DISTRIBUTION OF INTERLEUKIN-2 RECEPTORS IN ACTIVATED T-LYMPHOCYTE SUBSETS IN VITRO

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SYNOPSIS

Following activation in vitro, peripheral blood mononuclear cells (PBMC) express cell-associated interleukin-2 receptors (IL-2R). The present study was undertaken to define the proportion of B and T lymphocyte subsets that express the IL-2R (CD25 antigen) upon pokeweed mitogen stimulation. Double immunofluorescence staining with different fluorochromes, fluorescein isothiocyanate and physoethyrin, was applied for identification of IL-2R positive cells and individual lymphocyte subset. The exact percentage of individual activated lymphocyte subet bearing IL-2R was enumerated by photographic counting. There was paucity of IL-2R in freshly isolated, unstimulated peripheral blood, PBMC cultured without mitogen, and cultured B lymphocytes. Following pokeweed mitogen stimulation in vitro, twenty percent of cultivated CD4 (T-helper) lymphocytes and fifteen percent of activated CD8 (T-suppressor/cytotoxic) lymphocytes expressed IL-2R. Contrary to the reported data of Tac-positive cells in human lymphoid tissues, our study revealed that, upon pokeweed mitogen stimulation, approximately 55 percent of IL-2R positive PBMC were CD4 lymphocytes, and 45 percent of them were CD8 lymphocytes. These observations imply the plausible notion that interleukin-2 mediated immune activation of T lymphocytes in PBMC is different from that in local lymphoid organs.

Key Words: T lymphocytes, Interleukin-2 receptors, peripheral blood, lymphocyte culture.

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INTRODUCTION

Recent studies have revealed the multiple biological effect of the lymphokine termed interleukin-2 (IL-2).[1] Following cellular activation, newly synthesized receptors for IL-2 are expressed on the surface of T cells[1, 2] and B cells.[3] The interaction of interleukin-2 receptors (IL-2R) and soluble IL-2 leads to the proliferation and further differentiation of these lymphocytes.[1, 4] The human lymphocytic receptor for IL-2 plays a critical role in the growth of T cells and is required for full expression of the normal immune response.[5] Although the molecular biology of interleukin-2 receptor and its expression in normal T lymphocytes have been recently investigated,[5, 6] the expression of IL-2R in individual T lymphocyte subset has been fully studied. The present study was undertaken to find out the percentage of human T-helper and T-suppressor/cytotoxic lymphocytes that express IL-2R upon mitogenic stimulation.

Lymphocyte Culture

Peripheral blood mononuclear cells (PBMC) were separated from heparinized whole blood by density gradient centrifugation using Ficoll Hypague. After three washes in HBSS, the cells were resuspended at a concentration of 1 × 10^6cells/ml in complete culture medium (CCM) consisting of RPMI 1640 [Gibco, Chargrin Falls, U.S.A.] supplemented with 10% heat-inactivated fetal calf serum, 2mM, L-glutamine, 100 unit/ml pencillin, 100 ug/ml streptomycin and 2.5 ug/ml amphotericin B. Ten millilitres aliquots of this cell suspension were dispensed into 90 mm culture dishes [Coster,Cambridge, U.S.A.] and cultured in quadruplicators without mitogen, and with pokeweed mitogen (PWM) [Sigma Chemicals, St. Louis, U.S.A.] 1 ug/ml. Incubation was at 37°C in humid of 5% CO_2 in air. The dosage of mitogens was determined with a dose-response curve. The cells were incubated for five days before they were centrifuged at 400g. The cells were washed thrice in HBSS, the cell count was adjusted to 6 × 10^7/ml in HBSS containing 2% fetal calf serum, and 100 ul of the lymphocyte suspension was placed in microtubes. The tubes were then incubated for 30 min at 4°C with the anti-leu-2a, anti-leu-3a, anti-leu 12, and anti-IL-2R antibodies as described above. After incubation, the cells were washed thrice with HBSS and resuspended gently with a Pasteur pipette, and one drop examined with a Leitz Orthoplan microscope. Photographs were taken during examinations with different wavelength excitation as mentioned above. Appropriately 300-350 cells per slide were counted by examination of the colour photographs. Lymphoblasted cells with immunofluorescence staining for IL-2R were recorded and expressed as the percentage of individual T lymphocyte subset or B lymphocyte. When duplicate samples of same subject were performed, the results obtained agreed within 5% with preceding values.

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MATERIALS AND METHODS

Seventeen healthy subjects, ten men and seven women with an average age of 32 years (range 21-42 years) were studied. They did not have any systemic infection within four weeks prior to the study. Blood collected from the antecubital vein was anti-coagulated in ethylenediaminetetraacetic aid. Leukocyte count was performed by a Coulter ZF particle counter, and differential count was done manually by examination of 250 leucocytes on Wright's stained blood smears.

Direct immunofluorescence assay for lymphocyte subsets

Ten milliliters of venous heparinized blood were diluted with

equal volume of phosphate buffer saline (PBS) pH 7.4 and layered over a Ficoll-Hypaque [Nyegaard, Oslo, Norway] density gradient. All blood samples were processed for the immunofluorescence assay on the same day of collection. After centrifugation, the interface layer containing lymphocytes was washed thrice in Hank's balanced salt solution (HBSS) [Sigma Chemicals, St. Louis, U.S.A.], and the cell count was adjusted to 1 × 10^6 cells/ml in suspension medium (HBSS containing 2% fetal calf serum). One hundred ul of the lymphocyte suspension was placed in each of five tubes. The tube were then incubated for 30 minutes at 4°C with 10 ul of appropriate dilutions of Phycoerythrin (PE) conjugated anti-leu-2a, or anti-leu-3a murine monoclonal antibodies [Becton-Dickson, Mountain View, CA, U.S.A.]. After incubation with monoclonal T cell reagents, the cells were washed thrice in cold HBSS. The lymphocytes were then incubated for 30 minutes at 4°C with 10 ul of appropriate dilution of Fluorescein conjugated (FITC) anti-IL-2 receptor monoclonal antibody [Becton-Dickson, Mountain View, CA, U.S.A.]. After incubation with the two monoclonal antibodies, the cells were washed thrice in cold HBSS, resuspended in one to two drops of PBS pH 7.4 containing 30% glycerol (vol/vol). The Costained sections were examined by a fluorescopic microscope with epi-ilumination [Leitz, FRG] equipped with filters for narrow-band excitation and barrier filters LP520 for fluorescein and LP575 for phycoerythrin visualization. Alternated immunofluorscence examination was permitted by switching one filter consecutively to the other. In order to minimize the observer variation, photographs were taken during examinations with different wavelength excitation using Ektachrome film 400 ASA. Individual subset of lymphocytes expressing the IL-2R was identified by mononuclear cells demonstrating both orange-red and yellow-green fluorescence. Approximately 300 cells per slide were counted. The results were expressed as the percentage of each T-cells subset or mononuclear cells bearing IL-2R with respect to the total number of mononuclear cells presented in each field. The ratio between CD4 (leu-3a positive) and CD8 (leu-2a positive) T cell percentages was then deduced, and used as the index of the test results in each subject. When duplicate samples of some subjects were performed, the results agreed within 5% with preceding values.

The results are expressed as mean ± standard deviation. The data were analysed with Student's t-test as appropriate.

RESULTS

Absolute lymphocyte count of the 17 healthy subjects ranged from 1.2 to 2.2 × 10^9/L. All smears contained more than 300 lymphocytes for enumeration. Table 1 summarized the lymphocyte subset data in the freshly collected peripheral blood and the peripheral blood mononuclear cells cultured for 5 days. The mean percentages of CD4 (T-helper) and CD8 (T-suppressor/cytotoxic) lymphocytes in peripheral blood were 34.2 and 24.9 percent rspectively. The averaged CD4/8 ratio determined by the leu-3a and leu-2a antibodies was 1.48. An apparently lower CD4/CD8 ratio was obtained from the stimulated lymphocyte cultures but the changes failed to reach statistical significance. There was a good linear correlation between the CD4/8 ratio in the frshly isolated lymphocytes and the cultured lymphocytes activated in vitro ($r = 0.61$, $p < 0.02$).

The distribution of CD25 antigen (IL-2R) in different subsets of lymphocytes is depicted in Table 2. Interleukin-2 receptors were demonstrated on the cell surface in less than five percent of freshly isolated, unstimulated peripheral lymphocytes. There was a similar paucity of IL-2R on the cell surfaces of perpheral blood mononuclear cells cultured with no mitogen. With pokweed mitogen stimulation, there was a significant increase of activated lymphocytes demonstrating IL-2R on their cell surface. With double direct immunoflourescence technique using two monoclonal antibodies, the distribution of IL-2R in different lymphocyte subsets was measured by counting the cultured lymphocytes that expressed both antibodies. Twenty percent of CD4 lymphocytes and 15 percent of CD8 lymphocytes demonstrated cellular activation with expression of IL-2R after culturing with pokeweed mitogen for five days. IL-2R was demonstrated in less than five percent of CD20 (leu-12 positive B) lymphocytes. Serial measurements were performed in six healthy subjects and the percentages of T cell subpopulations expressing IL-2R remained relatively constant.

Table 1
T-LYMPHOCYTE SUBSETS IN PERIPHERAL BLOOD AND LYMPHOCYTE CULTURE AS DEFINED BY DIFFERENT ANTIBODIES
Subsets, % (mean ± SD)[a]

Antibody	Peripheral blood	Lymphocyte culture (PWM-stimulated)
Leu-3a	34.2 ± 5.6	44.8 ± 10.4
Leu-2a	24.9 ± 6.6	37.4 ± 11.9
Leu-3a/ Leu-2a ratio	1.48 ± 0.59	1.30 ± 0.38

a — expressed as a percentage of total cultured mononuclear cells.

Table 3 tabulates the percentage of T lymphocyte subsets expressing IL-2R in individual healthy subjects. The sum of the CD4 and CD8 lymphocytes expressing IL-2R approximately closely with the percentage of cultured mononuclear cells expressing IL-2R measured separatedly ($r = 0.86$, $p < 0.001$). These findings confirmed the paucity of IL-2R in B lymphocytes and, in practice, IL-2R was expressed only on the cell surface of activated T lymphocytes.

DISCUSSION

In the present study, the expression of IL-2R in human circuating lymphocytes was investigated. Previous studies reported zero to 15 percent of freshly isolated, unstimulated human peripheral blood lymphocytes reacted with anti-Tac antibody.[7, 8, 9] The majority of T lymphocytes express IL-2R 24 to 48 hours after activation with antigen.[10] Both purified CD4 and CD8 lymphocytes express CD25 antigen (IL-2R) when activated with mitogen[11, 12] and the quantatative amounts of soluble IL-2R and cellular IL-2R in CD4 and CD8 subsets were reported to be comparable.[11, 12] Evaluation of human lymphoid tissue with anti-Tac showed localization of Tac-positive cells in paracortical and interfollicullar regions of lymph nodes and tonsils. By dual staining, 80 percent of these cells were T-helper and 20 percent T-suppressor/cytotoxic lymphoctes.[13] Nevertheless, the pattern of expression of CD25 antigen (IL-2R) in different lymphocyte subsets in peripheral blood has not been studied previously.

In the present study, the expression of CD25 antigen in human circulating T lymphocytes was examined by a double immunofluorescence staining method.[13] Although this technique is not as sensitive as cytofluorographic studies, observer error was minimized by photographic examination of large number of lymphocytes. The mean percentages of CD4 and CD8 lymphocytes, and the CD4/CD8 ratio as determined by leu-3a and leu-2a monoclonal antibodies (34%, 24%, and 1.48 respectively) were similar to our previously reported values of 37%, 24% and 1.67 determined in 37 healthy subjects using OKT4 and OKT8 monoclonal.[14] The

Table 2
DISTRIBUTION OF CD25 ANTIGEN (IL-2R) IN FRESHLY ISOLATED AND CULTURED PERIPHERAL BLOOD MONONUCLEAR CELLS

Cells	Percent Positive cells
Fresh peripheral lymphocytes	$< 5^a$
Peripheral blood mononuclear cells cultured for 5 days without mitogen	$< 5^a$
Peripheral blood mononuclear cells cultured for 5 days with PWM	
Preparation constained with Leu-2a	16.2 ± 8.6^a
Preparation constained with Leu-3a	17.2 ± 10.2^a
CD4 (Leu-3a positive) Lymphocytic cells from peripheral blood mononuclear cells cultured for 5 days with PWM	19.8 ± 10.4^b
CD8 (Leu-2a positive) lymphocytic cells from peripheral blood mononuclear cells cultured for 5 days with PWM	15.4 ± 6.4^b
CD20 (Leu-12 positive) lymphosytic cells from peripheral blood mononuclear cells cultured for 5 days with PWM	$< 5^c$

a — expressed as a percentage of total cultured mononuclear cells.
b — expressed as a percentage of individual cultured T-lymphocyte subset.
c — expressed as a percentage of culutred B lymphocytes.

Table 3
PERCENTAGE OF ACTIVATED T LYMPHOCYTE SUBSETS BEARING IL-2R IN INDIVIDUAL PATIENTS

Case Number	Leu-3a cells with IL-2R (%)*	Leu-2a cells with IL-2R (%)*	T subsets + with IL-2R (%)*	Lymphocytes with IL-2R (%)*
1	3.6	4.7	8.3	9.4
2	3.4	2.7	6.1	7.6
3	9.0	5.1	14.1	11.6
4	8.4	6.5	14.9	16.0
5	7.3	9.3	16.6	16.4
6	7.5	9.4	16.9	18.5
7	3.0	3.5	6.5	7.9
8	6.5	3.6	9.1	8.4
9	3.5	10.2	13.7	13.7
10	7.6	9.3	16.9	18.1
11	13.9	12.6	26.5	24.5
12	13.8	8.3	22.1	22.1
13	15.2	3.7	19.2	28.1
14	6.2	6.7	12.9	18.0
15	5.4	4.8	10.2	8.7
16	17.2	6.5	23.7	19.7
17	2.8	2.5	5.3	6.7
mean ± SD	7.9 ± 4.9	6.5 ± 2.9	14.3 ± 6.1	15.0 ± 6.3

* — expressed as a percentage of total mononuclear of total mononuclear cells.
+ — summation of leu-2a and leu-3a positive lymphocytes with IL-2R.

The percentage of culture lymphocytes with IL-2R were determined from the average value obtained from two separate preparations.

CD4/CD8 ratio determined in the mitogen-stimulated lymphocyte culture correlated well with that determined in freshly isolated, unstimulated peripheral blood suggesting these circulating lymphocyte subsets preserved their immunoregulatory function when stimulated with pokeweed mitogen.

We confirmed that paucity of IL-2R positive cells in the freshly isolated, unstimulated peripheral blood[13, 15] and similarly we observed a paucity of IL-2R on the cell surface of peripheral blood mononuclear cells cultured with no mitogen. Although in vitro release of soluble IL-2R by B lymphocytes had been reported,[12] we could detect IL-2R in fewer than five percent of activated B lymphocytes using double immunofluorescence technique. On the contrary, in vitro stimulation with pokeweed mitogen revealed that IL-2R were

present in 20 and 15 percent of CD4 and CD8 lymphocytes respectively. As neither the activated B lymphocytes nor moncytes expressed significant amount of IL-2R,[12] one would expect the percentage of cultured peripheral blood mononuclear cells expressing IL-2R approximated the sum of individual subset of activated T lymphocyte bearing IL-2R. This was confirmed by our findings and further supported the accuracy of our double immunofluorescence technique. We could infer from these findings that approximately 55 percent of the cultured peripheral mononuclear cells expressing IL-2R are CD4 (T-helper) cells and, similarly, 45 percent are CD8 (T-suppressor/cytotoxic) cells. Hence, the immunologic function of IL-2R positive lymphocyte in peripheral blood is apparently different from that of lymphoid tissue in which 80 percent are leu-3a (T-helper) lymphocytes.[13]

We conclude from this study, that approximately 20 percent of CD4 lymphocyte and 15 percent of CD8 lymphocytes express IL-2R on their cell surface after culturing with pokeweed mitogen for 5 days. Since the human lymphocytic receptors for IL-2R plays a critical role in the proliferation of T lymphocytes and is required for full expression of normal immune response, analysis of IL-2R in lymphocyte subsets could be of interest in studying different autoimmune disorder, congenital and acquired immunodeficiency, and lymphoproliferative malignancies.

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