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Agar Plating Technique for Enumeration of IL-2 Producing Cells in Human Peripheral Blood Mononuclear Leukocytes

Chakrit Hirunpetcharat, Watchara Kasinrerk, and Sanit Makonkawkeyoon

SUMMARY **An agar plating technique for detection and enumeration of IL-2 producing cells from human peripheral blood mononuclear leukocytes (PBML) has been developed. This method is based on the principle that PHA-stimulated PBML, as effector cells, secrete interleukin 2 (IL-2) into soft agar containing mouse 3-day Con A blasts as IL-2 dependent responder cells. The IL-2 dependent Con A blasts proliferating around the IL-2 producing cells form colonies or clusters of cells and are easily visualized under a dissecting microscope. The development of IL-2 producing cells was optimum when 1 × 10^6 cells/ml PBML were stimulated with 2 μg/ml PHA-P for 4 hours, and when 2.5 × 10^5 cells were co-cultured with 6 × 10^6 Con A blasts in soft agar for 5 days. The average number of IL-2 producing cells in 10 normal healthy controls were 754 ± 94 (mean ± S.E.M.) cells/10^6 PBML. The numbers of IL-2 producing cells and the levels of IL-2 produced were highly correlated (r = 0.929). The subpopulation of lymphocytes in the colonies was shown to be mostly murine T-cells, since they were mostly Thy 1.2 positive, CD3 negative and surface immunoglobulin negative. This technique is very simple to perform and provides an accurate and straightforward means to enumerate IL-2 producing cells from human PBML in a variety of human immunologic disorders.**

Human interleukin 2 (IL-2) is an antigen non-specific, genetically unrestricted soluble factor produced by T cells stimulated with antigens, mitogens, or alloantigens.[1,2] Human IL-2 is a protein with a molecular weight of approximately 15,000 and an isoelectric point of approximately pH 6.5.[3] IL-2 plays a key immunoregulatory role through its ability to induce proliferation and differentiation of certain types of effector cells. *In vitro,* IL-2 has many effects, including enhancing the proliferative response of human peripheral blood mononuclear leukocytes or murine thymocytes;[4-6] supporting growth of continuous T cell lines;[7,8] enhancing the development of antigen-specific cytotoxic cells by alloantigen-stimulated T cells;[9,10] augmenting the plaque-forming cell responses of nude mouse spleen cells to sheep erythrocytes;[9,11] and inducing the production of γ-interferon.[12]

The production of IL-2 from human PBML,[13] murine or rat spleen cells,[1] and murine or human tumor cell lines[14] has been studied intensively in many laboratories. There are a number of methods for quantitation of IL-2 levels in tissue culture fluid including a bioassay, a radioreceptor assay and an ELISA-based assay.[15] However, these quantitative assays of IL-2 level in the tissue culture fluid have only measured the amount of IL-2 per unit of cells; they have not determined the number of cells producing IL-2. We have developed a simple technique for quantitative determination of the numbers of IL-2 producing cells from spleen cells of rat based on the principle that when IL-2 producing cells are cultured with IL-2 dependent cells in semisolid agar medium, IL-2 from IL-2 producing cells will stimulate IL-2 dependent cells to proliferate around IL-2 producing cells, which can be seen as "colonies" under a dissecting microscope.[16] Each colony represents an IL-2 producing cell. This agar

From the Section of Microbiology and Immunology, Research Institute for Health Sciences, and the Department of Clinical Immunology, Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai, Thailand.

Correspondence : Dr. Sanit Makonkawkeyoon, Department of Clinical Immunology, Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai 50002, Thailand.

plating technique is modified in this study to use for enumeration of IL-2 producing cells in human peripheral blood mononuclear leukocytes.

MATERIALS AND METHODS

Isolation and stimulation of PBML

Peripheral blood from laboratory personnel was drawn into test tubes containing glass beads and mixed well to eliminate platelets. PBML were isolated by Ficoll-Hypaque density gradient centrifugation.[17] PBML were washed twice with RPMI 1640 medium and resuspended with RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS, GIBCO), 10 mM HEPES, 100 units/ml penicillin G, 100 μg/ml streptomycin, 1 mM sodium pyruvate and 5×10^{-5} M 2-mercaptoethanol. PBML in supplemented RPMI 1640 medium were adjusted to 1×10^6 cells/ml, and then cultured with phytohemagglutinin-P (PHA-P, Wellcome) at 37° C, 5% CO_2. After an appropriate period of incubation, PBML were washed three times with RPMI 1640 medium and resuspended to the appropriate concentration with supplemented RPMI 1640 medium.

Preparation of 3-day Con A blasts

Three-day Con A blasts were prepared from BALB/c mice (8-12 weeks old) with some modifications of the methods of Granelli-Piperno *et al.*[18] and Makonkawkeyoon *et al.*[16] Briefly, mouse spleens were removed with aseptic technique, crushed and passed through a stainless steel mesh into RPMI 1640 medium. Erythrocytes were lysed by 0.83% ammonium chloride solution, and spleen cells were washed twice and resuspended in supplemented RPMI 1640 medium to a concentration of 1×10^6 cells/ml. One milliliter of spleen cell suspension was stimulated with 5 μg/ml concanavalin A (Con A, Sigma) at 37° C, 5% CO_2 for 3 days. The cells were washed twice, resuspended and adjusted to 6×10^6 cells/200 μl with supplemented RPMI 1640 medium[16].

Assay of IL-2 producing cells

The method used for enumeration of IL-2 producing cells from human PBML was based on the method described by Makonkawkeyoon *et al.*[16] Briefly, each tissue culture plate, 35 × 10 mm (Falcon), contained 1.0 ml of lower agar layer composed of 0.5 ml double strength RPMI 1640 with supplements and 0.5 ml of 1% agarose (Accurate Chemical, Hicksville, NY) in distilled water. The upper agar layer, with a volume of 1.0 ml, was composed of 0.28 ml of 1% agarose in distilled water, 0.28 ml of double strength supplemented RPMI 1640 medium, 0.14 ml RPMI 1640 medium with supplements, 0.2 ml responder cell suspension (3-day Con A blasts), and 0.1 ml effector cell suspension (PHA-P-stimulated PBML). The mixture was permitted to gel at room temperature for 15 min. For controls, cultures were set up in which the upper agar layer consisted of only effector cells or only responder cells. All culture plates were incubated in a CO_2 incubator at 37° C, 5% CO_2 in air with humidified atmosphere for 5 days. IL-2 producing cells were enumerated by searching for "colonies" under a dissecting microscope with 30 magnification.

The precision of this method was evaluated by the determination of the "allowable limits of error", "standard deviation" and "coefficient of variation".

Determination of optimal conditions for induction of IL-2 producing cells

1. Number of effector cells. Various cell numbers of PHA-P-stimulated human PBML: 0, 1.25×10^5, 2.5×10^5, 5.0×10^5, 7.5×10^5 and 1×10^6 cells were plated with 6×10^6 responder cells (3-day Con A blasts). The number of IL-2producing cells were enumerated after 5 days of incubation.

2. Concentrations of PHA-P. Human PBML at 1×10^6 cells/ml were stimulated with 0, 0.5, 1, 2 or 4 μg/ml PHA-P for 4 hours. The cell cultures were washed twice and resuspended in supplemented RPMI 1640 medium. The effector cells, 2.5×10^5 cells in 100 μl, were mixed with 6×10^6 responder cells in 200 μl. The number of IL-2 producing cells was counted after 5 days of incubation.

3. Optimal period for PHA-P stimulation. Human PBML at 1×10^6/ml were stimulated with 2 μg/ml PHA-P for periods of 0, 1, 2, 4 or 6 hours. After each incubation period, cultured cells were washed twice and resuspended in supplemented RPMI 1640 medium, and 2.5×10^5 cells of PHA-P-stimulated PBML were agar plated with 6×10^6 responder cells. IL-2 producing cells were enumerated after 5 days of incubation.

4. Optimal incubation period for development of IL-2producing colonies. After stimulation with 2 μg/ml PHA-P for 4 hours, PBML were washed twice and 2.5×10^5 cells/100 μl were mixed with 6×10^6 responder cells in 200 μl. IL-2 producing colonies in agar plates were counted every day for 7 days.

Correlation between number of IL-2 producing cells and level of IL-2

Various numbers of PHA-P-stimulated human PBML, 1.25×10^5, 2.5×10^5, 5.0×10^5, 7.5×10^5 and 1×10^6/ml, were mixed with 6×10^6 responder cells and agarose as described above. Another set of the same concentrations of effector cells was cultured at 37° C, 5% CO_2 for 24 hours under the same conditions. Culture supernatant fluid was collected by centrifugation and kept at -70° C for assay of IL-2. Correlation between numbers of IL-2producing cells and

levels of IL-2 production was determined by Pearson's product moment (correlation coefficient).

Assay of IL-2

The levels of IL-2 in the cultured supernatant fluids were determined by IL-2 dependent, 3-day Con A blast cells.[18] After two-fold serial dilutions of each cultured supernatant fluid were done in 100 μl of supplemented RPMI 1640 medium in a 96-well culture plate (Linbro, New Haven, CN), 100 μl containing 2×10^4 3-day Con A blasts was added into each culture well. After 24 hours of incubation at 37° C, 5% CO_2, each microtiter well was pulsed with 0.2 μCi of [^{3}H]-TdR (Amersham). Cell cultures were harvested 18 hours later onto glass fiber strips. The [^{3}H]-TdR incorporation was determined by liquid scintillation counting (LS 3801, Beckman Liquid Scintillation Counter). Levels of IL-2 were expressed in units/ml by comparison with commercial standard human IL-2 (Human interleukin 2, ultrapure; Genzyme, Boston, MA, USA).

Morphological and cell surface markers studies

Colonies in each culture were collected by a capillary pipette into 0.5 ml warm (37° C) RPMI 1640 medium. In each experiment, 10 colonies from one plate of culture were pooled. The morphology of cells was freshly examined by phase contrast microscopy and by light microscopy after staining with Wright's stain. Cells from colonies were stained with OKT3 antibody or (rabbit) anti-mouse immunoglobulins-fluorescein conjugated serum or (goat) anti-mouse Thy 1.2-fluorescein conjugated serum (Becton Dickinson, Mountain View, CA) for detection of human T cells or mouse B cells or mouse T cells, respectively.

RESULTS

Optimal conditions for induction of IL-2 producing cells

An IL-2producing cell appears as a "colony" derived from the proliferative response of 3-day Con A blasts (IL-2dependent cells) to IL-2 secreted by IL-2producing cell (Fig. 1). The optimal concentration of PHA for stimulation of IL-2producing cells was 2 μg/ml as shown in Fig. 2. Figure 3 shows that using 2 μg/ml PHA, maximal numbers of human IL-2producing cells in PBML were obtained with a stimulation time of 4 hr. The appropriate concentration of effector cells was 2.5×10^5 cells/culture for co-culture with 6×10^6 responder cells as depicted in Fig. 4. Optimal incubation time for development of colonies of IL-2 responder cells surrounding IL-2 producing cells was 5 days as shown in Fig. 5.

Correlation between number of IL-2 producing cells and level of IL-2

The numbers of IL-2producing cells and the levels of IL-2 secretion in supernatant fluids were determined in parallel from cells cultured under the same conditions in order to determine the correlation as described in Materials and Methods. Fig. 6 shows a very good correlation between the numbers of IL-2producing cells and the levels of IL-2 in the culture supernatant fluid ($r = 0.929$).

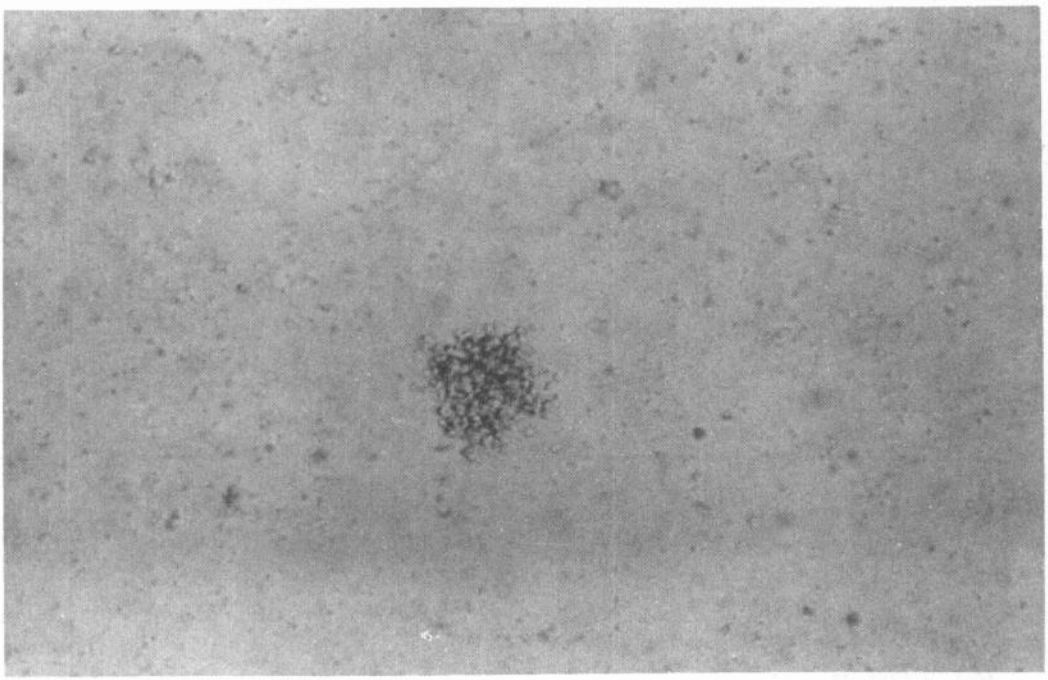

Fig. 1 An IL–2 producing cell identified by a "colony" resulting from the proliferative response of 3–day Con A blasts to IL–2 secreted by the IL–2 producing cell.

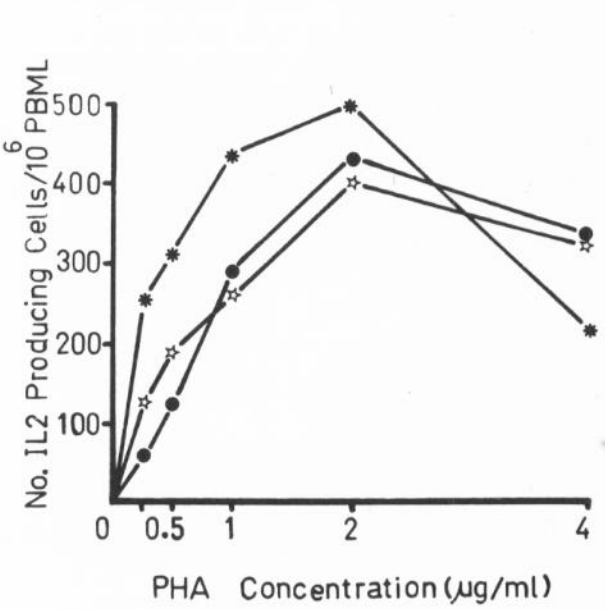

Fig. 2 Determination of optimal concentration of PHA to stimulate PBML for IL–2 producing cells (3 experiments).

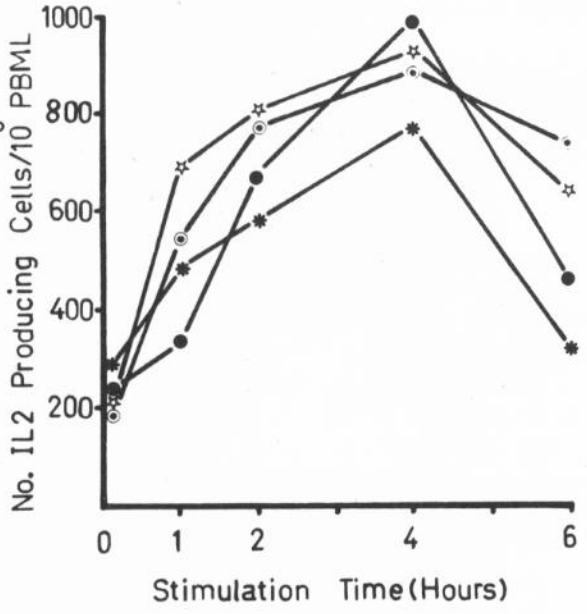

Fig. 3 Determination of optimal stimulation time of PHA for PBML to develop IL–2 producing cells (4 experiments).

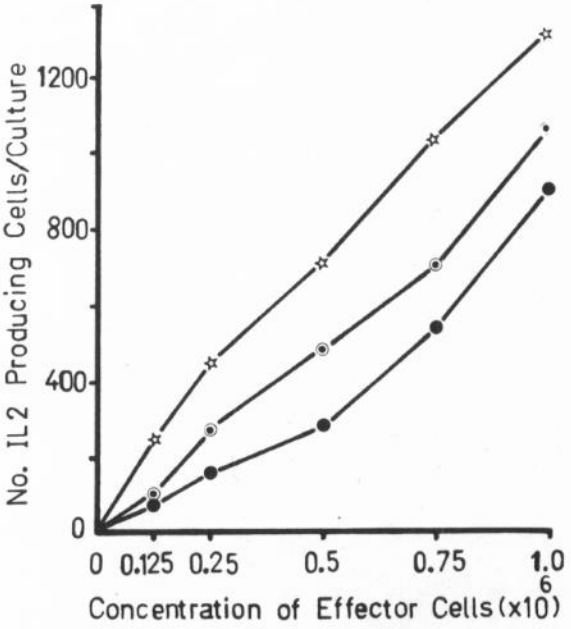

Fig. 4 Optimal concentration of effector cells for development of IL–2 producing cells (3 experiments).

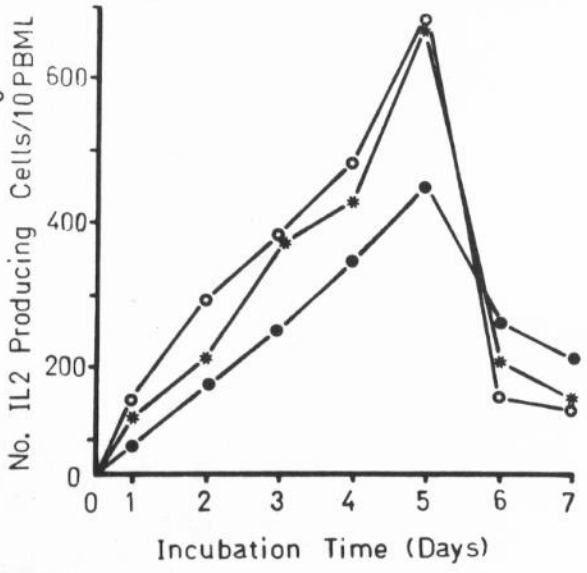

Fig. 5 Optimal incubation time for maximum development of IL–2 producing cells (3 experiments).

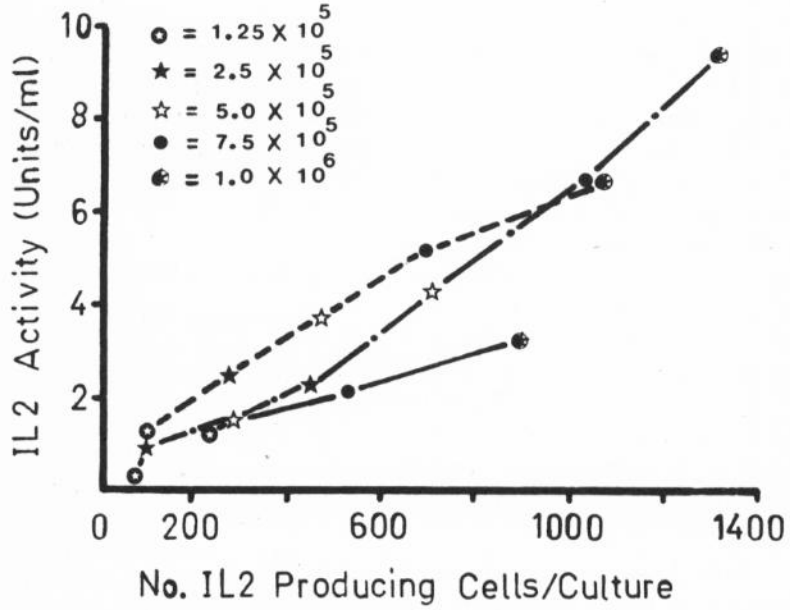

Fig. 6 Correlation between levels of IL–2 activities and numbers of IL–2 producing cells (3 experiments).

Number of IL-2 producing cells in healthy normal individuals

The numbers of IL-2producing cells per 1×10^6 PBML were determined from 10 healthy Thai individuals (Table 1). The numbers of IL-2producing cells ranged from 405 to 1319 cells/10^6 PBML with an average of 754 ± 94 (mean $\pm$ S.E.M.) cells/10^6 PBML. When 6×10^6 responder or 2.5×10^5 effector cells were cultured alone in soft agar plate, the average numbers of spontaneous colonies were 67 colonies/6×10^6 responder cells (mean $\pm$ S.E.M. $= 67 \pm 25$ colonies) and 0 colonies/10^6 effector cells, respectively. The allowable limits of error, standard deviation and coefficient of variation were $\pm 30.3\%$, 105.9 and $\pm 14\%$ respectively. From the statistical analysis, it was concluded that the precision of this method was acceptable to be used for the determination of IL-2producing cells.

Table 1. Enumeration of IL–2producing cells from human peripheral blood mononuclear leukocytes (PBML)

Donor no.	IL–2producing cells per 10^6 PBML
1	630
2	405
3	625
4	880
5	808
6	460
7	440
8	1,319
9	1,070
10	898
$\bar{X} \pm$ S.E.M	754 ± 94

Morphology and cell surface markers.

When cells from 10 pooled colonies of IL-2producer and IL-2 responder cells were stained by OKT3, (rabbit) anti-mouse immunoglobulins-fluorescein conjugate or (goat) anti-mouse Thy 1.2-fluorescein conjugate, almost all cells were positive for Thy 1.2, suggesting that most were mouse T cells. However, when the same cell suspension was stained with OKT3 sera, only a few cells were positive, suggesting that comparatively fewer were human T cells. There were no mouse B cells in the cell suspension since all cells were negative to (rabbit) anti-mouse immunoglobulins.

DISCUSSION

Interleukin 2 plays a key role in immunoregulation both *in vivo* and *in vitro*. Its ability to stimulate continuous growth of T lymphocytes has attracted considerable scientific attention. IL-2 is not only a useful reagent for the maintenance of large numbers of effector T lymphocytes, but also a potentially potent immunoregulatory molecule. It could be of therapeutic usefulness in a number of clinical conditions. Many investigators have successfully worked on the production, isolation and purification of human-, rat- and murine IL-2.[1,19,20]

There are many methods used for detecting IL-2 levels in cell culture fluid, and the ability of T lymphocytes to produce IL-2 in various diseases has been evaluated using these procedures.[21] The unit amount of IL-2 per amount of cell culture could be determined by these IL-2 assays. However, the number of T lymphocytes that can produce and

secrete IL-2 cannot. Recently, we have developed a simple and reliable technique which can enumerate IL-2 producing cells from rat spleen using a soft agar system.[16] In this study, we used the same principle with slight modification of our previous technique to detect and enumerate IL-2producing cells in human PBML.

We have determined the optimal conditions for enumeration of IL-2 producing cells in human PBML by agar plating technique. The numbers of IL-2producing cells and IL-2 levels were highly correlated ($r = 0.929$). We have also studied the kinetics of IL-2 secretion by PHA-stimulated human PBML. The early IL-2 level in cultured supernatant could be detected after 4 hours of PHA stimulation. IL-2 level increased after 6, 8 and 12 hours of PHA stimulation. The maximum IL-2level was detected at 18 hours and only slightly declined at 24 hours; after that the levels of IL-2rapidly decreased and became nearly zero at 72 hours (data not shown).

Gillis *et al.*[22] attempted to identify IL-2producer and responder T lymphocyte subpopulations using monoclonal antibody specific to IL-2 and immunoperoxidase staining. They identified a T cell subpopulation in PHA-stimulated human PBML cultures which they believed to be IL-2producer T lymphocytes. Their interpretation was supported by a similar staining pattern of Jurkat FHCRC cell line known to be a human IL-2 producer. Their IL-2 producer cells also had intense cytoplasmic staining surrounding the IL-2 deposit-free nucleus.

In this study we found that maximal clonal expansion of the responder cells occurs 5 days after the incubation period. However, these clusters of cells began to degenerate after 6 days of incubation. This phenomenon was comparable to the time course found in the formation of T cell colonies.[23] It was suggested that the development of T cell colonies consisted of 3 phases: (1) logarithmic phase during the first 5 days; (2) stationary phase; (3) gradual degeneration of colonies due to cell lysis.[23] The majority of cells in colonies or cell clusters in this study were mouse T-cells (Thy 1.2 positive cells), therefore the development and degeneration of colonies should have a similar time course as T cell colonies. From our previous study, we have shown that 3-day Con A blasts are truly IL-2 dependent cells.[16] It is likely that the secreted product is IL-2, and its proof could be made by demonstration of the suppression of colony formation in the preparation containing anti-IL-2 MAb.

Some investigators have tried to enumerate IL-2producing T cells. Miller and Stutman[24] tried to enumerate IL-2secreting T helper cells in spleen cell populations of mice by limiting dilution analysis (LDA). After alloantigen stimulation, there was about one T cell in every 30 to 300 that could generate detectable levels of IL-2. Using LDA method, Moretta *et al.*[25] and Vie and Miller[26] reported that approximately 40% and 16.3% of PBML could secrete IL-2, respectively. Our agar plating method, therefore, seems to detect a far lower percentage of IL-2producing cells (average numbers of 754 cells/10^6 PBML). The sensitivity of the LDA method was very high in their studies because [^{3}H]-thymidine incorporation was used in the assay system, while our method depended on the proliferative response of the responder cells to secreted IL-2. Although the LDA method seems to be a very sensitive method for enumeration of IL-2producing cells, the methodology is quite complicated to perform and the calculation of IL-2 producing cell numbers depends on statistical analysis and estimation. Our procedure, on the other hand, determines the actual number of IL-2 producing cells which are surrounded by IL-2dependent cells forming as a colony, providing a simple, accurate and straightforward means to enumerate IL-2producing cells in a variety of immunologic disorders. This procedure can also be used for studying the mechanisms of cellular immunoregulation of IL-2production for the determination of cellular abnormality in various immunological diseases.

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