Title: AN AGENT FOR SELECTIVE SUPPRESSION DISEASE-ASSOCIATED AUTO-REACTIVE B-CELLS

Abstract: The invention relates to an agent for selective suppression of disease associated B-lymphocytes, more precisely of disease-associated DNA-specific B-cells in systemic lupus erythematosus. The agent is a bispecific chimeric monoclonal antibody capable of cross-linking an inhibitory and an immunoglobulin receptor on the surface of autoreactive B lymphocytes. According to the invention a method for production of the same chimeric antibody is disclosed.
An agent for selective suppression disease-associated B-lymphocytes

Field of invention

This invention relates to an agent for selective suppression of disease-associated B-cells in autoimmune diseases, especially in systemic lupus erythematosus.

Background of the invention

It is known that autoimmune diseases develop in approximately two percent of human population. In these diseases, the patients' own tissues and organs are attacked by the immune system with autoantibodies or with autoreactive T lymphocytes. As a consequence of the autoimmune attack, an inflammatory reaction is provoked that leads to a serious damage or destruction of the targeted organ. Depending on the number of organs targeted, autoimmune diseases are divided into two groups – systemic and organ-specific.

Systemic lupus erythematosus is a typical systemic autoimmune disease. It is characterized by the appearance of IgG antibodies against double-stranded (ds) DNA and nucleoprotein complexes (1,2). Serious complications of lupus include glomerulonephritis, psychosis, arthritis and others. The anti-inflammatory and immunosuppressive drugs used for treating lupus erythematosus delay the development of kidney disease and the other complications, but do not affect the primary disease itself. Even more, such medicines have serious and even life-threatening side-effects.

DNA-specific B-cells in lupus patients are a logical target for selective therapeutic intervention. Most scientists accept the braking of tolerance to DNA as starting point of the disease. Because of the importance of the anti-DNA antibody producing cells in the pathogenesis of lupus the efforts for their selective suppression and/or elimination look well founded.

It was shown that the disease-associated antibodies in lupus-prone (NZBx NZW)F1 mice are produced by B-1 cells. The continuous elimination of these cells from the very earliest age prevented the development of glomerulonephritis and increased significantly the animal’s
life-span (3). This therapeutic approach is, however, not effective in MRL/lpr lupus mice and in lupus patients as their DNA-specific B-lymphocytes belong to the B-2 type.

The studies of Coutts, S. M. et al. (1996) and of R. Furier et al. (2001) showed that selective inducing single signal-type of anergy resulted in selective inactivation of disease-associated B cells. For this aim, the surface immunoglobulin receptor was engaged by an artificial molecule, containing ologovalent B cell epitopes, linked to a non-immunogenic carrier. T-cell epitopes should not be present on it. An example for such a molecule is LJP394, which was shown to be able to suppress in vivo DNA-specific B cells in BXSB mice developing spontaneously a lupus-like disease. The administration of this immunomodulator resulted in diminished kidney damage and in increased time of survival. LJP394 has already shown encouraging results in phase II of clinical trials. (4,5).

A therapeutic approach was published based on the administration of a phosphorilated peptide from the self-antigen ribonucleoprotein. This treatment resulted in suppression of serum anti-DNA antibody levels and of proteinuria in lupus-prone MRL/lpr mice (6). An agonistic anti-CD137 monoclonal antibody has been shown to down-regulate lupus activity in the same mouse strain (7).

Publications for PCT patent applications WO 9941285A1, WO 00011732 and for European patent applications EP1210375A1, EP1292621A1 described agents and methods for diagnostic and therapy of systemic lupus erythematosus that target the immunoglobulin Fc receptors. These include receptors Fcα (FcαR) or Fcγ (FcγR).

PCT patent publication WO 01/09186 (PCT/US00/20158) described a therapeutic agent, composed of at least one part that binds to a Fc receptor, e.g. FcαR or FcγR and at least one other part that binds to another target – e.g. a tumor cell or a pathogen. These molecules could be used for induction of an immune response against weakly immunogenic molecules.

None of these patents and patent applications, however, is dealing with targeting to the inhibitory FcγRIIb receptor on the surface of B cells.

Monoclonal antibodies exist binding only to the human FcγRIIb, but not to the FcγRIIa isoform of the receptor (e.g. the Ly17.2 antibody, Haemerling, U., 1975, (8)).

It is well known that when an immune complexes, composed of an IgG antibody and an antigen, cross-link the FcγR for IgG, type IIb (FcγRIIb) and the surface immunoglobulin receptor of B lymphocytes, their activity is specifically suppressed (9). B cells express on
their surfaces only one type of FcγRII receptors—FcγRIIib (Fig.2). When Fc-gammaRIIb lacking B lymphocytes were transfected with this receptor the cells were suppressed by the mechanism described above. Mice lacking Fc-gammaRIIb were shown to react with heightened antibody production to T-independent and T-dependent antigens (11).

The DWEYSVWLSN peptide is shown to mimic antigenically native DNA. The peptide is bound by disease-associated anti-DNA antibodies. Non-autoimmune mice, immunized with the peptide, produced IgG anti-native DNA antibodies and developed renal lesions, typical for lupus (12).

The hybridoma 2.4G2, ATCC No. HB-197, producing a monoclonal antibody to both isoforms of mouse receptor CD32 - FcγRIIa and FcγRIIb is well known, too.

Description

The invention relates to a chimeric monoclonal antibody, that suppresses selectively the disease-associated B cells’ activity. As it is mentioned above, some autoimmune diseases, including systemic lupus erythematosus, are characterized by the production of autoantibodies to native DNA. A novel agent is developed with the aim of suppressing selectively targeted autoreactive B cells. It is a chimeric antibody molecule, that can cross-link the inhibitory FcγRIIib and B cells’ immunoglobulin receptors with a desired specificity.

In order to design the agent, according to the invention, a pretreated synthetic decapeptide is used, specifically the DWEYSVWLSN peptide which is an antigenic DNA mimotope, to the C-end of which a linker (a hexamethylene group) is added (Fig.1).

At the same time, the rat 2.4G2 hybridoma, producing a monoclonal antibody specific to mouse CD32 (FcγRII) is cultured in the CHO protein-free medium (Gibco, Gaithersburg, MD) at 37 °C in an atmosphere with a relative humidity of 90% and containing 5% CO2. The monoclonal antibody is isolated from the medium at the end of the cultivation by ammonium sulfate precipitation. The antibody thus obtained does not contain immunoglobulins with an unrelated specificity and non-immunoglobulin impurities and is 90-95% pure.

After the purification stage the monoclonal antibody is coupled to the modified decapeptide DWEYSVWLSN, using 1-ethyl-3(3’-dimethylaminopropyl) carboimidine.HCl (EDC). 0.4 mg of the peptide is dissolved in dimethylphormamide/ phosphate buffer in ratio 1:9 and EDC in a phosphate buffer pH 6.0 - 0.3 mg/ml. The EDC solution, containing the peptide, is
added to the antibody solution and the volume is brought to 150 ml with phosphate buffered saline pH 6.0. This mixture is incubated for 16 hours at +4°C with continuous mixing. After that it is dialyzed at +4°C against 200 volumes of PBS pH 7.0 for 16 hours and concentrated to a final volume of 20 ml using ultrafiltration through a 5 kD-pore membrane (Amicon, Millipore Corp.). This preparation contains hybrid antibody molecules, composed of the monoclonal antibody against mouse CD32 (FcγRIIa and FcγRIIb isoforms) and of the chemically modified DWEYSVWLSN peptide. The chimeric molecule is capable to bind to the inhibitory B cell receptor FcγRIIB, and to the immunoglobulin receptors of B-lymphocytes with an anti-DNA specificity. This bi-specificity of the chimeric molecule is retained in cases when it includes a whole immunoglobulin molecule, as well as its F(ab’)2 and Fab fragments. It is manifested also in such cases, when portions of the animal immunoglobulin molecule are replaced with human ones.

The protein content of chimera solution is determined spectrophotometrically at 280 nm. The purity of the constructed chimera is analysed by SDS-PAGE under non-reducing conditions.

Figure 3 shows the reverse-phase HPLC curves of unconjugated monoclonal antibody and of the chimeric antibody. The change in the elution curve of the latter proves that coupling of peptides to the immunoglobulin molecule has indeed taken place.

Flow cytometry analysis provides an evidence that the conjugation of the 2.4G2 monoclonal antibody to the peptide does not affect its binding to the mouse CD32 molecule (Figure 4).

The ability of the chimeric antibody according to this invention to down-regulate DNA-specific B lymphocytes was studied in experiments in vitro and in vivo.

The immunomodulatory activity of the chimera was initially studied on in vitro cultured spleen cells from MRL/lpr mice with lupus. These cells produce and secrete into the culture medium IgG anti-dsDNA antibodies. The cells were cultured in the presence of bacterial lipopolysasharide (positive control) or in the presence if increasing concentrations of the chimera according to the invention composed of the 2.4G2 antibody coupled to the DWEYSVWLSN peptide, or in the presence of the same concentrations of a control chimera. The results show that the chimera according to the invention suppresses the production of IgG anti-dsDNA antibodies (Figure 5).

The serum level of IgG anti-dsDNA antibodies correlates with the activity of the disease. The level of proteinuria depends on the severity of the kidney involvement. The aim of the in vivo experiments was to prove that the chimera according to the invention binds to and suppresses
the disease-associated DNA-specific B cells in mice that develop lupus spontaneously. The results obtained prove that the administration of the chimera according to the invention, to animals with systemic lupus, results in down regulation of the disease activity, suppresses their kidney involvement and prolongs their lives.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1. Scheme of the chimeric antibody molecule, according to the invention. The linker part is represented with a small circles and a decapptide – with a small squares.

Fig. 2. Cross-linking of the immunoglobulin and of the inhibitory FcγRIIb receptor on B-cells’ surface suppresses the activity of the cells.

Fig. 3. Reverse-phase HPLC analysis of unconjugated 2.4G2 antibody and of the chimeric antibody, according to the invention.

Fig. 4. The chimeric antibody retains its capability to bind to the CD32 molecule (flow cytometric analysis).

A – mouse spleen cells + isotype control;

B – cells stained with a commercial 2.4G2 antibody + biotinilated anti-rat Ig- antibody + streptavidin-FITC;

C – cells stained with the chimera according to the invention + biotinilated anti-rat Ig-antibody + streptavidin-FITC;

D - cells stained with a commercial 2.4G2 antibody (0.5 ug/tube) + commercial 2.4G2 antibody-FITC (10 ug/tube);

E – cells stained with the chimeric antibody, according to the invention (0.5 ug/tube) plus commercial 2.4G2 antibody-FITC (10 ug/tube).

Fig. 5. Effect of the antibody chimera, according to the invention on the in vitro production of anti-DNA IgG antibodies.

diamonds - kinetics of IgG anti-DNA antibodies in the culture supernatant of MRL/lpr mouse spleen cells, cultured in presence of the chimeric antibody, according to the invention;

squares – control chimera-treated cells;

triangles – in the presence of medium only;

circles - in the presence of 10 ug/ml bacterial lipopolysaccharide.
Fig. 6. Serum levels of IgG anti-DNA antibodies in 7-weeks old female MRL/lpr mice, treated for 6 weeks twice weekly intravenously with:

squares – PBS;
triangles – with a control chimera
circles – with the chimeric antibody, according to the invention.

Fig. 7. Proteinuria in 7-weeks old female MRL/lpr mice, treated for 6 weeks twice weekly intravenously with:

squares – PBS;
triangles – with a control chimera
circles – with the chimeric antibody, according to the invention.

Fig. 8. Levels of IgG anti-DNA antibodies in the sera of 17-weeks old female MRL/lpr mice, treated 6 weeks twice weekly intravenously with:

squares – PBS;
triangles – control chimera;
circles – chimeric antibody, according to the invention.

Fig. 9. Proteinuria levels in 17-weeks old female MRL/lpr mice, treated for 6 weeks twice weekly intravenously with:

squares – PBS;
triangles – control chimera;
circles – chimeric antibody, according to the invention.

The following Examples illustrate the present invention and are not intended to limit the same.

Example 1: Construction of the chimeric monoclonal antibody

$10^8$ cells of rat hybridoma 2.4G2 (ATCC HB-197), producing a monoclonal antibody binding to mouse CD32, was grown in the protein-free medium CHO (GIBCO, Gaithersburg, MD) at 37°C in air, containing 5% CO₂ and at 90% humidity.

After the end of cultivation, the monoclonal antibody secreted into the medium was purified by precipitation with ammonium sulfate. Equal volumes of culture supernatant and of a saturated solution of ammonium sulfate were slowly mixed and left for 16 hours at +4°C with constant stirring. After centrifugation at 5 000 g for 30 min the supernatant was discarded,
PBS pH 7.0 in a 10 times smaller volume than the starting one was added and the dissolved precipitate was dialyzed twice against 500 volumes of the same buffer.

After purification, the monoclonal antibody was coupled using 1-ethyl-3(3’-dimethylaminopropyl)carboimidine.HCl (ECL) to the decapeptide, modified in advance as it was mentioned before. The following solutions were made in advance: 1.5 mg/ml solution of the monoclonal antibody in phosphate buffer pH 6.0; 0.4 mg/ml solution of the peptide in dimethylformamide/ phosphate buffer pH 6.0 and 0.3 mg/ml EDC in phosphate buffer pH 6.0. 7.5 ml of the peptide solution was mixed with 3.75 ml EDC and 10 ml of the antibody solution. The mixture was incubated for 16 hours at +4 °C with constant stirring. After that it was dialysed for 16 hours at +4°C against 200 volumes of phosphate-buffered saline pH 7.0. The solution of the chimeric antibody was concentrated to a final volume of 20 ml using ultrafiltration with a 5 kDa pore membrane (Amicon, Millipore Corp.)

The protein content of the solution was determined spectrometrically at 280 nm. The purity of the constructed chimeric antibody was determined by SDS-PAGE under non-reducing conditions.

The reverse-phase HPLC elution curves on Fig.3 proved that peptides were coupled to the monoclonal antibody. The chimeric monoclonal antibody retained its ability to bind to mouse CD32 as shown by flow cytometric analysis (Fig. 4).

Example 2: Determination of immunomodulatory effect of the constructed hybridoma in vitro.

Spleen cells obtained from MLR/lpr mice with lupus, cultured in vitro, produced and secreted into the medium IgG DNA-specific antibodies. The cultivation was carried out in the presence of 10 ug/ml lipopolysascharide from E. coli, or in the presence of 100 ng/ml of the chimera, according to the invention, or in presence of the same concentration of control chimera, composed of monoclonal antibody 2.4G2, conjugated to the peptide WSLDYWNESV, which contains the same aminoacids as the peptide DWEYSVWLSN, but in a shuffled order. Inhibition of the production of IgG antibodies to native DNA was observed when the cultivation was carried out in the presence of the chimera, according to the invention (Fig. 5).
Example 3: Methods for the measurement of the levels of native DNA-specific IgG antibodies in sera and of the protein concentration in the urine.

The levels of native DNA-specific IgG antibodies were determined by fluid-phase ELISA, according to a known technique (13). Briefly, calf thymus DNA (from Sigma-Aldrich) was treated with nuclease S1 (from Amersham Pharmacia Biotech) in order to remove the inevitably present single-stranded DNA portions. The pre-treated DNA was biotinilated using a kit for nucleic acids biotinilation (from Vector laboratories, UK). Serial dilutions of the investigated fluids (cell culture supernatants and sera) starting with 1:50 were mixed with a standard amount of the biotinilated DNA and after incubation, the mixture was transferred to the wells of a second plate, coated in advance with avidin. After subsequent incubation and washing steps, the presence of adsorbed mouse IgG was determined by adding a alkaline phosphatase conjugate of anti-mouse IgG antibody.

The quantity of protein, excreted with the urine, which is a criterion for the severity of the kidney injury in lupus, was measured using a dry test for urine analysis (Bayer, UK).

Example 4: Study of the immunomodulatory activity of the chimeric monoclonal antibody in young lupus-prone animals.

For this purpose a group of fifteen 7 weeks-old female MLR/lpr mice was set up. The animals were still healthy at this age, but the appearance of the first signs of lupus autoimmune disease was imminent. A group of five mice was treated intravenously for 6 weeks twice weekly with 0,1 ml PBS. A second group of five animals was treated with 20 ug of the chimera, according to the invention, and another group was treated with 20 ug of the control chimera, according to Example 2. Once in two weeks the animals were bled and the sera were kept frozen at -20°C. Sera from all groups and all bleedings were tested by ELISA on a single day.

The results showed that the appearance of anti-DNA IgG antibodies and of proteinuria was prevented in the next 6 weeks in the group treated intravenously with the chimeric antibody, according to the invention (Figs. 6 and 7).

Example 5. Analysis of the immunomodulatory activity of the chimeric monoclonal antibody in adult mice with advanced stage of lupus.
A group of fifteen 17-weeks MLR/lpr mice was divided into three subgroups with 5 animals each. At this age they were in an advanced stage of the disease. The first group of five mice was injected twice weekly intravenously as in Example 4 with 0.1 ml PBS. A second group of five animals was treated with 20 μg of the chimera, according to the invention, and a third group of five animals - with 20 μg control chimera, according to Example 2.

In animals, which received PBS intravenously, the levels of anti-DNA IgG antibodies increased sharply, maximal levels were reached around week 19. At the end of experiment (23nd week), only animal from this group survived.

The intravenous administration of the chimera, according to the invention, resulted in maintenance of a flat level of the disease-associated IgG anti-DNA antibodies in the first 4 weeks after the start of the infusions (Fig.8). Moreover, the increase of proteinuria was prevented by the application of the chimera, according to the invention, whereas in the PBS-treated group it reached very high levels (15 g/l) before the death of the animals.
CLAIMS

1. An agent for selective suppression of disease-associated B cells, especially those with specificity to DNA in systemic lupus erythematosus, characterized in that the agent is a chimeric monoclonal antibody, capable of cross-linking an inhibitory and the immunoglobulin receptor on the surface of autoreactive B lymphocytes.

2. An agent for selective suppression of disease-associated B cells, according to claim 1, characterized in that the targeted inhibitory receptor is FcγRIIb.

3. An agent for selective suppression of disease-associated B cells, according to claim 1, characterized in that the immunoglobulin receptor on the surface of autoreactive B lymphocytes is DNA-specific.

4. Method for constructing the agent for selective suppression of disease-associated DNA-specific B cells according to claim 1, characterized in that the chemical coupling of a modified synthetic peptide, which is an antigenic mimotope of DNA, to a monoclonal antibody to an inhibitory B cell receptor in carried out in the presence of 1-ethyl-3(3'-dimethylaminopropyl) carboimidine.HCl (EDC) and subsequent incubation, dialysis and concentration.

5. Method for constructing the agent for selective suppression of disease-associated DNA-specific B cells according to claims 1 and 4, characterized in that the antigenic mimotope of DNA is composed of 10 amino acids.

6. Method for constructing the agent for selective suppression of disease-associated DNA-specific B cells according to claims 1, 4 and 5, characterized in that the sequence of the antigenic mimotope of DNA is DWEYSSVWLSN.

7. Method for constructing the agent for selective suppression of disease-associated DNA-specific B cells according to claims 1, 4, 5 and 6, characterized in that a hexamethylene group is added to the C-end of the antigenic mimotope of DNA.

8. Method for constructing the agent for selective suppression of disease-associated DNA-specific B cells according to claims 1 and 4, characterized in that the monoclonal antibody is produced by the rat hybridoma ATCC HB-197.

9. Method for constructing the agent for selective suppression of disease-associated DNA-specific B cells according to claims 1, 4 and 8, characterized in that the
monoclonal antibody used is in its intact form, in the form of F(\text{ab}')_2 or Fab fragments or part of its molecule is replaced with parts of a human immunoglobulin molecule.

10. Use of chimeric monoclonal antibody according to claims 1 to 9 for selective suppression of pathological DNA-specific B cells.
Bibliography


Figure 2
Figure 3
Figure 4
### A. CLASSIFICATION OF SUBJECT MATTER

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According to International Patent Classification (IPC) or to both national classification and IPC

### B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database consulted during the international search (name of database and/or where practical, search terms used)

- EPO-Internal
- EMBASE
- BIOSIS
- PAJ
- WPI Data
- CHEMABS Data

### C. DOCUMENTS CONSIDERED TO BE RELEVANT

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Further documents are listed in the continuation of box C

Patent family members are listed in annex

* Special categories of cited documents:
  * A document defining the general state of the art which is not considered to be of particular relevance
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*X* document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the international search: 31 January 2005

Date of mailing of the international search report: 18/02/2005

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk
Tel: (+31-70) 340-2040, Fax: (+31-70) 340-3016

Authorized officer: Ulbrecht, M
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INTERNATIONAL SEARCH REPORT

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 10
   because they relate to subject matter not required to be searched by this Authority, namely:
   Although claim 10 is directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:  
   because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:  
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant’s protest.

[ ] No protest accompanied the payment of additional search fees.
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