PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12N 5/08

A1

(11) International Publication Number: WO 90/13629

(43) International Publication Date: 15 November 1990 (15.11.90)

(21) International Application Number: PCT/US90/02649

(22) International Filing Date: 9 May 1990 (09.05.90)

(30) Priority data:
350,392 11 May 1989 (11.05.89) US

(71) Applicant: THE PUBLIC HEALTH RESEARCH INSTITUTE OF THE CITY OF NEW YORK, INC. [US/US]; 455 First Avenue, New York, NY 10016 (US).

(72) Inventors: BUSHKIN, Yuri ; 2600 Netherland Avenue, 922 Riverdale, NY 10463 (US). DEMARIA, Sandra ; Via Breglio, 140, I-10147 Turin (IT).

(74) Agents: RYAN, M., Andrea et al.; White & Case, 1155 Avenue of the Americas, New York, NY 10036 (US).

(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GB (European patent), HU, IT (European patent), JP, KR, LU (European patent), NL (European patent), NO, SE (European patent), SU.

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: A METHOD FOR THE ACTIVATION OF T CELLS

(57) Abstract

This invention relates to a method for activating CD8-positive T cells. The activation is achieved by forming an association between two T cell surface molecules such as CD8 and HLA-A, HLA-B, or HLA-C.

+ See back of page

DESIGNATIONS OF "DE"

Until further notice, any designation of "DE" in any international application whose international filing date is prior to October 3, 1990, shall have effect in the territory of the Federal Republic of Germany with the exception of the territory of the former German Democratic Republic.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

Code	State	Code	State	Code	State
AT	Austria	ES	Spain	MC	Monaco
AU	Australia	FI	Finland	MG	Madagascar
BB	Barbados	FR	France	ML	Mali
BE	Belgium	GA	Gabon	MR	Mauritania
BF	Burkina Fasso	GB	United Kingdom	MW	Malawi
BG	Bulgaria	GR	Greece	NL	Netherlands
BJ	Benin	HU	Hungary	NO	Norway
BR	Brazil	IT	Italy	RO	Romania
CA	Canada	JP	Japan	SD	Sudan
CF	Central African Republic	KP	Democratic People's Republic of Korea	SE	Sweden
CG	Congo			SN	Senegal
CH	Switzerland	KR	Republic of Korea	SU	Soviet Union
CM	Cameroon	LI	Liechtenstein	TD	Chad
DE	Germany, Federal Republic of	LK	Sri Lanka	TG	Togo
DK	Denmark	LU	Luxembourg	US	United States of America

DESCRIPTION

5 A METHOD FOR THE ACTIVATION OF T CELLS

BACKGROUND OF THE INVENTION

10 This work was partially supported by a grant from the National Institute of Health.

This invention relates to a method for activating human T cells. The activation of T cells is believed to be of therapeutic importance in treating and 15 fighting diseases such as cancer, viral infections, and autoimmune disorders.

Immunity in vertebrates including man is the result of many complex cellular interactions. Although a variety of cells and humoral factors play a role in 20 protecting an individual against foreign antigens, lymphocytes are the primary cells involved in generating an immune response.

There are two principal classes of lymphocytes. One major class are the B cells which interact with soluble 25 antigens, and produce and secrete specific antibodies.

The other class, T cells, consists of several subpopulations which regulate the immune response by direct cell-cell interactions and by producing and releasing in the blood a variety of lymphokines.

30 In mammals, B cells differentiate from hematopoietic stem cells, probably in the bone marrow. T cells differentiate from hematopoietic stem cells which have migrated from the bone marrow to the thymus. There are several steps in the maturation of T cells. In the thymus,

differentiating T cells are known as thymocytes and express unique cell surface protein "markers" which are characteristic for every differentiation step, Reinherz and Schlossman, "The Differentiation and Function of Human T Lymphocytes", Cell, 19:821-827 (1980).

Mature T cells leave the thymus as long-lived small lymphocytes which circulate in the blood stream, lymphatic system and intercellular spaces. Such T cells have immunologic specificity, they can recognize and react with antigens and also distinguish between "self" and "non-self" during cell-mediated immune response (e.g., graft rejection). These T cells express high levels of the cell surface marker T cell receptor (TCR) and are segregated into two major subclasses of CD4-positive and CD8-positive cell populations. When T cells encounter an antigen which is recognized by the T cell receptor on their surface, they are stimulated to divide and proliferate so that many more T cells of the same antigen specificity are produced. This process of T-cell activation enables the activated T cells to perform different tasks.

Several subclasses of mature T cells have been recognized based on the different tasks they perform. Helper cells (T_h) are required for promoting or enhancing B-cell antibody production. Cytotoxic/effector or killer cells (T_k) directly kill their target cells, usually virally infected, malignant or otherwise altered cells. Suppressor cells (T_s) suppress or down-regulate immunological reactions. Most of the T_h cells express the CD4 antigen whereas the T_k and T_s cells express the CD8 antigen, Swain, "T-cell Subsets and the Recognition of MHC Class", Immunol. Rev., 7:129 (1983).

T cells respond specifically to the antigen on the surface of target cells which is associated with major histocompatibility complex (MHC) antigens. MHC antigens are

expressed on most human cells. MHC is divided into three classes (I-III) of antigens, Steinmetz et al., "Genes of the Major Histocompatibility Complex in Mouse and Man", Science, 222:727 (1983). MHC class I antigens include human
5 leukocyte antigens (HLA-A, -B and -C). Recent reports suggest that on many cell types studied, there is a physical association between MHC antigens and other cell surface proteins with receptorial activities. The review article entitled, "Function by Association?", M. Edidin, Immunology
10 Today, 9:218-219 (1988) concludes that although there may be spatial associations between MHC Class I antigens and other T cell surface proteins, the function of these associations remains unknown.

The majority of human cytotoxic and suppressor
15 T cells express the cell surface protein CD8. T cells expressing CD8 are herein designated CD8 cells. CD8 is thought to be involved in the process of T-cell recognition and activation, serving as an important element in MHC class I-restricted immune response. For review see, Engleman
20 et al., "Activation of Human T Lymphocyte Subsets: Helper and Suppressor/Cytotoxic T cells Recognize and Respond to Distinct Histocompatibility Antigens", J. Immunol., 127:2124 (1981); Meuer et al., "Clonal Analysis of Human Cytolytic T Lymphocytes: $T4^+$ and $T8^+$ Effector T Cells Recognize Products
25 of Different Major Histocompatibility Complex Regions", Proc. Natl. Acad. Sci. USA, 79:4395 (1982); Swain, "T-cell Subsets and the Recognition of MHC Class", Immunol. Rev., 74:129 (1983). T cells are known to be activated when CD8 interacts on the cell surface with the T cell receptor
30 complex, Emmrich et al., "Synergism in the Activation of Human CD8 T cells by Cross-linking the T-cell Receptor Complex With the CD8 Differentiation Antigen", Proc. Natl. Acad. Sci. USA, 83:8298 (1986). Nevertheless, the precise

role of CD8 in T-cell recognition and activation remains unclear.

Recently, CD8 and the MHC Class I heavy chain antigens have been found to be noncovalently associated on the surface of antigen- or mitogen-activated human T cells but the functional role of this molecular complex was not determined, Bushkin et al., "Physical Association Between CD8 and HLA Class I Molecules on the Surface of Activated Human T Lymphocytes", Proc. Nat'l. Acad. Sci. USA, 85:3985-3989 (1988); and Blue et al., "Evidence for Specific Association Between Class I Major Histocompatability Antigens and the CD8 Molecules on Human Suppressor/Cytoxic Cells", 54:413-421 (1988).

SUMMARY OF THE INVENTION

It has now been found that the cross-linking of at least two molecules on the surface of a mature human T cell results in activation of this T cell and subsequent vigorous proliferation in the presence of interleukin-2.

BRIEF DESCRIPTION OF THE DRAWING

Figure 1 shows the kinetics of proliferative responses of CD8-MHC class I cross-linked CD8 cells.

DETAILED DESCRIPTION OF THE INVENTION

This invention is based on the finding that noncovalent complexes of CD8 and MHC class I antigens are found on activated human cytotoxic/suppressor T cells.

Accordingly, it has been found that noncovalent cross-linking of CD8 and MHC class I antigens on the surface of resting CD8 cells can activate these T cells and stimulate their proliferation in response to for example interleukin-2 (IL-2).

Cross-linking of T cell surface molecules can be accomplished by any known means of associating molecules of this type. For example, the use of primary antibodies or parts thereof which bind to T cell surface proteins and subsequent cross-linking of the primary antibodies with secondary antibodies serves to noncovalently associate T cell surface proteins. The same results could be achieved by the use of hetero-conjugate (hybrid) antibodies. Hybrid antibodies can be generated by chemical cross-linking of anti-CD8 and anti-MHC class I antibodies, or by "hybrid" hybridomas obtained by the fusion of parental hybridomas secreting anti-CD8 and anti-MHC class I antibodies.

In a preferred embodiment of this invention, CD8 and MHC class I antigens on the same activated CD8 cell are noncovalently associated. However, it is believed that any means of covalent association such as that achieved by chemical cross-linking could also be used.

Preferably cross-linking of CD8 and MHC class I antigens on a CD8 cell is accomplished by a two step method. The first step is to incubate a CD8 cell sample with at least one of the monoclonal antibodies (mAb) specific for CD8 (C8, OKT8A and anti-Leu-2a) and with at least one of the mAb specific for MHC class I antigens (PA2.6, BB7.7 and A1.4). The first set, or primary, mAb are allowed to bind to their respective antigens (CD8 and MHC class I) and then the excess, unbound, mAb is washed away from the cells. In the second step, the mAbs bound to the CD8 and MHC class I antigens on the cell surface are brought into close proximity to cause a noncovalent association of CD8 and MHC class I antigens. This is accomplished by addition of a secondary antibody to the cells coated with primary mAb. A suitable secondary antibody is a polyclonal affinity-purified goat anti-mouse IgG (GaMIg) antibody which recognizes and binds to the unbound Fc-portion of the

primary mAb. The noncovalent association between CD8 and MHC class I antigens approximated by anti-CD8, anti-MHC class I and secondary antibodies is due to some degree of structural homology between CD8 and MHC class I antigens, Littman et al., "The Isolation and Sequence of the Gene Encoding T8: a Molecule Defining Functional Classes of T Lymphocytes", Cell, 40:237-246 (1985); and Anderson et al., "Regulatory Interactions Between Members of the Immunoglobulin Superfamily", Immunol. Today, 9:199-203 (1988).

Cross-linking of CD8 and MHC class I antigens as described above results in a marked proliferation of resting human CD8 cells in the presence of IL-2. To a lesser extent, CD8 cell activation and proliferation can also be induced by cross-linking of MHC class I antigens to themselves (Fig. 1 and Table I). Activation of CD8 cells by cross-linking of CD8 and MHC class I antigens is accompanied by expression of both IL-2 receptor and the MHC class II (HLA-DR and -DQ) antigens as demonstrated by immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. The presence of these antigens is indicative of the T-cell activation. Activation of CD8 cells by cross-linking is apparently independent of accessory monocytes.

Various anti-CD8 and anti-MHC class I mAb which recognize nonpolymorphic antigenic determinants can be used to activate CD8 cells. Surprisingly, not all mAb induce proliferation to the same extent. Among mAb specific for the MHC class I antigens, PA2.6, BB7.7 and A1.4 mAb together with anti-CD8 mAb, markedly stimulated the proliferation of CD8 cells, W6/32 mAb showed a weak stimulation, whereas BBM.1, Q1/28 and HC10 mAbs failed to induce proliferation (Table II). In contrast to the anti-MHC class I mAb, all anti-CD8 mAb examined (C8, OKT8A and anti-Leu-2a) induced

the proliferation of CD8-MHC class I cross-linked cells with similar efficacy. These results suggest that physical interaction between CD8 and at least one specific determinant of MHC class I antigens can trigger the activation of resting human CD8 cells.

It is believed that CD8 cells activated in such a way become functionally competent cytotoxic and/or suppressor T cells. This novel method of T-cell activation could therefore be of great importance in generating activated T cells for therapeutic purposes, similar to the use of lymphokine-activated killer and tumor-infiltrating T lymphocytes in cancer therapy, Oehoa et al., "Lymphokine-Activated Killer Activity in Long-Term Cultures with Anti-CD3 Plus Interleukin 2: Identification and Isolation of Effector Subsets", Cancer Research, 49:963-968 (1989); and Rosenberg et al., "Use of Tumor-Infiltrating Lymphocytes and Interleukin-2 in the Immunotherapy of Patients with Metastatic Melanoma", N. Engl. J. Med., 319:1676-1680 (1988). Thus, activated cytotoxic effector T cells generated by cross-linking of CD8 and MHC class I antigens may be used in the treatment of cancer and viral infections in mammals including humans, whereas suppressor T cells activated in this manner may be effective in the treatment of autoimmune diseases.

In addition, the presence of CD8-MHC class I molecular complex on the T cell surface may serve as a marker for certain stages of T-cell activation, and as such, be useful in clinical diagnosis of various disease states and in the follow-up control of various anti-cancer and immunodeficiency treatments.

One obvious advantage of our method is that T cells could be activated both in vitro and in vivo in mammals including humans. However, activation of human T cells in vivo would require some modifications, for

example, hybrid anti-CD8/MHC class I mAb, if used, would need to be produced by human hybridomas rather than by murine hybridomas.

This invention will be better understood by reference to specific examples which are given for purposes of illustration and are not meant to limit the invention.

Example 1

Purification of CD8 Cells

Peripheral blood mononuclear cells were isolated from buffy coats of healthy donors by gradient centrifugation on Ficoll-Hypaque (Isolymph, Gallard-Schlesinger Chemical Mfg. Corp., Carle Place, NY). T lymphocytes were separated from non-T cells by rosetting with neuraminidase (Sigma)-treated sheep red blood cells at 4°C for 1 hr., Kansas et al., "Functional Characterization of Human T Lymphocyte Subsets Distinguished by Monoclonal Anti-Leu8", J. Immunol., 134:2995 (1985). After separation on a Ficoll-Hypaque gradient, rosetted cells were recovered by treatment with Tris-buffered ammonium chloride. Purified CD8 cells were prepared by negative selection using a panning technique as previously described, Wysocki et al., "'Panning' for Lymphocytes: A Method for Cell Selection", Proc. Natl. Acad. Sci. USA, 75:2844 (1978). Briefly, unfractionated T cells were treated with anti-Leu-3a mAb, and then washed and incubated in affinity-purified GaMIg-coated panning dishes at 4°C for 2 hr as described by Engleman et al., "Activation of Human T Lymphocyte Subsets: Helper and Suppressor Cytotoxic Cells Recognize and Respond to Distinct Histocompability Antigens", J. Immunol., 127:424 (1981).

Nonadhered CD8 cells were collected, and contaminating monocytes were depleted by repeated adherence to tissue culture flasks at 37°C. Monocytes were further

depleted by treatment with L243 mAb (IgG2a) at 4°C for 30 min, and then with 10% rabbit complement (Low-Tox-H, Cedarlane Laboratories, Ontario, Canada) at 37°C for 1 hr with frequent agitation. The phenotypic profile of the
5 purified CD8 cells was determined by fluorescence activated cell sorter (FACS) analysis (Ortho System 50H Cytofluorograph, Ortho Diagnostics Systems, Inc., Westwood, MA) and was determined to be as follows: ≥90% $CD8^+$, ≥93% $CD3^+$, <8% $CD4^+$, and <1.5% Leu-$M3^+$ cells. After exhaustive depletion
10 of monocytes with L243 mAb plus complement, the Leu-$M3^+$ cells were reduced to <0.5%, and the CD8 cells thus obtained failed to proliferate when cultured for 3 days in the presence of OKT3 mAb.

15 Example 2

Cell Culture and Proliferation Assay

The purified CD8 cells prepared according to Example 1 were suspended at 10^6 cells/ml in RPMI 1640 medium supplemented with 10% fetal bovine serum, glutamine (0.06
20 mg/ml), streptomycin (0.1 mg/ml) and penicillin (100 U/ml) (hereafter, culture medium) and were incubated with 10-15 μg/ml of various mAb for 1 hr at room temperature.

Various anti-CD8 and anti-MHC class I antigen mAb were used. OKT8A (IgG2a, Ortho Diagnostics, Raritan,
25 NJ), anti-Leu-2a (IgG1, Becton Dickinson, Mt. View, CA) and C8 (IgG2a, Dr. C.Y. Wang, United Biomedical, Lake Success, NY) are specific for human CD8. W6/32 (IgG2a), PA2.6 (IgG1), BB7.7 (IgG2b) (American Type Culture Collection (ATCC), Rockville, MD), A1.4 (IgG1), HC10 (IgG2a, Dr. N.J.
30 Stam, the Netherlands Cancer Institute, the Netherlands) and Q1/28 (IgG2a, Dr. C. Russo, Cornell University Medical College, New York, NY) are directed against the MHC class I HLA-A, -B and -C antigens. BBM.1 (ATCC) is an IgG2b mAb recognizing β_2-microglobulin (β_2m). All anti-CD8 and anti-

MHC class I antigen mAb were equilibrated with respect to the concentration of mouse immunoglobulin.

After precoating with mAb, cells were washed twice and seeded in flat-bottomed 96-well microtiter plates at $5x10^4$ or $1x10^5$ cells/ well in 200 μl culture medium. Unless noted otherwise, affinity-purified GaMIg (Organon Teknika Corp., West Chester, PA) (2.5 μg/ml) and/or recombinant human IL-2 (Boehringer Mannheim, W. Germany) (10 U/ml) were added to the cell cultures. The cells were cultured at 37°C for 5 days, and pulsed with 1 μCi/well of $[^3H]$thymidine ($[^3H]$TdR), (6.7 Ci/mmol; New England Nuclear Corp., Boston, MA) 16 hr prior to harvest. Results were expressed in counts per minute (cpm) as mean $[^3H]$TdR uptake of triplicate determinations ±SD.

Example 3

Cross-linking of CD8 and MHC Class I Antigens

In order to induce the proliferation of CD8 cells in response to IL-2, CD8 and MHC class I molecules on the surface of CD8 cells were cross-linked. This was done by first incubating CD8 cells prepared according to Example 1 with anti-CD8 and/or anti-MHC class I mAb, and then reacting with GaMIg as described in Example 2.

As seen in Table I, CD8-MHC class I cross-linked cells markedly proliferated in response to IL-2 as determined by $[^3H]$TdR uptake. Both GaMIg and IL-2 are essential for the proliferation of CD8 cells. The effect of IL-2 on CD8 cell proliferation is dose-dependent: an increase in IL-2 concentration from 10 U/ml to 100 U/ml resulted in an up to 8-fold increase in cell proliferation. Likewise, the proliferation of CD8 cells also depends on the concentration of GaMIg added, with as little as 0.25 μg/ml of GaMIg inducing a 3-fold increase in T cell proliferation. As was shown for CD4 cells, Geppert et al., "Activation of

Human T Cells by Cross-linking Class I MHC Molecules", J. Immunol. 140:2155 (1988), cross-linking of MHC class I antigens alone by A1.4 mAb and GaMIg resulted in a moderate proliferation of CD8 cells, which was significantly lower than that of CD8-MHC class I cross-linked CD8 cells. In contrast, cross-linking of CD8 molecules alone using C8 mAb and GaMIg failed to induce significant proliferation (Table I and Figure 1).

In Table I, uncoated CD8 cells or cells precoated with mAb C8, A1.4 or C8 and A1.4 in combination, were cultured at 5×10^4 cells/well in culture medium in 96-well plates for 5 days in the presence or absence of GaMIg and/or IL-2 as described in Example 2.

TABLE I

Stimulation of CD8 Cell Proliferation by Cross-Linking of CD8 and MHC Class I Antigens

Addition of		[^{3}H]TdR uptake (mean cpm ±SD) of CD8 cells coated with			
GaMIg	IL-2	none	C8	A1.4	C8+A1.4
–	–	170±85	183±75	144±37	122±12
–	+	227±105	ND	ND	167±8
+	–	201±53	ND	ND	111±21
+	+	309±50	623±119	2136±184	7861±133

The CD8 cell population vigorously depleted of monocytes by treatment with L243 mAb and complement, also proliferated after cross-linking of CD8 and MHC class I

antigens, suggesting that the activation of CD8 cells is independent of accessory cells. It was noted, however, that addition of a small quantity of autologous monocytes to these cells dramatically increased their proliferative response, perhaps due to an IL-2-mediated, monocyte-dependent T cell proliferation.

Kinetic studies, shown in Figure 1, demonstrated that cross-linking of CD8 and MHC class I antigens induced a marked proliferative response of CD8 cells as early as day 3, and reached a plateau on day 5. Cross-linking of MHC class I antigens alone showed similar kinetics with a less marked proliferative response. Cross-linking of CD8 alone failed to stimulate the proliferation of CD8 cells.

In Figure 1, CD8 cells were first incubated in culture medium alone (+), with OKT3 (open triangle), C8 (open square), A1.4 (black triangle) or a mixture of C8 and A1.4 mAb (black square), then washed and cultured in the presence of GaMIg and IL-2 as described in Example 2. IL-2 was omitted in CD8 cells cross-linked with OKT3 mAb.

Example 4

Expression of IL-2R and MHC Class II Antigens by Activated T Cells

It is known that T cells activated by antigen or mitogen express IL-2 receptor (IL-2R) and MHC class II antigens, Cotner et al., "Simultaneous Flow Cytometric Analysis of Human T-cell Activation Expression Antigen Expression and DNA Content", J. Exp. Med., 157:461 (1983); and Robbins et al., "Activated T Cells and Monocytes Have Characteristic Patterns of Class II Antigen Expression", J. Immunol., 141:1281 (1988). To determine whether cross-linking of CD8 and MHC class I antigens also induces the expression of these activation markers, CD8-MHC class I cross-linked CD8 cells and control, untreated CD8 cells were cultured in the presence or absence of GaMIg and/or IL-2 for 5 days as

described in Example 2. The cells were then radioiodinated, and the cell lysates were reacted with anti-Tac (anti-IL-2R), and 1D1 and anti-Leu-10 (anti-MHC class II) mAb followed by SDS-PAGE analysis. Radioiodination,
5 immunoprecipitation and SDS-PAGE procedures are described in more detail as follows.

Aliquots of 10^7 cells were radioiodinated by the lactoperoxidase method according to the manufacturer's instructions with 2 mCi of [^{125}I] (100 mCi/ml; Amersham
10 Corp., Arlington Heights, IL). Cells were then lysed with 2 ml of isotonic phosphate-buffered saline, pH 8.3/0.5% Nonidet P-40/1 mM phenylmethylsulfonyl flouride/10 mM iodoacetamide according to Bushkin et al., "A New HLA-linked T Cell Membrane Molecule, Related to the β Chain of the
15 Clonotypic Receptor, is Associated With T3", J. Exp. Med., 164:458 (1986). After depletion of free iodide by passage through a 0.2 ml column of AG1X8Cl$^-$ (Bio-Rad, Richmond, CA), the lysates were absorbed twice with 300 μl of goat anti-mouse IgG-agarose beads (Sigma) alone and immunoprecipitated
20 with 60 μl of goat anti-mouse IgG-agarose beads coated with specific mAb. The materials were eluted from the beads with Laemmli sample buffer, as previously described, Laemmli, "Cleavage of Structural Proteins During the Assembly of the Head of Bacteriophage T4", Nature, 227:680 (1970). The
25 immunoprecipitated proteins were then analyzed by SDS-PAGE on vertical slab gels of 13% acrylamide under reducing conditions.

Results of this experiment showed that only CD8-MHC class I cross-linked CD8 cells cultured in the
30 presence of IL-2 were induced to express IL-2R, and the MHC class II HLA-DR and HLA-DQ antigens. These activation markers were not expressed in control CD8 cells, CD8 cells cultured with IL-2 alone, or CD8-MHC class I cross-linked CD8 cells cultured without IL-2.

Example 5

Effects of Different Anti-MHC Class I mAb on T Cell Activation

As shown in Table II, there is a marked variation in the ability of different anti-MHC class I mAb to stimulate the proliferation of CD8-MHC class I cross-linked cells. In this Table, CD8 cells precoated with mAb C8 and/or various mAb specific for MHC class I antigen, were cultured at $5x10^4$ or $1x10^5$ per well in culture medium in 96-well plates for 5 days in the presence of GaMIg and IL-2 as described in Example 2. PA2.6, BB7.7 and A1.4 mAb induced potent proliferation of CD8-MHC class I cross-linked CD8 cells. W6/32 mAb exerted a weak stimulatory effect. On the other hand, BBM.1, HC10 and Q1/28 mAb did not stimulate the cells. It is unlikely that the difference in the ability of various anti-MHC class I mAb to stimulate the proliferation of cells was due to different binding affinity of the antibodies, as all the anti-MHC class I mAb were found to precipitate similar amount of MHC class I antigen from radioiodinated cell lysates. The ability of PA2.6, BB7.7 and A1.4 mAb to stimulate cross-linked CD8 cells is possibly related to the antigenic region (α2 domain of MHC class I antigen) these mAb recognize. Stimulation of cross-linked CD8 cell proliferation appears to be a highly specific process, which requires direct participation of CD8 protein and at least one epitope of MHC class I antigen which is accessible upon binding of PA2.6, BB7.7 and A1.4 mAb. Therefore, it is likely that other mAb which recognize the MHC class I α2 domain could work in conjunction with anti-CD8 mAb to activate CD8 cells.

TABLE II

Relevance of nonpolymorphic epitopes of MHC class I antigen to the activation of cross-linked CD8 cells

Cells/well	C8	[^{3}H]thymidine uptake (mean cpm $\pm$SD) of CD8 cells cross-linked with							
		none	BBM.1	HC10	Q1/28	W6/32	A1.4	PA2.6	BB7.7
$5x10^4$	-	122±2	260±48	ND	ND	460±11	724±45	2204±136	ND
	+	167±8	295±36	ND	ND	753±109	2483±74	5845±21	ND
$1x10^5$	-	332±50	510±47	355±63	372±65	576±12	1262±14	1905±183	1206+89
	+	658±53	652±53	385±44	463±52	1002±137	2453±161	4070±159	2540±146

CLAIM:

5

1. A method of activating mammalian T cells comprising forming an association between at least two of the molecules located on the surface of the T cell.

2. The method according to claim 1 wherein the molecules are selected from the group consisting of CD8, HLA-A, HLA-B and HLA-C.

10

3. A method of activating mammalian T cells comprising cross-linking T cell surface molecules using a primary antibody or parts thereof which recognizes T cells or parts thereof and subsequently noncovalently cross-linking the primary antibody attached to the T cell or parts thereof using a secondary antibody which recognizes and binds to the primary antibodies.

15

4. The method according to claim 3, wherein the T cell surface molecules are selected from the group consisting of CD8, HLA-A, HLA-B and HLA-C and the primary antibodies to T cells or parts thereof, are anti-CD8 antibody and anti-MHC class I antibody, and the secondary antibodies to the primary antibodies attached to the T cell surface molecules are antibodies to some region held in common by the primary antibodies.

20

25

5. The method according to claim 4 wherein the primary antibodies to T cells or parts thereof are mouse anti-human CD8 antibody and mouse anti-human MHC class I antibody, and the secondary antibody to these primary antibodies is a goat antibody recognizing the Fc region of mouse antibody.

30

6. The method according to claim 5 wherein the primary antibodies are monoclonal antibodies, whereas the secondary antibody is a polyclonal antibody.

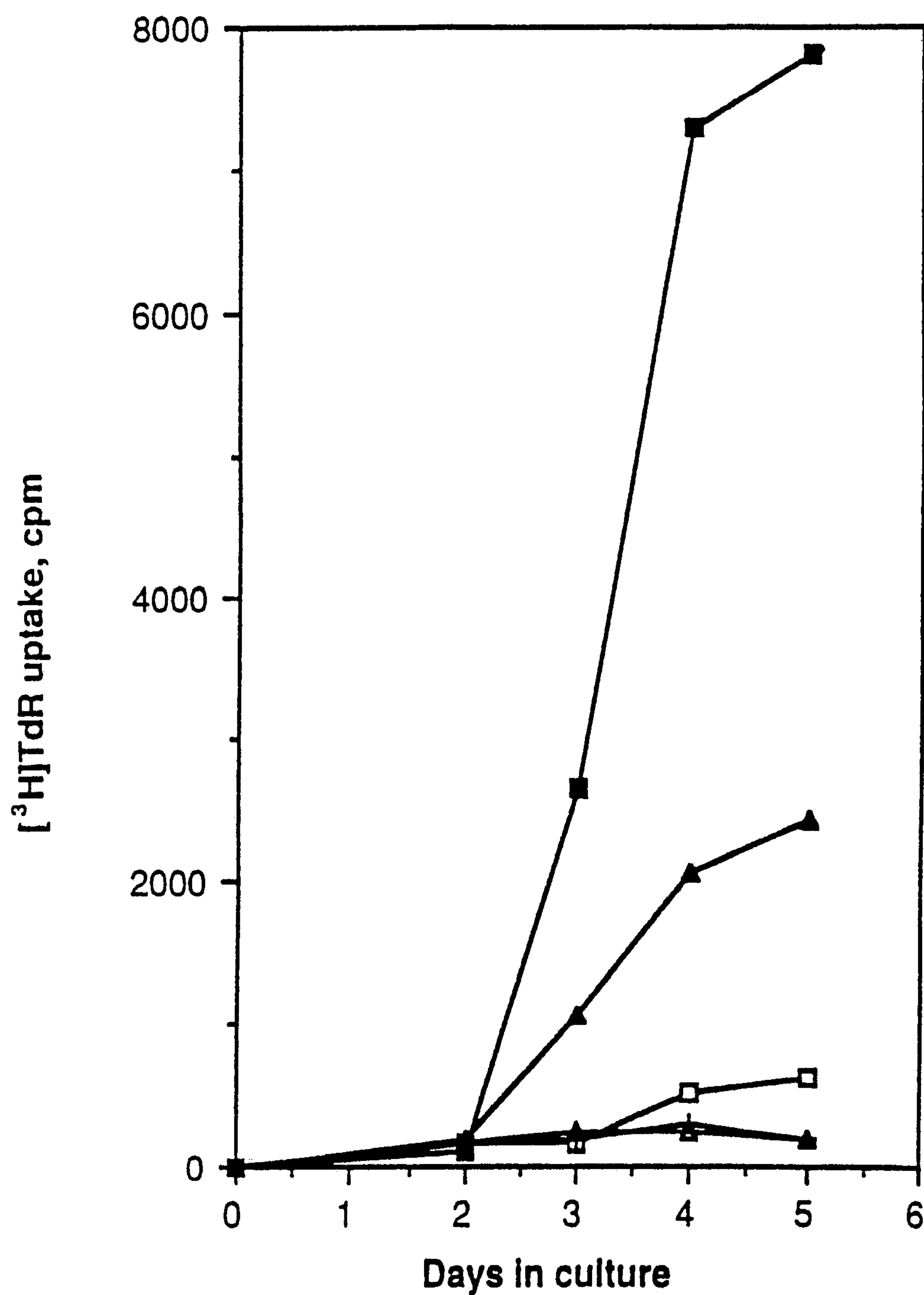
35

7. The method according to claim 6 wherein the monoclonal antibodies are selected from the group consisting of PA2.6, BB7.7, A1.4, C8, OKT8A and anti-Leu-2a.

8. An activated mammalian T cell wherein at least two of the cell surface molecules selected from the group consisting of CD8, HLA-A, HLA-B and HLA-C are cross-linked.

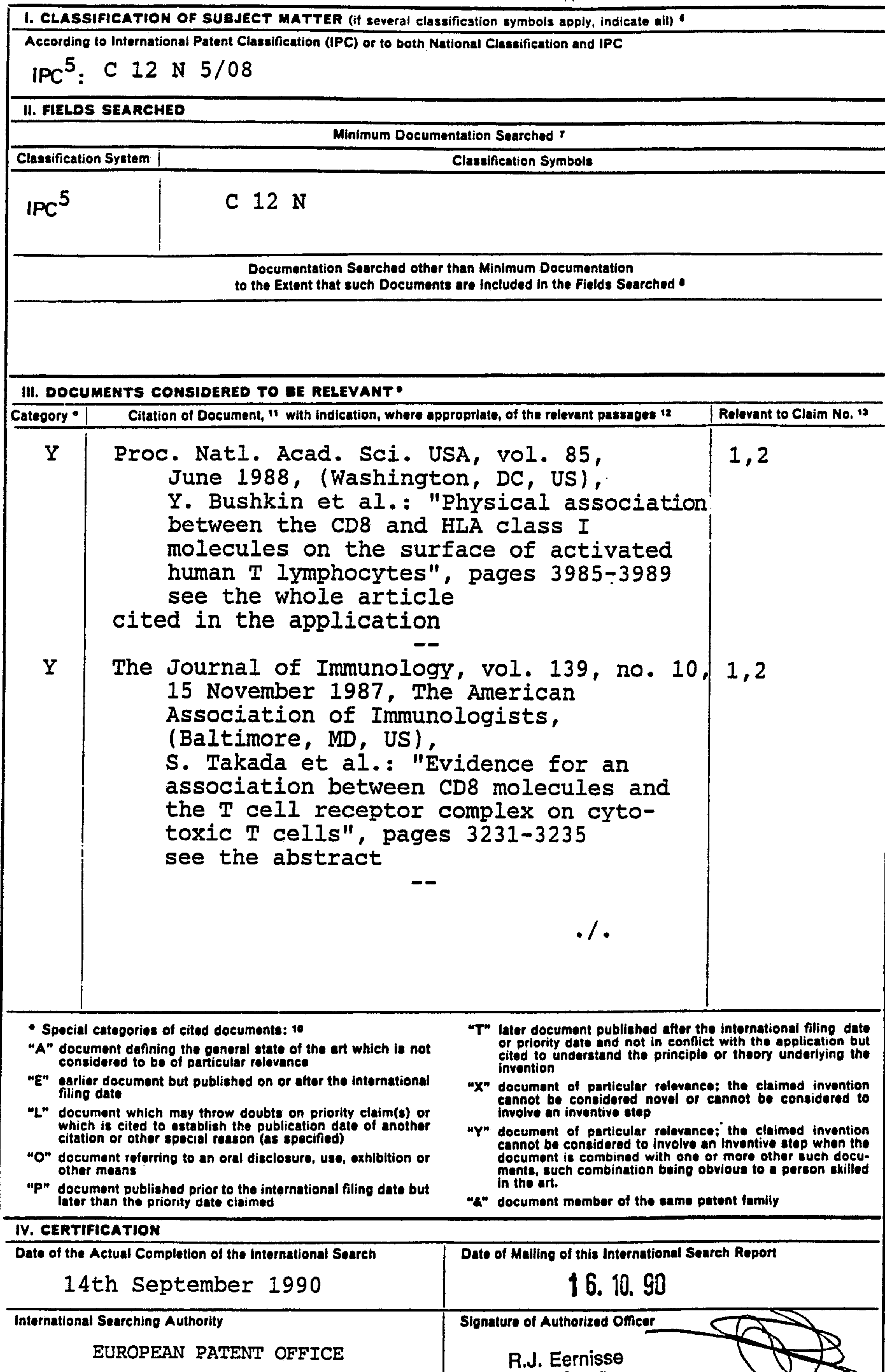
9. An activated mammalian T cell according to claim 8 wherein the molecules are cross-linked using a primary antibody or parts thereof which recognizes T cells or parts thereof and subsequently noncovalently cross-linking the primary antibody attached to the T cell or parts thereof using a secondary antibody which recognizes and binds to the primary antibodies.

FIG. 1



INTERNATIONAL SEARCH REPORT

International Application No PCT/US 90/02649



I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

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

IPC^5: C 12 N 5/08

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC^5	C 12 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	Proc. Natl. Acad. Sci. USA, vol. 85, June 1988, (Washington, DC, US), Y. Bushkin et al.: "Physical association between the CD8 and HLA class I molecules on the surface of activated human T lymphocytes", pages 3985-3989 see the whole article cited in the application --	1,2
Y	The Journal of Immunology, vol. 139, no. 10, 15 November 1987, The American Association of Immunologists, (Baltimore, MD, US), S. Takada et al.: "Evidence for an association between CD8 molecules and the T cell receptor complex on cyto-toxic T cells", pages 3231-3235 see the abstract --	1,2

./.

* Special categories of cited documents: [10]

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

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

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

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"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	Date of Mailing of this International Search Report
14th September 1990	16. 10. 90
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	R.J. Eernisse

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages	Relevant to Claim No.
P,X	The Journal of Immunology, vol. 142, no. 12, 15 June 1989, The American Association of Immunologists, (Baltimore, MD, US), M.C. Wacholtz et al.: "Patterns of costimulation of T cell clones by cross-linking CD3, CD4/CD8, and class I MHC molecules", pages 4201-4212 see the whole article --	1-9
P,X	The Journal of Immunology, vol. 142, no. 11, 1st June 1989, The American Association of Immunologists, (Baltimore, MD, US), T.D. Geppert et al.: "Activation of human T cell clones and Jurkat cells by cross-linking class I MHC molecules", pages 3763-3772 see the abstract; table III ----------	1-3,8,9