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(54) Title: MONOCLONAL ANTIBODY TO INTRACELLULAR EPITOPE OF HUMAN T CELL RECEPTOR ZETA CHAIN AND METHOD OF PREPARATION

(57) Abstract

A monoclonal antibody of class IgG selectively binds to permeabilized but not unpermeabilized T cells. The monoclonal antibody TIA-2 binds to an intracellular epitope of the zeta chain of the T cell receptor complex. A method of screening a population of monoclonal antibodies for a monoclonal antibody that selectively binds to permeabilized but not unpermeabilized lymphocytes includes the steps of stabilizing a population of lymphocytes by contacting the cells with a mild fixative; permeabilizing the cells by contacting them with a mild glycosidic detergent; contacting the population of monoclonal antibodies with the stabilized, permeabilized lymphocytes; contacting the population of monoclonal antibodies with unpermeabilized lymphocytes; and selecting a monoclonal antibody that reacts with the permeabilized lymphocytes but not with the non-permeabilized lymphocytes.

* See back of page

DESIGNATIONS OF "DE"

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Monoclonal Antibody to Intracellular Epitoe of Human T Cell Receptor Zeta Chain and Method of Preparation

Background

This invention relates to monoclonal antibodies, and specifically to monoclonal antibodies to lymphocytes.

Monoclonal antibodies reactive with lymphocyte surface structures are useful as therapeutic and diagnostic agents and as reagents in studies of lymphocyte biology. A prominent structure on peripheral blood T lymphocytes is the T cell antigen receptor complex. The receptor portion of the complex consists of primarily extracellular, variable, clone-specific heterodimers (composed of the subunit proteins alpha, beta, gamma, or delta) which determine the specificity of the receptor and function as the binding site for antigen. The heterodimers are noncovalently associated with the proteins of the CD3 complex - gamma, delta, epsilon, zeta, and eta. It is believed that the proteins of the CD3 complex, which have primarily invariant regions, act as intracellular transducers of the ligand-binding signals.

The individual proteins of the complex are synthesized intracellularly, and the complex is assembled and then transported to the plasma membrane. The zeta chain, whose nine amino acid extracellular domain is identical in man and mouse, is synthesized in rate limiting amounts and appears to play a unique role in intracellular targeting and assembly and the efficient expression of the complex on the cell surface. In the absence of zeta, 95% of the receptor complexes are rapidly transported to and degraded in liposomes. A low expression of the CD3 zeta chain can lead to clinical symptoms of immunodeficiency.

Monoclonal antibodies reactive with a particular antigen are prepared by standard techniques: immunizing a mammal, e.g., a mouse, with the antigen; fusing splenocytes from the immunized mammal to myeloma cells for the production of hybridomas; obtaining viable clones of the hybridomas; and screening the clones for their reactivity with the antigen (e.g., by flow cytometric analysis).

Summary of the Invention

The invention features, in one aspect, a monoclonal antibody of class IgG, which antibody selectively binds to permeabilized T cells but not to permeabilized B cells nor to unpermeabilized T cells.

In the preferred embodiment the monoclonal antibody is produced by a hybridoma formed by fusion of cells from a mammalian myeloma line (specifically NS-1 myeloma cells) and spleen cells from a mammal (specifically a Balb/c mouse) previously immunized with permeabilized human T cells (specifically from Ficoll purified peripheral blood mononuclear cells); the antibody, TIA-2, recognizes a T cell restricted 32 kd homodimer, a dimer of the zeta chain of the T cell receptor complex.

In another aspect, the invention features a method of screening a population of monoclonal antibodies for a monoclonal antibody that selectively binds to permeabilized but not unpermeabilized lymphocytes; the method includes the steps of stabilizing a population of lymphocytes by contacting the cells with a mild fixative; permeabilizing the cells by contacting them with a mild glycosidic detergent; contacting a population of monoclonal antibodies with the stabilized, permeabilized cells; contacting the same population of monoclonal antibodies with unpermeabilized cells; and selecting a monoclonal antibody that reacts with the permeabilized

lymphocytes but not with the non-permeabilized lymphocytes. In the preferred embodiment, the lymphocytes are T cells; the fixative is formaldehyde at a concentration of from 0.005 - 0.1%, preferably 0.01%; and the mild glycosidic detergent is digitonin at 1 to 100 μg/ml, preferably 10 μg/ml.

In another aspect, the invention features a method of determining the state of T cell activation of a patient that involves contacting serum, urine, or circulating peripheral blood cells from the patient with a monoclonal antibody that recognizes the zeta chain of the T cell receptor complex (e.g., TIA-2) and measuring the extent of binding of the antibody with the zeta chain; to determine the extent of T cell infiltration of a specific tissue, a fixed tissue section is contacted with the monoclonal antibody.

By providing a method of permeabilizing lymphocytes that both preserves the integrity of intracellular structures and stabilizes the cells to withstand the repeated washings that are necessary for flow cytometric analysis, the invention provides a way of screening a population of monoclonal antibodies for an antibody that binds specifically to an intracellular target as opposed to a surface molecule of a specific lymphocyte population. The specific monoclonal antibody described, TIA-2, was unlikely to be isolated by screening methods that analyzed only for antibodies to cell surface molecules as the bulk of the zeta protein chain resides intracellularly.

As the zeta chain is believed to be sloughed off of activated T cells into a patient's biological fluids, e.g., serum or urine, TIA-2 can be used as a rapid diagnostic reagent for the assessment of the state of lymphocyte activation, for example in patients with autoimmune diseases such as systemic lupus erythematosas,

rheumatoid arthritis, vasculitis, and polymyositis. In patients who are recent allograft recipients, T cell activation could indicate imminent graft rejection. In addition, TIA-2 can be used as a stain to locate lymphocytes in fixed tissues. TIA-2 is also likely to be a valuable research reagent in determining the specific role of the zeta chain in T cell activation.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiment and from the claims.

Description of the Preferred Embodiment

We first briefly describe the drawings.

Figs. 1 and 2 are autoradiograms of SDS polyacrylamide gels.

Monoclonal antibody TIA-2 selectively recognizes permeabilized but not unpermeabilized T lymphocytes and is directed at an intracellular epitope of the T cell receptor zeta chain. TIA-2 was isolated by the following procedure.

Hybridomas suitable for screening for production of antibodies reactive with intracellular antigens were prepared by immunizing 6 week old Balb/c mice with permeabilized T lymphocytes (25-30 x 10^6) at 21 day intervals over a 9-12 week period. The immunogen was prepared using Ficoll purified peripheral blood mononuclear cells obtained from plateletpheresis residues that were rosetted with sheep erythrocytes (Lay et al., Nature 300:267(1971)). Purified T lymphocytes were washed three times in PBS, resuspended at 5 x 10^6 cells/ml and permeabilized by the addition of digitonin (10 μg/ml) for 5 minutes on ice. The adequacy of permeabilization was monitored by determining trypan blue uptake, which was typically greater than 90%. Permeabilized lymphocytes were pelleted, resuspended at 25-30 x 10^6 cells/ml in sterile PBS and injected

intraperitoneally into Balb/c mice. Splenocytes from immunized mice were fused to NS-1 myeloma cells for the production of hybridomas (Kohler et al., Nature 256:495(1975)).

Individual clones of the hybridomas as prepared above were screened for reactivity to permeabilized T lymphocytes by a modification of the flow cytometric method. In order to permeabilize the cells without causing cellular damage or the loss of intracellular constituents and in order to protect the permeabilized cells against disintegration during the many washes required in preparation for flow cytometric analysis, T lymphocytes purified by sheep erythrocyte rosetting were first stabilized by mild fixation with 0.01% formaldehyde in PBS for 20 min. on ice. Cells were then washed four times with ice cold PBS, resuspended at 5×10^6 cells/ml in PBS and permeabilized by the addition of digitonin (10 μg/ml) for 5 minutes on ice. After the adequacy of permeabilization had been confirmed by trypan uptake, cells were pelleted and resuspended in PBS at 20×10^6 cells/ml. Hybridoma supernatants were added to permeabilized cells in a 1:1 ratio. After 30 minutes on ice, cells were washed three times with PBS containing 0.05% Tween-20 to remove unbound antibody, further incubated with goat anti-mouse-FITC, washed, resuspended in PBS and 1% formaldehyde, and analyzed flow cytometrically, using an Epics 752 flow cytometer.

Alternatively, the lymphocytes could be stabilized by any other mild fixative, e.g., glutaraldehyde at 0.1-1%. Also, any mild detergent having the properties of digitonin would be a suitable permeabilizing agent.

Monoclonal antibodies that selectively reacted with intracellular T cell specific structures were selected by screening the hybridoma supernatants on digitonin treated T cells and B cells as well as on

native T cells. TIA-2 was one such monoclonal antibody selected. The specificity of binding of TIA-2 is indicated in the following table:

RESTRICTED EXPRESSION OF TIA-2

CELL TYPE	RELATIVE FLUORESCENCE
T lymphocytes	+++ (>95%)
CD4+ lymphocytes	+++ (>95%)
CD8+ lymphocytes	+++ (>95%)
T4Cl clone	+++ (>95%)
T4T8Cl clone	+++ (>95%)
B lymphocytes	-
REX	+++ (>95%)
Jurkat	+++ (>95%)
HPB-ALL	+++ (>95%)
HUT-78	+++ (>95%)
CEM	+++ (>95%)
Raji	-
BJAB	-
Daudi	-
U937	-

Analysis of the specificity of binding of TIA-2 revealed that it recognizes an intracellular epitope on the zeta chain of the T cell receptor complex. Peripheral blood T lymphocytes that had been permeabilized by the method described previously were radioiodinated using lactoperoxidase. After solubilization of the cells in NP-40 lysis buffer, immunoprecipitates were formed using TIA-2, separated on SDS polyacrylamide gels, and subjected to autoradiography. As shown in Fig. 1, immunoprecipitates formed using TIA-2 (lanes 2 and 5) included a prominent band at 32 kd that was reduced to 16 kd in the presence of 2-mercaptoethanol. Immunoprecipitates formed using Protein A-Sepharose™ with rabbit anti-mouse immunoglobulin (lanes 1 and 4) alone, or an isotype

matched control antibody (12T4D11, anti-CD4) (lanes 3 and 6) did not contain these structures. Similarly, when T cell lysates prepared in the presence or absence of 2-mercaptoethanol were separated on SDS polyacrylamide gels, transferred to nitrocellulose, and probed with TIA-2, these same 32 kd and 16 kd structures were specifically recognized (Fig. 2). Taken together, these results show that TIA-2 binds to a 32 kd disulfide linked homodimeric structure, the same structure as that of the zeta chain.

Use

As the monoclonal antibody to the zeta chain recognizes an internal epitope and not a surface structure, it will bind to antigen in both its native and denatured state and consequently can recognize its antigen in tissue sections that have been fixed, e.g., in formaldehyde, or glularaldehyde, or in Western blot analysis. TIA-2 is a highly sensitive probe for T cells in fixed tissues; therefore, TIA-2 can be used to monitor the extent of T cell infiltration into specific tissues.

When T cells are activated, the zeta chain is sloughed off the surface of the T cell or disappears into the internal cytoplasm. Consequently, a detection of an increase of zeta chain in a speciman of a biological fluid from a patient, e.g., serum or urine, or a decrease in surface zeta chain in circulating peripheral blood T lymphocytes would indicate T cell activation, e.g., in a patient with an autoimmune disease. In an allograft recipient, the detection of T cell activation could indicate imminent graft rejection.

Other embodiments are within the following claims. For example, lymphocytes other than T cells can be fixed and permeabilized by the method described above and then used to screen a population of monoclonal antibodies prepared against the specific lymphocyte under

analysis. In addition, fixed and permeabilized lymphocytes of any type could be analyzed for selective binding to a monoclonal antibody by an enzyme-linked ELIZA assay instead of by flow cytometry.

Claims

1. A monoclonal antibody of class IgG, which antibody:

selectively binds to permeabilized T cells but not permeabilized B cells; and

selectively binds to permeabilized but not unpermeabilized T cells.

2. The monoclonal antibody of claim 1 which is produced by a hybridoma formed by fusion of cells from a mammalian myeloma line and spleen cells from a mammal previously immunized with permeabilized human T cells.

3. The monoclonal antibody of claim 2 wherein said mammal is mouse.

4. The monoclonal antibody of claim 1 which is produced by a hybridoma formed by fusion of NS-1 myeloma cells and spleen cells from a Balb/c mouse previously immunized with permeabilized human T cells from Ficoll purified peripheral blood mononuclear cells.

5. The monoclonal antibody of claim 1, which antibody further binds to a T cell restricted 32 kd homodimer.

6. The monoclonal antibody of claim 1, which antibody further binds to the zeta chain of the T cell receptor complex.

7. The monoclonal antibody TIA-2.

8. A method of screening a population of monoclonal antibodies for a monoclonal antibody that selectively binds to a permeabilized but not an

unpermeabilized lymphocyte, said method comprising the steps of:

stabilizing a population of lymphocytes by contacting said cells with a mild fixative;

permeabilizing said lymphocytes by contact with a mild glycosidic detergent;

contacting said population of monoclonal antibodies with said stabilized and permeabilized lymphocytes;

contacting said population of monoclonal antibodies with unpermeabilized lymphocytes; and

selecting a monoclonal antibody that binds to said permeabilized lymphocytes but not to said non-permeabilized lymphocytes.

9. The screening method of claim 8 wherein said lymphocyte is a T cell lymphocyte.

10. The screening method of claim 8 wherein said fixative is an aqueous solution containing 0.005 - 0.1% formaldehyde at 0-5°C and said mild glycosidic detergent is an aqueous solution containing to 100 μg/ml digitonin at 0-5°C.

11. The screening method of claim 8 wherein said fixative is an aqueous solution containing 0.01% formaldehyde at 0-5°C and said mild glycosidic detergent is an aqueous solution containing 10μg/ml digitonin at 0-5°C.

12. A method of determining the state of T cell activation of a patient, said method comprising

contacting serum, urine, or circulating peripheral blood cells from said patient with the monoclonal antibody of claim 6 or claim 7; and

measuring the extent of binding of said antibody with zeta chain in said serum or urine, or on said circulating peripheral blood cells.

13. A method of determining the extent of T cell infiltration into a specific tissue of a patient, said method comprising

contacting a fixed tissue section from said specific tissue of said patient with the monoclonal antibody of claim 6 or claim 7; and

measuring the extent of binding of said antibody with zeta chain in said fixed tissue section.

FIG. 1

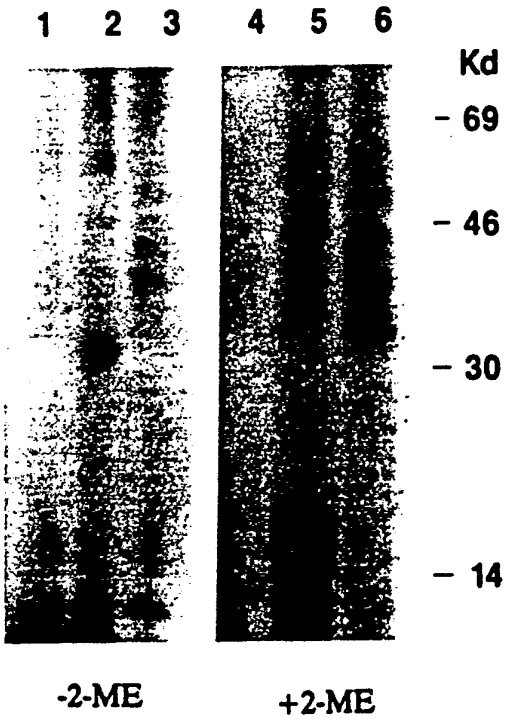
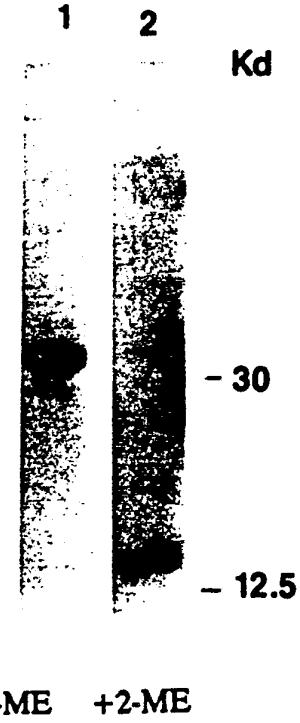


FIG. 2



INTERNATIONAL SEARCH REPORT

International Application No PCT/US90/03403

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5): C07K 15/28; G01N 33/535; C12P 21/08
U.S.: 530/387,388; 435/7, 70.21

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
US	530/387,388; 435/7, 240.27, 70.21

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

Databases: CAS; Biosis; APS. For: monoclonal antibody, zeta, T cell permeabilized, receptor, complex.

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
X Y	The Journal of Immunology, vol. 134, no. 2, issued February 1985, "Co-expression of an epitope on human free k-light chains and on a cytoplasmic component in activated T cells", (WALKER et al.) pp.1059-1064, see pp. 1059, 1060.	1-4 8-11
Y	Proceedings of the National Academy of Sciences USA vol. 85, issued December 1988, "Molecular cloning and chiomosoman localization of the human T-cell receptor zeta chain: distinction from the molecular CD3 complex" (WEISSMAN et al) pp. 9709-9713 see p. 9710	1-13
Y	Cell, vol. 52, issued 15 January 1988, "Failure to synthesize the T cell CD3 - zeta chain: structure and function of a partial T cell receptor complex" (SUSSMAN et al.), pp 85-95, see p. 93	1-13
Y	Proceedings of the National Academy of Sciences USA, vol. 77, no. 6 issued June 1980, "The cytoskeleton of digitonin-treated rat hepatocytes" (FISKUM et al) pp 3430-3434, see entire document	8-11
Y	Nature, vol. 324, issued 04 December 1986, "A new subunit of the human T cell antigen receptor complex"	1-13

* Special categories of cited documents: [15]

"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

"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.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
17 July 1990	09 OCT 1990
International Searching Authority [1]	Signature of Authorized Officer [20]
ISA/US	Paula Hutzell

Form PCT/ISA/210 (second sheet) (May 1986)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

	(WEISSMAN et al) pp 480-482, see entire document	
Y	WEIR, D.M. "Handbook of experimental immunology," volume 4, published 1986 by Blackwell Scientific Publications (OXFORD) see pages 108.1-108.9	1-13
Y	New England Journal of Medicine, vol. 319, no. 18, issued 03 November 1988, "Familial defect in the surface expression of the T cell receptor. CD3 complex", (ALARCON et al) pp. 1203-1208, see pp. 1203, 1207-8.	12, 13

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

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

1. ☐ Claim numbers . because they relate to subject matter [1] not required to be searched by this Authority, namely:

2. ☐ Claim numbers..... , 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 [1], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

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 of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ 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 claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.