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(54) Title: TREATMENT OF AUTOIMMUNE DISEASES BY INDUCING TOLERANCE TO CELLS, TISSUES AND ORGANS

(57) Abstract

Cells genetically engineered with DNA encoding a plurality of antigens of a cell, tissue, or organ to which tolerance is to be induced. The cells are free of co-stimulatory antigens, such as B7 antigen. Such cells induce T-cell anergy against the proteins encoded by the DNA, and may be administered to a patient in order to prevent the onset of or to treat an autoimmune disease, or to induce tolerance to a tissue or organ prior to transplantation.

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TREATMENT OF AUTOIMMUNE DISEASES BY INDUCING TOLERANCE TO CELLS, TISSUES AND ORGANS

This invention relates to the treatment of autoimmune diseases. More particularly, this invention relates to the treatment of autoimmune diseases by administering genetically engineered cells which induce tolerance to a particular protein, cell, or organ.

The immune system protects an individual from invading organisms by attacking and destroying such invading organisms, while at the same time recognizing and tolerating the individual's own proteins, cells, and organs.

In order for the T-cells of the immune system to mount an immune response against a given foreign antigen, two types of interactions between T-cells and antigen presenting cells are required. The first interaction, or "first signal," occurs between the T-cell antigen receptors of T-cells and the antigen itself, which is carried upon the major histocompatibility molecule complexes of antigen presenting cells, whereby the T-cells recognize foreign antigens. The second interaction, or "second signal", occurs when the B7 molecule (which is a co-stimulatory antigen) of antigen presenting cells is recognized by its cognate receptor, CD28, on the membrane of T-cells. This "second signal" is the one which signals the T-cells to

proliferate, and to attack and destroy the cells which express the foreign antigen.

It has been postulated that autoimmune diseases, such as Type I diabetes, Graves or Hashimoto's disease, rheumatoid arthritis, autoimmune male and female infertility, myasthenia gravis, and multiple sclerosis, are due to the failure of specific proteins in an individual (sometimes referred to as "self" proteins) to be recognized as such by the immune system. Instead, such proteins and the cells which express such proteins are recognized as foreign, which results in the activation of autoimmune pathogenic lymphocytes. Once such lymphocytes are activated, they are continuously produced.

In an autoimmune disease, an antigen presenting cell induces a T-cell response against "self" proteins or "self" cells by providing two signals. The "first signal" enables the T-cells to recognize the "self" protein or "self" cell which expresses the protein as foreign. This signal stimulates T-cells known as CD4+ cells. The "second signal" is one which signals the T-cells to proliferate, and to attack and destroy the cells which express the "self" protein. The second signal, such as B7, is recognized by the T-cells. Upon recognition by the T-cells of B7, the CD4+ cells are induced to produce IL-2, and expand clonally, followed by attack and destruction of the target cells.

In the absence of the second signal (eg., B7), the T-cells recognize the antigen displayed upon the antigen presenting cell; however, interactions of this T-cell with this antigen does not stimulate clonal expansion, but instead stimulates a long-lasting tolerance to the antigen, known as anergy.

It is therefore an object of the present invention to treat or prevent autoimmune diseases by inducing tolerance to a "self" protein or "self" cells.

In accordance with an aspect of the present invention, there are provided cells which are genetically engineered with DNA

encoding a plurality of antigens of a cell, tissue, or organ to which tolerance is to be induced. The genetically engineered cells are free of co-stimulatory antigens, such as B7 or heat stable antigen, also known as HSA.

The term "co-stimulatory antigen" as used herein, means that upon recognition of such antigen by a cognate receptor, T-cells are signaled to proliferate, and to attack and destroy cells which express a foreign antigen.

Applicant has found that when such cells, which include DNA encoding a plurality of antigens of a particular cell, tissue, or organ, yet are free of co-stimulatory antigens, such as B7 or HSA, are administered to an individual, there is induced a T-cell anergy whereby the target cells, tissues or organs which normally express such antigens are not attacked by the T-cells.

Such a treatment to induce T-cell anergy leads to a "re-education" of the immune system, resulting in the induction of tolerance to the target cell antigens, whereby the T-cells will not respond when they come in contact with proteins, cells, or organs that contain these antigens.

In one embodiment the cells from which the antigens may be derived may be any type of cells which may be attacked or destroyed by T-cells during the course of events in an autoimmune disease. Such cells include, but are not limited to, pancreatic beta cells, thyroid follicular cells, cells of synovial joints, ovarian cells, testicular cells, muscle cells, and nerve cells. The antigens may be obtained by obtaining a purified population of the desired cells from a patient, and then collecting mRNA from the purified cells. The mRNA may then be converted to cDNA. The cDNA may then be cloned into an appropriate expression vector(s) which is then transfected into cells obtained from the patient. Appropriate expression vectors are those which may be employed for transfecting DNA into eukaryotic cells. Such vectors include, but are not limited to, eukaryotic vectors, such

as, for example, yeast vectors and fungal vectors, and viral vectors, such as, but not limited to, retroviral vectors. Examples of retroviral vectors which may be employed include, but are not limited to, those derived from Moloney Murine Leukemia Virus, Moloney Murine Sarcoma Virus, Rous Sarcoma Virus and Spleen Necrosis Virus.

In one alternative, a single cDNA library may be obtained for a particular cell, tissue, or organ. This cDNA library may then be used to engineer cells which may be used for the treatment of any and all patients.

In one embodiment, the genetically engineered cells which are free of co-stimulatory antigens such as B7 antigen or HSA are parenchymal cells; i.e., cells which are contained within an organ or a gland.

Parenchymal cells which may be genetically engineered include, but are not limited to, keratinocytes, hepatocytes, endothelial cells, muscle cells, or fibroblasts. In one embodiment, the parenchymal cells are keratinocytes. The genetically engineered cells may be obtained from the patient.

The genetically engineered cells may then be administered to the patient in an amount effective to prevent the onset of or to treat an autoimmune disease in a patient. Depending upon the severity of the disease, the dosage regimen will vary. In one embodiment, once a patient is diagnosed with the particular disease, 10^8 cells are administered subcutaneously daily for about 2 weeks. Levels of circulating antibodies directed against the protein, cell, or organ are monitored routinely thereafter. The cells may be administered intravenously or injected into the thymus.

Autoimmune diseases which may be prevented or treated with the genetically engineered cells of the present invention include, but are not limited to, Type I diabetes (wherein the parenchymal cells are engineered with DNA encoding pancreatic

beta cell antigens), Graves or Hashimoto's disease (wherein the parenchymal cells are engineered with DNA encoding thyroid follicular cell antigens), rheumatoid arthritis (wherein the parenchymal cells are engineered with DNA encoding antigens from cells of synovial joints), myasthenia gravis (wherein parenchymal cells are engineered with DNA encoding antigens from muscle cells), multiple sclerosis (wherein the parenchymal cells are engineered with DNA encoding antigens from nerve cells), or autoimmune infertility (wherein the parenchymal cells are engineered with DNA encoding antigens from ovarian cells or testicular cells).

In one illustrative embodiment, keratinocytes are obtained from a patient with an autoimmune disease, or a patient who may be susceptible to an autoimmune disease. The genes encoding the antigens of a target cell (such as, for example, a pancreatic beta cell) are obtained by collecting mRNA from a purified preparation of target cells. The mRNA is converted to cDNA, which is cloned into an appropriate expression vector, such as, but not limited to, a simian virus 40 vector or a bovine papilloma virus vector. The expression vector may contain a selectable marker such as, for example, a G418 resistance marker.

The expression vector is then transfected into the keratinocytes. Transfected keratinocytes which express the target cell proteins are then selected with an appropriate selection agent, such as G418. Expression of autoreactive epitopes may be confirmed with FACS analysis.

The selected cells are then cultured in vitro and treated with Vitamin D, TNF-α, or gamma-interferon to increase expression of the MHC antigens of the target cells that display the processed target cell proteins. Expression of such antigens may be confirmed with FACS analysis. The keratinocytes may then be administered to a patient in an amount effective for preventing the onset of or for treating an autoimmune disease. Such

administration may be by intravenous administration or alternatively, the keratinocytes may be administered directly into the thymus. After administration, peripheral blood samples may be obtained and quantitated for changes in antibodies directed against "self" epitopes. Also, spleen and lymph node T-cells may be tested in vitro for auto-reactivity against target cells.

The genetically engineered cells may also be useful in the prevention of transplant rejection disease. Thus, in one embodiment, the cells, such as keratinocytes, may be engineered with DNA encoding a plurality of antigens from cells or an organ which is to be transplanted into a patient. The cells or organ may be derived from the same species as the host, or may be derived from a different species. For example, if one desired to transplant porcine pancreatic beta cells into a human patient in order to treat Type I diabetes, one may engineer parenchymal cells with DNA encoding a plurality of porcine beta cell antigens, and administer the engineered parenchymal cells to the patient prior to the transplantation of the of such cells into a human patient, and such porcine pancreatic beta cells may be transplanted into the human patient with a decreased risk of rejection. It is to be understood, however, that this embodiment of the present invention is not to be limited to the prevention of the rejection of transplanted porcine beta cells into a human patient.

The invention will now be described with respect to the following example; however, the scope of the present invention is not intended to be limited thereby.

Example

A. mRNA extraction from cells to be used in the production of a cDNA library

Total RNA is isolated from pancreatic Beta cells by the method described in Maniatis, et al., Molecular Cloning - A Laboratory Handbook (1982).

The cells are washed with 10 volumes of ice cold phosphate buffered saline without magnesium and calcium ions. The cells are then pelleted by centrifugation. The cells are then resuspended in 10-20 volumes of RNA extraction buffer (NaCl, $MgCl_2$, Tris, Nonidet, dithiothreitol, and placental RNase inhibitor). An equal volume of proteinase digestion buffer is added (Tris, EDTA, NaCl, SDS). The solution is vortexed, and the lysate is drawn into a 21 gauge needle and rapidly expelled into a polypropylene tube. Proteinase K is added, and the solution is incubated for 30 minutes at 37°C. Proteins are extracted with an equal volume of phenol:chloroform. The aqueous phase is collected and 2.5 volumes of ethanol are added. RNA is recovered by centrifugation. The pellet is then washed with 70% ethanol, and then dried at room temperature. The pellet is then redissolved in Tris buffer with EDTA. $MgCl_2$, dithiothreitol, and RNase inhibitor are then added, followed by addition of RNase - free pancreatic DNase I, EDTA, and SDS. The solution is then extracted with phenol:chloroform. Ice cold ethanol is then added to the aqueous phase, and the RNA is collected by centrifugation. The pellet is then stored at -70°C.

B. Selection of mRNA

A column of oligo(dT)-cellulose is poured and washed with sterile water. The column is then washed with Tris, NaCl, EDTA, and sodium lauryl sarcosinate. The RNA is then dissolved in sterile water, heated, and 2X buffer is added. The solution is applied to the column. The eluate is collected, heated, and re-applied to the column. 1ml fractions are then collected, and absorbance of each fraction is read at OD260. PolyA and RNA are eluted with Tris, EDTA and SDS. 3M sodium acetate is then added to the eluted RNA, and RNA is recovered by centrifugation. mRNA

is then fractionated by gel electrophoresis to insure that the majority of the mRNA appears between 500 bases and 8 kb.

C. Synthesis of a DNA and engineering expression vector

Synthesis of first and second strands of cDNA are performed according to classical methods described in Maniatis, et al., Molecular Cloning - A Laboratory Handbook (1982). Double stranded cDNA is blunt-ended and synthetic linkers are added for insertion into a eukaryotic expression vector known as pSVT7 (Maniatis, 1982). This vector includes an SV40 origin of replication, a bacteriophage T7 promoter, and a multiple cloning site. The vector also includes a neomycin resistance marker. replication, a bacteriophage T7 promoter, and a multiple cloning site. The vector includes a neomycin resistance marker.

D. Insertion of expression vector into tolerance-inducing cell

Keratinocytes are obtained from the patient to be treated. The cells then are propagated in vitro with standard tissue culture methods. Expression vectors containing the double stranded cDNA are transfected into the keratinocytes by the calcium phosphate precipitated DNA method. Cells that have taken up the expression vector are selected by culture in the presence of G418.

E. Administration of engineered cells

The engineered cells are expanded such that 10^8 cells can be injected into the patient daily for 2 weeks. Circulating antibody levels are routinely quantitated in vitro by FITC analysis for activity directed against the cells from which the mRNA was obtained. During this time, peripheral blood leukocytes are obtained from the patient and tested by a mixed leukocyte reaction for their ability to respond to the cell from which the mRNA was obtained.

Within two weeks, the response to the target cells will be reduced due to the induction of tolerance afforded by the

presentation of the target antigens by the host's antigens without the co-stimulaltory angtigen.

It is to be understood, however, that the scope of the present invention is not to be limited to the specific examples described above. The invention may be practiced other than as particularly described and still be within the scope of the accompanying claims.

WHAT IS CLAIMED IS:

1. Cells genetically engineered with DNA encoding a plurality of antigens of a cell, tissue, or organ to which tolerance is to be induced, said cells being free of co-stimulatory antigens.
2. The cells of Claim 1 wherein the DNA encodes antigens from pancreatic beta cells.
3. The cells of Claim 1 wherein the DNA encodes antigens from thyroid follicular cells.
4. The cells of Claim 1 wherein the DNA encodes antigens from cells of synovial joints.
5. The cells of Claim 1 wherein the DNA encodes antigens from muscle cells.
6. The cells of Claim 1 wherein the DNA encodes antigens from nerve cells.
7. The cells of Claim 1 wherein the DNA encodes antigens from ovarian cells.
8. The cells of Claim 1 wherein the DNA encodes antigens from testicular cells.
9. The genetically engineered cells of Claim 1 wherein said cells are parenchymal cells.
10. The cells of Claim 9 wherein said parenchymal cells are keratinocytes.
11. A method of treating an autoimmune disease, comprising:

administering to a patient cells genetically engineered with DNA encoding a plurality of antigens of a cell, tissue, or organ to which tolerance is to be induced, said cells being free of co-stimulatory antigens, and said cells being administered in an amount effective to treat an autoimmune disease in a patient.

12. The method of Claim 11 wherein said autoimmune disease is Type I diabetes and the DNA encodes antigens from pancreatic beta cells.

13. The method of Claim 11 wherein said autoimmune disease is Graves disease and the DNA encodes antigens from thyroid follicular cells.

14. The method of Claim 11 wherein said autoimmune disease is rheumatoid arthritis and the DNA encodes antigens from cells of synovial joints.

15. The method of Claim 11 wherein said autoimmune disease is myasthenia gravis and the DNA encodes antigens from muscle cells.

16. The method of Claim 11 wherein said autoimmune disease is multiple sclerosis and the DNA encodes antigens from nerve cells.

17. The method of Claim 11 wherein said autoimmune disease is female autoimmune infertility and the DNA encodes antigens from ovarian cells.

18. The method of Claim 11 wherein said autoimmune disease is male autoimmune infertility and the DNA encodes antigens from testicular cells.

19. The method of Claim 11 wherein said genetically engineered cells are parenchymal cells.

20. The method of Claim 19 wherein said parenchymal cells are keratinocytes.

21. A method of preventing the onset of an autoimmune disease, comprising:

administering to a patient cells genetically engineered with DNA encoding a plurality of antigens of a cell, tissue, or organ to which tolerance is to be induced, said cells being free of co-stimulatory antigens, and said cells being administered in an amount effective to prevent the onset of an autoimmune disease in a patient.

22. The method of Claim 21 wherein said autoimmune disease is Type I diabetes and the DNA encodes antigens from pancreatic beta cells.

23. The method of Claim 21 wherein said autoimmune disease is Graves disease and the DNA encodes antigens from thyroid follicular cells.

24. The method of Claim 21 wherein said autoimmune disease is rheumatoid arthritis and the DNA encodes antigens from cells of synovial joints.

25. The method of Claim 21 wherein said autoimmune disease is myasthenia gravis and the DNA encodes antigens from muscle cells.

26. The method of Claim 21 wherein said autoimmune disease is multiple sclerosis and the DNA encodes antigens from nerve cells.

27. The method of Claim 21 wherein said autoimmune disease is female autoimmune infertility and the DNA encodes antigens from ovarian cells.

28. The method of Claim 21 wherein said autoimmune disease is male autoimmune infertility and the DNA encodes antigens from testicular cells.

29. The method of Claim 21 wherein said genetically engineered cells are parenchymal cells.

30. The method of Claim 29 wherein said parenchymal cells are keratinocytes.

31. A method of preventing transplant rejection disease comprising:

administering to a patient cells genetically engineered with DNA encoding a plurality of antigens of said cells, tissue, or an organ to be transplanted into said patient, said cells being free of co-stimulatory antigens, said cells being administered

in an amount effective to induce tolerance to said cells or said tissue or said organ in a patient.

32. The method of Claim 31 wherein said genetically engineered cells are parenchymal cells.

33. The method of Claim 32 wherein said parenchymal cells are keratinocytes.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/10534

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 37/00
US CL :424/93+

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)

U.S. : 424/93+

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

none

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Dialog, Keratinocytes, tolerance autoantigens, transplantation

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	European Journal of Immunology, volume 20, issued 1990, V. Bal et al., "Antigen presentation by keratinocytes induces tolerance in human T cells", pages 1893-1897, see entire article.	31-33
Y	Nature, volume 336, issued 01 December 1988, J. Markmann, et al., "Antigen presenting function of class II MHC expressing pancreatic beta cells", pages 476-479, see entire article.	1-3

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

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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

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

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Date of the actual completion of the international search	Date of mailing of the international search report
06 JANUARY 1994	JAN 31 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer SUZANNE ZISKA
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/10534

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Nature, volume 342, issued 30 November 1989, L.C. Burkly et al., "T-cell tolerance by clonal anergy in transgenic mice with nonlymphoid expression of MHC class II I-E", pages 564-566, see entire article.	1-30
Y	International Immunology, volume 4, no. 5, issued 1992, G. Schonrich et al., "Distinct mechanisms of extrathymic T cell tolerance due to differential expression of self antigen", pages 581-590, see entire article.	1-30

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/10534

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

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

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

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 II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

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

Please See Extra Sheet.

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.

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

Group I, claims 1-20, drawn to a first product, and a first method of using the first product: claims 1-10, drawn to cells genetically engineered with DNA encoding a plurality of antigens of a cell, tissue or organ to which tolerance is to be induced, classified in Class 435, subclass 172.3, for example and claims 11-20, drawn to a method of treating an autoimmune disease by administering to a patient cells which have been genetically engineered with DNA, classified in Class 424, subclass 93+, for example.

Group II, claims 21-30, drawn to a method of preventing the onset of an autoimmune disease comprising administering to a patient cells genetically engineered with DNA, classified in Class 424, subclass 93+, for example.

Groups III, claims 31-33, drawn to a method of preventing transplantation rejection, classified in Class 424, subclass 93+, for example.

The inventions listed as Groups I-III do not meet the requirements for Unity of Invention for the following reasons:

Each grouping of claims forms a separate invention not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.