid sequence.

Description of the Figures

Figure 1 Rate of particle size increase per time unit in a solution of 30 mg/ml anti-IL13Rα1 antibody (a) and the reverted variant antibody (b) both in 20 mM histidine buffer, pH 6, with 0, 140 and 500 mM NaCl at 50 °C, determined by dynamic light scattering.

Figure 2 Analytical size exclusion chromatograms of two solutions of 30 mg/ml anti-IL13Rα1 antibody and the reverted form both in the presence of 500 mM NaCl for 15 h at 10 °C.

Figure 3 Kyte-Doolittle plot of the heavy chain residues 1 to 130 with an analysis window of 9 residues of parent anti-IL13Rα1 antibody.

Figure 4 Kyte-Doolittle plot of the heavy chain residues 1 to 130 with an analysis window of 9 residues of variant anti-IL13Rα1 antibody.

Figure 5 Kyte-Doolittle plot of the heavy chain residues 1 to 130 with an analysis window of 9 residues of parent anti-OX40L antibody.

Figure 6 Kyte-Doolittle plot of the heavy chain residues 1 to 130 with an analysis window of 9 residues of variant anti-OX40L antibody.

Materials and Methods

Analytical size exclusion chromatography

The content of high molecular weight species (HMWs), antibody monomers and low molecular weight species (LMWs) was determined by analytical size-exclusion chromatography using a TSK 3000S WXL 7.8 x 300 mm column (Tosoh, Stuttgart, Germany). As eluent, a buffer solution containing 200 mM KH_2PO_4 and 250 mM KCl at pH 7.0 was used at a flow rate of 0.5 ml/min. An amount of 150 µg of protein was loaded per run. Detection was accomplished via UV absorption at 280 nm.

Dynamic light scattering (DLS)

DLS is a non-invasive technique for measuring particle size, typically in the sub-micron size range. In the current invention the Zetasizer Nano S apparatus (Malvern Instruments, Worcestershire, UK) with a temperature controlled quartz cuvette (25 °C) was used for monitoring a size range between 1 nm and 6 µm. The intensity of the back scattered laser light was detected at an angle of 173°. The intensity fluctuates at a rate that is dependent upon the particle diffusion speed, which in turn is governed by particle size. Particle size data can therefore be generated from an analysis of the fluctuation in scattered light intensity (Dahneke, B.E. (ed), Measurement of Suspended Particles by Quasielectric Light Scattering, Wiley Inc. (1983); Pecora, R., Dynamic Light Scattering: Application of Photon Correlation Spectroscopy, Plenum Press (1985)). The size distribution by intensity was calculated using the multiple narrow mode of the DTS software (Malvern).

Example 1

Production of variant anti-IL13Rα1 antibody

The anti-IL13Rα1 antibody light and heavy chain encoding genes were separately assembled in mammalian cell expression vectors. Thereby the gene segments encoding the anti-IL13Rα1 antibody light chain variable region (V_L) and the human κ-light chain constant region (C_L) were joined as were gene segments for the anti-IL13Rα1 antibody heavy chain variable region (V_H) and the human γ1-heavy chain constant region (C_{H1}-Hinge-C_{H2}-C_{H3}). General information regarding the nucleotide sequences of human light and heavy chains from which the codon usage is given in: Kabat, E. A., Wu, T. T., Perry, H. M., Gottesman, K. S., and Foeller, C. Sequences of Proteins of Immunological Interest, Fifth Ed., NIH Publication No. 91-3242 (1991). The transcription unit of the anti-IL13Rα1 antibody κ-light chain is composed of the following elements:

- the immediate early enhancer and promoter from the human cytomegalovirus (HCMV),
- a synthetic 5'-UT including a Kozak sequence,
- a murine immunoglobulin heavy chain signal sequence including the signal sequence intron,

- the cloned anti-IL13Rα1 antibody variable light chain cDNA arranged with a unique BsmI restriction site at the 5' end and a splice donor site and a unique NotI restriction site at the 3' end,
- the genomic human κ-gene constant region, including the intron 2 mouse Ig-κ enhancer (Picard, D., and Schaffner, W., Nature 307 (1984) 80-82), and
- the human immunoglobulin κ-polyadenylation ("poly A") signal sequence.

The transcription unit of the anti-IL13Rα1 antibody γ1-heavy chain is composed of the following elements:

- the immediate early enhancer and promoter from the human cytomegalovirus (HCMV),
- a synthetic 5'-UT including a Kozak sequence,
- a modified murine immunoglobulin heavy chain signal sequence including the signal sequence intron,
- the cloned anti-IL13Rα1 antibody variable heavy chain cDNA arranged with a unique BsmI restriction site at the 5' and a splice donor site and a unique NotI restriction site at the 3' end,
- the genomic human γ1-heavy gene constant region, including the mouse Ig μ-enhancer (Neuberger, M.S., EMBO J. 2 (1983) 1373-1378),
- the human γ1-immunoglobulin polyadenylation ("poly A") signal sequence.

Besides the anti-IL13Rα1 antibody κ-light chain or γ1-heavy chain expression cassette these plasmids contain

- a hygromycin resistance gene,
- an origin of replication, oriP, of Epstein-Barr virus (EBV),
- an origin of replication from the vector pUC18 which allows replication of this plasmid in E. coli, and
- a ß-lactamase gene which confers ampicillin resistance in E. coli.

An expression plasmid encoding the variant anti-IL13Rα1 antibody γ1-heavy chain was created by site-directed mutagenesis of the parent antibody expression plasmids using the QuickChange™ Site-Directed mutagenesis Kit (Stratagene). Amino acids are numbered according to EU numbering (Edelman, G.M., et al., Proc. Natl. Acad. Sci. USA 63 (1969) 78-85; Kabat, E. A., Wu, T. T., Perry, H. M.,

Gottesman, K. S., and Foeller, C., (1991) Sequences of Proteins of Immunological Interest, Fifth Ed., NIH Publication No. 91-3242).

A stably transfected CHO clone produces the variant anti-IL13Rα1 antibody at 130 mg/liter. The downstream processing was conducted by employing three sequential chromatographic steps: Protein A chromatography, cation exchange chromatography and anion exchange chromatography.

Example 2

Determination of the rate of aggregate size increase via dynamic light scattering

In order to follow aggregation over time, dynamic light scattering (DLS) measurements were conducted at regular time intervals. High salt concentrations stabilize hydrophobic interactions; hence hydrophobicity-related aggregation is expected to be more pronounced at high salt concentrations. The change of average particle size (Z-average radius) was monitored as a metric for protein aggregation (Figure 1). Samples were dialyzed in buffer containing various amounts of NaCl (20 mM His/His-HCl at pH 6.0 + 0/140/500 mM NaCl) at a protein concentration of 30 mg/ml. DLS measurements were carried out on a Wyatt DynaPro plate reader in 394-well micro titer plates at a temperature of 50 °C.

Example 3

Stability testing of variant anti-IL13Rα1 antibody

Induction of high molecular weight compounds (HMWs) was performed by dialyzing samples in 20 mM His/His-HCl at pH 6.0, containing 0 or 500 mM NaCl, followed by incubation at 10 °C for 15 h. The formation of HMWs compared to the untreated samples was monitored by SEC HPLC (Figure 2).

Patent Claims

1. Method for providing a nucleic acid sequence encoding an immunoglobulin comprising the following steps

   a) aligning the amino acid sequence of the fourth heavy chain framework region of an immunoglobulin with the reference sequence xxxxTTxTxSS (SEQ ID NO: 01) or xxxxTxxTxxx (SEQ ID NO: 11) to achieve maximal level of amino acid sequence identity,

   b) identifying aligned amino acid positions with different amino acid residues in the fourth heavy chain framework region and the reference sequence,

   c) modifying the immunoglobulin by substituting an amino acid residue in the fourth heavy chain framework region at a position identified in b), which is threonine or serine in the reference sequence and which is not threonine and not serine in the fourth heavy chain framework region, for the respective threonine or serine residue as in the reference sequence,

   d) providing a nucleic acid sequence encoding the immunoglobulin.

2. Method for humanizing an immunoglobulin comprising the following steps

   a) aligning the amino acid sequence of the fourth heavy chain framework region of the immunoglobulin with the reference sequence xxxxTTxTxSS (SEQ ID NO: 01) or xxxxTxxTxxx (SEQ ID NO: 11) to achieve maximal level of amino acid sequence identity,

   b) identifying aligned amino acid positions with different amino acid residues in the fourth heavy chain framework region and the reference sequence,

   c) humanizing the immunoglobulin by substituting an amino acid residue in the fourth heavy chain framework region at a position identified in b), which is threonine or serine in the reference sequence and which is not threonine and not serine in the fourth heavy chain framework

region, for the respective threonine or serine residue as in the reference sequence,

d) optionally providing a nucleic acid sequence encoding the humanized immunoglobulin.

3. Method for producing an immunoglobulin comprising the following steps

a) aligning the amino acid sequence of the fourth heavy chain framework region of the immunoglobulin with the reference sequence xxxxTTxTxSS (SEQ ID NO: 01) or xxxxTxxTxxx (SEQ ID NO: 11) to achieve maximal level of amino acid sequence identity,

b) identifying aligned amino acid positions with different amino acid residues in the fourth heavy chain framework region and the reference sequence,

c) modifying the immunoglobulin by substituting an amino acid residue in the fourth heavy chain framework region at a position identified in b), which is threonine or serine in the reference sequence and which is not threonine and not serine in the fourth heavy chain framework region, for the respective threonine or serine residue as in the reference sequence,

d) cultivating a mammalian cell comprising the nucleic acid encoding the modified immunoglobulin heavy chain amino acid sequence and a nucleic acid encoding a corresponding light chain amino acid sequence for the expression of the immunoglobulin heavy and light chain,

e) recovering the immunoglobulin from the mammalian cell or the cultivation medium and thereby producing an immunoglobulin.

4. The method according to any one of the preceding claims, characterized in that the reference amino acid sequence is xxxxTxxTxxx (SEQ ID NO: 11).

5. Method according to any one of the preceding claims, characterized in that the identifying and the modifying is of the threonine amino acid residue at position 5 and/or 8 of the reference amino acid sequence.

6. Method according to any one of the preceding claims, characterized in that the reference sequence is the sequence WGQGTLVTVSS (SEQ ID NO: 02), or the sequence WGRGTLVTVSS (SEQ ID NO: 03), or the sequence WGQGTMVTVSS (SEQ ID NO: 04), or the sequence WGQGTTVTVSS (SEQ ID NO: 05), or the sequence WGKGTTVTVSS (SEQ ID NO: 06).

7. Method according to any one of the preceding claims characterized in that the modifying step is

modifying the immunoglobulin by substituting an amino acid residue in the fourth heavy chain framework region at a position identified in the previous step which is threonine in the reference sequence and which is not threonine in the fourth heavy chain framework region for the respective threonine residue as in the reference sequence.

8. Method according to any one of the preceding claims, characterized in that the method comprises as first step

providing or determining the amino acid sequence of the fourth framework region of the immunoglobulin heavy chain.

9. Method according to any one of the preceding claims, characterized in that the immunoglobulin is a human or humanized immunoglobulin.

10. Method according to any one of the preceding claims, characterized in that the immunoglobulin is a chimeric immunoglobulin, or a CDR-grafted immunoglobulin, or a T-cell epitope depleted immunoglobulin, or a variant thereof.

11. Method according to any one of claims 3 to 10, characterized in that the mammalian cell is a CHO cell or a HEK cell.