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In vitro Propagation of Tissue-infiltrating Lymphoid Cells from Lesional Skin by Culture in IL 2-containing Medium

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Using an in vitro culture system, we have propagated lymphoid cells infiltrating the tissues of allergic contact dermatitis and several inflammatory dermatoses, and those drawn from the blister observed at the sites of allergic contact dermatitis. Approximately 10^7 cells were eventually obtained after 3 weeks from 3 mm punch-biopsied tissues by their culture in human IL 2-containing medium. Studies by flow-cytometrical analysis demonstrated that the proliferated cells were composed of 32–88 % Leu-1-bearing (Leu-1$^+$) cells, 70–90 % Leu-2a-bearing (Leu-2a$^+$) cells, 10–40 % Leu-3a-bearing (Leu-3a$^+$) cells, 70–90 % HLA-DR-bearing (HLA-DR$^+$) cells, and less than 10 % Tac (IL 2 receptor)-bearing (Tac$^+$) cells. Only the cultured cells derived from lesional tissue of pityriasis rosea and those from the blister content of contact dermatitis showed low percentage of T cell phenotype-bearing cells. These results suggest that the described method has opened a new system that enables us to culture possible effector lymphoid cells actually infiltrating various lesional skin. (Received February 4, 1986.)

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In many dermatological disorders, cellular immune reaction is suggested to play an important role in their pathogenesis. Various attempts have been made for analysis of in situ cellular immune reactions in dermatoses. Among them, the recent advance of immunohistopathologic technique using monoclonal antibodies specifying T lymphocyte, B lymphocyte, subsets of T lymphocyte, macrophage, and Langerhans' cell have greatly contributed to our understanding of the immunological mechanisms occurring in the lesional skin. However, they still do not offer satisfactory insight into specificity or unpredicted activities of the T lymphocyte subsets identified. In order to obtain more precise information about in situ immune reactions taking place in skin lesions, particularly about the cellular functional capability, we need a system that enables us to grow and proliferate pivotal T lymphocytes actually infiltrating the tissue without losing their in vivo specificity.

It is possible to maintain and observe the behavior of T lymphocytes infiltrating skin tumor-rejection sites in vitro, but only for a limited period of time (1). The discovery and characterization of T cell growth factor (IL 2) (2, 3) made it possible to easily maintain cultures of antigen-specific (4–6) and alloreactive T cells (7) for long periods of time in vitro. Based on this principle, Mayer et al. (8) succeeded to propagate the in vivo activated allospecific T lymphocytes from human renal allograft biopsies undergoing rejection.

In this study we have tried to propagate T lymphocytes infiltrating the lesional tissue of allergic contact dermatitis and other inflammatory dermatoses from 3 mm punch biopsy samples or cells collected from blisters developing at the site of contact dermatitis. After successful propagation, we have further analysed the surface phenotypes of such in vitro cultured cells.

MATERIALS AND METHODS

Reagents

The following reagents were used: RPMI 1640 (Gibco Laboratories, Grand Island, NY); L-glutamine, 2-mercaptoethanol, dinitrochlorobenzene (DNCB) (Wako Pure Chemical Industries, Ltd, Osaka, Japan); Kanamycin (Meiji Seika Co., Ltd, Tokyo, Japan); monoclonal antibodies Leu-1, Leu-2a, Leu-3a, and anti-HLA-DR (Becton Dickinson, Facs. System, Sunnyvale, Calf); anti-Tac antibody for interleukin 2 (IL 2) receptor was kindly provided by Dr T. Uchiyama, Department of Internal Medicine, Kyoto University School of Medicine; the $F(ab')_2$ fraction of FITC-conjugated anti-mouse immunoglobulin (Tago, Inc., Burlingame, Calf); IL 2 produced by Escherichia coli transformed with the gene for human IL 2 (recombinant IL 2) was generously provided by Shionogi Seiyaku Co. Inc., Osaka, Japan.

Biopsy samples

Three mm punch biopsy samples were obtained from the sites of the positive patch test of six DNCB-sensitized men and the lesions of each one case of psoriasis, pityriasis rosea, spontaneously regressing flat warts, and lichen planus. For the production of allergic contact dermatitis, normal volunteers were at first sensitized with topical application of 2% DNCB in acetone and two weeks later challenged with 0.2% DNCB in acetone.

Cells in blister fluid

Two days after DNCB challenge, two of six volunteers developed a blistering response at the site of the positive patch test. The blister fluid was drawn by a syringe and the cells were collected by centrifugation at 1100 rpm for 5 min.

Culture of cells

Biopsy specimens were washed in PBS and divided into approximately 1 mm × 1 mm pieces. Each piece was placed in a well of 96-well, flat-bottomed tissue culture plate (Costar no. 3596), and cultured in medium (RPMI 1640 supplemented with 10% human AB serum, 2-mercaptoethanol (5×10^{-5} M), L-glutamine (2×10^{-3} M), and kanamycin (60 μg/ml)) for the first day. On the following day, the half amount of culture medium of each well was changed. One or two wells, which contained cells exuding from the explant in greater numbers than others, were replenished and cultured again with the above mentioned medium, while others were cultured with the medium further supplemented with 400 U/ml recombinant IL 2. Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2 and observed daily on an inverted stage microscope to monitor cells that were migrating out of the tissue. The medium was replenished at least every third day, and the cells were transferred into the well of a 24-well, flat-bottomed tissue culture plate (Nunc no. 143982), whenever the well of a 96-well, flat-bottomed tissue culture plate was filled with them. The cells obtained from the blister fluid were cultured in the medium containing 400 U/ml recombinant IL 2 in the wells of a 24-well, flat-bottomed tissue culture plate from the start.

Analysis of the surface phenotype of in vitro-cultured cells

After 18 to 24 days of culture, the surface phenotypes of cells were analysed by fluorescence-activated cell sorter (FACS Analyzer). The cell suspension was incubated with 50 μl of monoclonal antibodies Leu-1, Leu-2a, Leu-3a, anti-HLA-DR, and anti-Tac or of the control non-reactive monoclonal antibody for 45 min at 4°C and then the cells were washed 3 times in PBS. The cells were further incubated with the $F(ab')_2$ fraction of FITC-conjugated goat anti-mouse immunoglobulin at a 1:20 dilution for 30 min at 4°C and washed 3 times in PBS. Five thousand cells per assay were analysed by passage through a fluorescence-activated cell sorter (FACS Analyzer).

RESULTS

In vitro culture of biopsy tissues

As shown in Fig. 1, a dense exudate of lymphoid cells developed adjacent to most of the tissue pieces after 24 h. These exudates were noted even in the absence of IL 2-containing medium, but only when exogenous IL 2 was provided did the number of cells increase after the first 24 h. After about 10 days, lymphoid cells in the IL 2 containing medium increased in number to fill the well of 96-well culture plate, showing the morphology of activated T lymphocyte (Fig. 2*a*). On the other hand, the culture of the tissue in the IL 2-

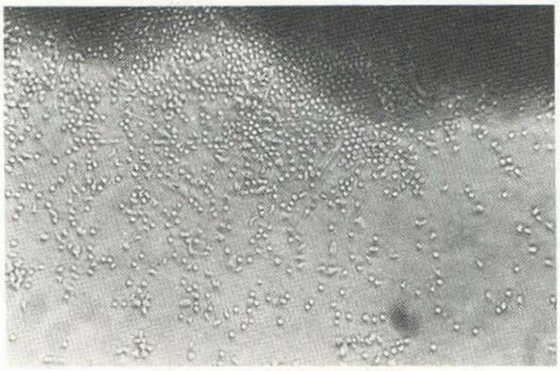

Fig. 1. Appearance of lymphoid cells exuding from the tissue piece after 24 h of IL 2-free culture. There are not a few round or polygonal lymphoid cells surrounding the tissue piece (×240).

free medium showed only the growth of fibroblast instead of the proliferation of lymphoid cells (Fig. 2 *b*). After approximately 3 weeks of culture, the cell growth became slow with the number of cells in the IL 2-containing medium usually reaching about 10^7.

In vitro culture of blister-derived lymphoid cells

The cultured cells obtained from the blisters of allergic contact dermatitis increased in number in the IL 2-containing medium. Their morphology was smaller but polygonal as compared with that of tissue-derived lymphoid cells. After 18 days, 1.2×10^7 cells could be collected from the culture of each blister content.

The surface phenotype analysis of cultured cells

The surface phenotypes of cells cultured in the IL 2-containing medium were summarized in Table I and the representative examples of cytofluorographs were illustrated in Fig. 3.

The cells obtained by the culture of the tissue or from contact dermatitis consisted of 70–90% of Leu-2a-positive (Leu-2a$^+$) cells, 10–40% of Leu-3a-positive (Leu-3a$^+$) cells, 70–90% of HLA-DR antigen-positive (HLA-DR$^+$) cells, and less than 10% Tac antigen-positive (Tac$^+$) cells. The percentage of Leu-1-positive (Leu-1$^+$) cells was varying from 32 to 88%. The surface phenotypes of the exudate cells from the culture of the tissues of psoriasis, lichen planus, and spontaneously regressing flat warts were almost the same as

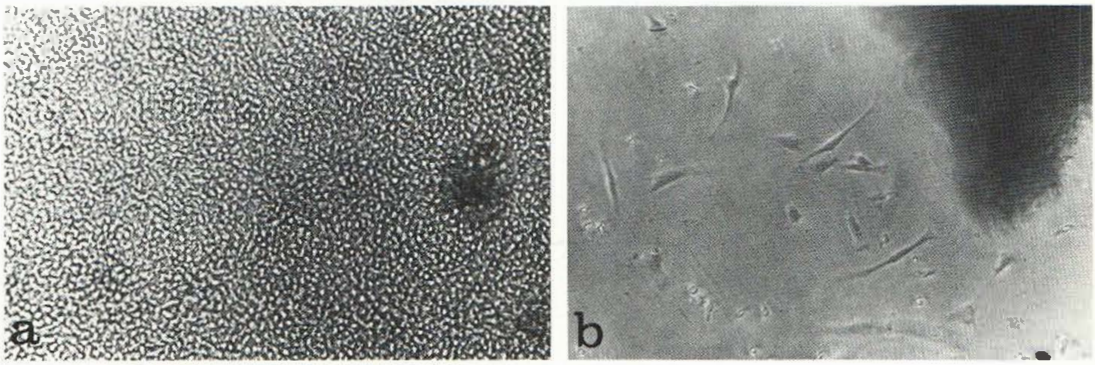

Fig. 2. Appearance of the tissue piece after 10 days of culture. (*a*) In IL 2-containing medium, many large lymphoid cells grow and proliferate (×240). (*b*) In IL 2-free medium, some fibroblasts begin to proliferate instead of lymphoid cells (×240).

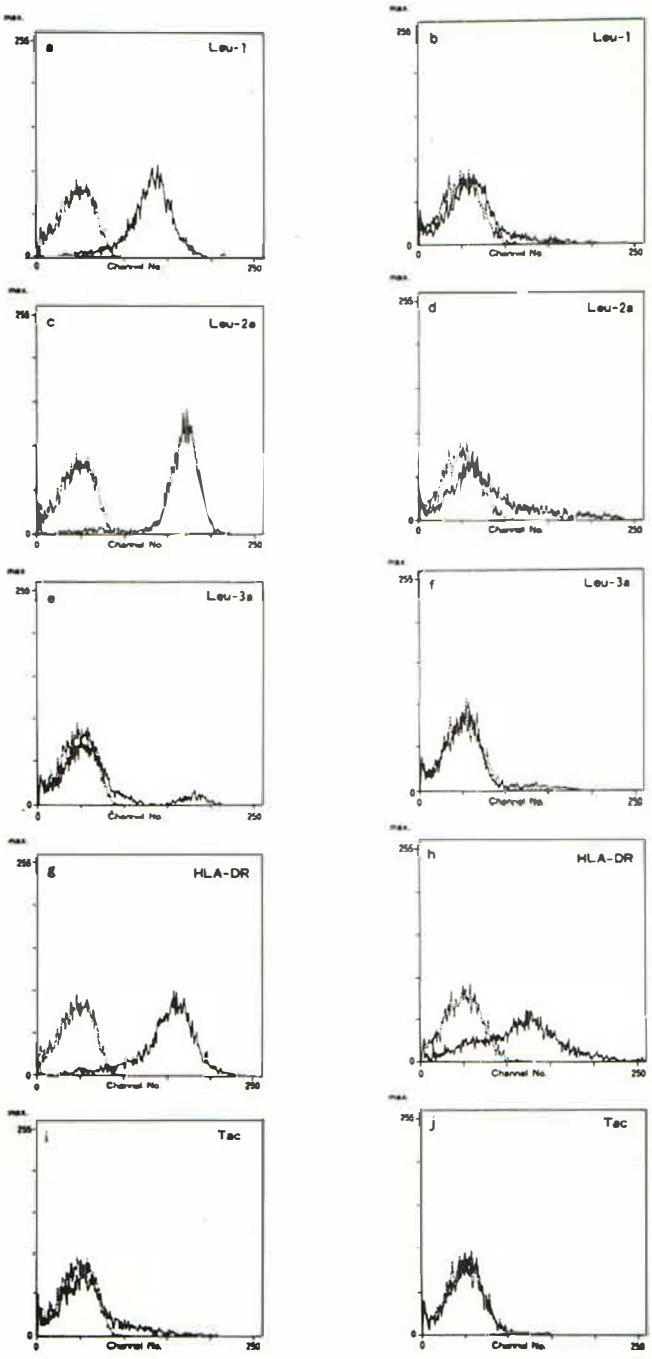

Fig. 3. The representative examples of the cytofluorographs of lymphoid cells cultured for 3 weeks in IL 2-containing medium. The solid lines show the cytofluorographs of the cells stained with monoclonal antibodies i.e., *a*, Leu-1; *c*, Leu-2a; *e*, Leu-3a; *g*, anti-HLA-DR antibody; *i*,anti-Tac antibody in case 4 and *b*, Leu-1; *d*, Leu2a; *f*, Leu-3a; *h*, anti-HLA-DR antibody; *j*, anti-Tac antibody in case 8. The dotted lines show the cytofluorographs of the cells stained with control unreactive monoclonal antibody. Fluorescence intensity (log unit) was expressed on the *X* axis and cell number on the *Y* axis.

those from the tissue of contact dermatitis. On the other hand, the cells from the tissue of pityriasis rosea were composed of smaller numbers of cells bearing T cell markers, i.e. 26% Leu-1$^+$, 25% Leu-2a$^+$, and 5% Leu-3a$^+$ cells with 13% HLA-DR$^+$ cells.

In contrast to the biopsy-derived cells, the cells derived from the content of the

vesicular lesions of contact dermatitis showed less numerous cells bearing T cell markers, whereas the percentage of HLA-DR$^+$ cells was 62%.

DISCUSSION

Using the above mentioned in vitro culture system, we have succeeded in proliferating possible effector lymphoid cells actually infiltrating inflammatory skin lesions. Within 3 weeks we could propagate the infiltrating cells from a biopsy tissue fragment at least to the level of 10^7 cells, which is just enough to carry out the analysis of the cell surface phenotype using fluorescence-activated cell sorter. The sum of respective percentage of Leu-2a$^+$ and Leu-3a$^+$ cells among the in vitro cultured cells was approximately 100% except for those from pityriasis rosea. Therefore it is reasonable to think that only T cells were selectively cultured in our culture condition. It is strange that the percentage of Leu-1$^+$ cells, which varied from 30% to 88%, was smaller than that of Leu-2a$^+$ cells or the sum of those of Leu-2a$^+$ and Leu-3a$^+$ cells. We suggest that by this in vitro culture of T cells in IL 2-containing medium, Leu-1 antigen might have disappeared from their surface.

It is noteworthy that more than 70% of the in vitro cultured T cells were Leu-2a$^+$ cells, while Leu-3a$^+$ cells were less than 30%. Although we could not perform a concomitant immunohistologic study of in situ infiltrating cells or a time course study about the phenotypes of cultured cells because of the limited amounts of the samples, this is probably in a striking contrast to the features noted at the beginning of the culture judged from the results of the in situ immunohistologic analysis already reported by many researchers. Those results indicate that the major infiltrating mononuclear cells are T cells bearing the surface marker of helper T cells, i.e. Leu-2a$^-$ and Leu-3a$^+$ cells or OKT4$^+$ and OKT8$^-$ cells, e.g., in allergic contact dermatitis (9), lichen planus (10), psoriasis (11), and pityriasis rosea (12). Such discrepancy is probably caused by unclarified factors

Table I. *The surface phenotypes of in vitro cultured cells*

ND = not performed

No.	Age	Sex	Disease	Leu-1 (%)	Leu-2a (%)	Leu-3a (%)	HLA-DR (%)	Tac (%)
1	26	M	Contact dermatitis (lesional tissue)	52	70	30	ND	ND
2	26	M	Contact dermatitis (lesional tissue)	84	70	38	ND	ND
3	25	M	Contact dermatitis (lesional tissue)	32	70	22	ND	ND
4	24	M	Contact dermatitis (lesional tissue)	88	92	11	91	10
5	25	M	Contact dermatitis (lesional tissue)	68	78	29	69	6
6	26	M	Contact dermatitis (lesional tissue)	37	79	14	83	8
7	30	M	Contact dermatitis (blister)	39	36	14	ND	ND
8	24	M	Contact dermatitis (blister)	9	24	5	62	3
9	56	M	Lichen planus	83	85	11	55	11
10	27	M	Pityriasis rosea	36	25	5	13	1
11	40	M	Psoriasis	70	70	10	ND	ND
12	27	F	Regressing warts	75	65	25	ND	ND

associated with the culture conditions in which Leu-2a$^+$ suppressor/cytotoxic T cells rather than Leu-3a$^+$ helper/inducer T cells could proliferate more easily.

Several investigators demonstrated that antigen-specific helper T cells, which were activated by the antigen in vivo or in vitro, could proliferate in the IL 2-containing medium (4–6). However, Gullberg et al. (13) showed different requirements for the activation and growth of unprimed cytotoxic and helper T lymphocytes; they noted that accessory cells were necessary for the proliferation of helper T cells. Furthermore, Lotze et al. (14) have found that peripheral blood lymphocytes cultured in IL 2-containing medium attain lytic activity against cultured and fresh autologous tumors. The similarity of our results with these findings suggests that since the lesional tissues of dermatoses contain the peripheral blood T cells which are not primed to the specific antigen in their infiltrate, it is possible for larger numbers of cytotoxic T cells or lymphokine-activated killer cells than helper T cells to be proliferated in the IL 2-containing culture of the tissues. Our data about the characteristics of the surface phenotypes of cultured T lymphocytes seem to indicate that the infiltrating T cells in the lesional skin are a mixture of the antigen-specific primed lymphocytes and the unprimed lymphocytes that are attracted into the lesional tissue by the effect of lymphokines released by the primed lymphocytes. Experiments in progress are analysing the function of these in vitro propagated cells to elucidate whether these cells are actually antigen-specific or not. However, since in many dermatoses the in situ infiltrating T cells are mainly composed of helper T cells as shown by immunohistopathologic studies and helper T cells are suspected to play an important role in the their pathogenesis, in order to analyse the in situ immune reaction in vitro much more properly and in a way closely simulating the in vivo state, it appears that we need an additional modification in our system to make it possible to propagate much more helper T cells.

The number of Tac$^+$ cells, activated T cells having IL 2 receptor (15), was very small among the in vitro cultured T cells after approximately 3 weeks of culture. Furthermore we observed that the growth rate of cells began to diminish after 3 weeks of culture. Deeper et al. (16) reported that human lymphocytes maintained in long-term culture with IL 2 showed a progressive decline in receptor number without restimulation with lectin or antigen. These facts also implicate that further modification such as the stimulation with lectin which has been used in propagating the allospecific cytotoxic T cells from renal allograft biopsies (8) is necessary in our system to obtain more numbers of cells.

As compared with Tac$^+$ cells, the percentage of HLA-DR$^+$ cells was much larger. Since activation of T cells with IL 2 induces HLA-DR antigen expression on their surface (17), our data imply that these HLA-DR$^+$ cells in our culture system are IL 2-activated proliferating T cells.

We are still unable to explain why the percentage of Leu-1$^+$ cells or the sum of Leu-2a$^+$ cells and Leu-3a$^+$ cells was less than 40% in the cells derived from the lesional tissue of pityriasis rosea, where the percentage of HLA-DR$^+$ cells is also remarkably low. Such a feature was also observed with the cultured cells from vesicular lesions of allergic contact dermatitis, although in this case, the percentage of HLA-DR$^+$ cells was not low. We are now undertaking a study to characterize these HLA-DR$^+$ cells lacking T cell markers. Despite the presence of several unclarified facts, this in vitro method to propagate possible effector lymphocytes from the lesions of different dermatoses might provide a valuable model for functional analysis of the infiltrating cells and in this way facilitate our understanding of the basic immune mechanisms underlying various skin disorders.

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