In Vitro Cell-mediated Immunity Assay for Helper or Suppressor Activities of Human T Lymphocytes*

Sanit Makonkawkeyoon, Ph.D.
Vicharn Vithayasai, M.D., Ph.D.

SUMMARY An *in vitro* method has been developed for evaluating helper or suppressor activity of human T lymphocytes in cell-mediated immunity (CMI). Mouse thymocytes from an inbred strain of mice were stimulated with human interleukin 1 (IL1), which then responded to phytohaemagglutinin (PHA) stimulation by active proliferation. These indicator cells, when co-cultured with human T-lymphocytes, become an *in vitro* CMI assay system. When T lymphocytes in the co-culture have suppressor activity or a high suppressor:helper ratio, there is a very low tritiated thymidine (^{3}H-thymidine) uptake by the indicator cells; however, when the T lymphocytes have helper activity or a high helper:suppressor ratio, there is high ^{3}H-thymidine uptake compared with normal controls. This *in vitro* CMI assay may be useful for evaluating helper or suppressor-T cell activity in various immunological abnormality.

ASIAN PACIFIC J ALLERG IMMUN 1985; 3:73-76.

It is known that the human immune system can be divided into two major parts, one involving humoral immunity (antibody and complement) and the other, cellular immunity (T lymphocytes). There are many useful *in vitro* methods for measuring humoral-mediated immunity (HMI), while there are some *in vitro* techniques for detecting cell-mediated immunity (CMI).[1,2] The common *in vitro* models for measuring CMI include the lymphocyte transformation test with specific antigens (such as PPD, *Candida*) or non-specific stimulants (such as PHA, Con A), determination of lymphocyte mediators (lymphokines), measurement of graft-versus-host (GVH) activity and mixed lymphocyte culture (MLC).[1-3]

Most of the above-mentioned *in vitro* methods for CMI study currently use unfractionated cells for assaying the CMI response, and it is quite complicated to interpret the results of each CMI test. However, many investigators have recently become interested in the immunoregulatory role of helper and suppressor T cells in both HMI and CMI. Most of the papers reporting this involve the investigation of the ratio of helper T cells to suppressor T cells.[4-6] The helper-to-suppressor T-cell ratio is usually obtained from the OKT4/OKT8 lymphocytes. Determination of the percentage of OKT4 and OKT8 cells reflects the phenotypic expression of T lymphocytes rather than the functional activities of these cells. It is, therefore, valuable to develop a method which can be used for measuring the helper or suppressor activity of the regulatory T cells in CMI responses *in vitro*.

This study reports on a simple and accurate *in vitro* CMI assay which has been developed for measuring helper or suppressor activity of the regulatory T cells in CMI response. Mouse thymocytes stimulated with human IL1 and PHA were used as indicator cells for measuring the helper or suppressor activity of T cells. This method should have wide application for measuring either helper or suppressor abnormalities of CMI in various diseases.

MATERIALS AND METHODS

Human peripheral blood mononuclear leukocytes

Twenty millilitres of heparinised (10 μ/ml blood) venous blood was obtained from normal volunteers and blood bank donors. The blood was diluted 1:2 with sterile 0.01 M phosphate buffer saline (PBS) pH 7.2 and concentration by Ficoll-

*From the Department of Clinical Immunology, Faculty of Associated Medical Sciences, and Department of Microbiology, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand.

Hypaque centrifugation by the method of Boyum (1968).[7] The peripheral blood mononuclear leukocyte (PBML) layer was collected, washed and the cell concentration adjusted with RPMI 1640 (Gibco, Grand Island Biological Co., Grand Island, NY, U.S.A.) containing 100 units/ml of penicillin, 100 μg/ml of streptomycin, 2 mM of L-glutamine and 5 per cent heat-inactivated foetal calf serum (FCS, Gibco).

Blood samples were also obtained from three patients with active tuberculosis; all patients were positive by chest X-ray and the culture of *Mycobacterium tuberculosis.*

Induction of suppressor T cells

PBML ($4x10^6$/4 ml) was stimulated with 50 μg of Concanavalin A (Con A), incubated for 18 hours and afterwards washed with RPMI 1640 medium. The cells were then cultured for another 42 hours in the same volume of culture media, then washed twice with RPMI 1640 and the cell concentration adjusted to 10^6/ml.

Purification of human T lymphocytes

Human T lymphocytes were isolated using AET (S2-aminoethyl-isothiouranium hydrobromide)-treated sheep erythrocytes (E_{AET}).[1] Briefly, PBML at a concentration of $1x10^6$/ml in 10% FCS in RPMI was mixed with 0.1 ml of 1% E_{AET}, incubated at 37°C for 10 minutes, centrifuged at 600xG for 2 minutes, and incubated overnight at 4°C. The cell pellet was resuspended by gentle aspiration. The rosette- and non-rosette-forming cells were separated by Ficoll-Hypaque density gradient centrifugation. The E_{AET} were lysed with an ammonium chloride buffer solution.[1] The remaining T cells were washed and counted.

Mitomycin C treated cells[8]

Both Con A- and non-Con A- stimulated PBML and T lymphocytes were treated with mitomycin using 10^6 cells/ml with 50 μg of mitomycin, incubated at 37°C for 30 minutes. The treated cells were washed three times with RPMI 1640, and the amount of cells was readjusted.

IL1 production

Ten millilitres of PBML having a concentration of $1x10^6$ cells/ml were incubated in a 100x15 mm plastic Petri dish (Falcon, Oxnard, CA, U.S.A.) for 2-4 hours. The nonadherent cells were removed by thrice rinsing them off with 5% FCS-RPMI. The resulting adherent mononuclear cells gave a positive test for nonspecific esterase 80-85 per cent by nonspecific esterase staining technique.[1] Ten millilitres of fresh 5% FCS-RPMI was added to the adherent cells with 20 μg/ml of lipopolysaccharide (LPS, *Escherichia coli* 055:B5, Difco, Detroit, MI, U.S.A.). Cultures were incubated at 37°C for 24 hours in a 5% CO_2 incubator, and the culture fluids were harvested by centrifugation (100xG for 10 minutes at 4°C). The supernatant fraction was dialysed against RPMI 1640 media overnight at 4°C, then sterilised by Millipore filtration (Swinnex 0.22 μ, 13 mm, Millipore Corporation, Bedford, Mass, U.S.A.) and stored in one millilitre aliquots at -70°C.

IL1 activity assay

Thymocytes from 8 to 10 week-old Balb/c mice were used in the IL1 activity assay. Animals were killed by cervical dislocation and the thymus gland was removed aseptically. Thymocyte suspensions were prepared by gently homogenising at least two thymus glands on a metal sieve, washed twice with 5% FCS-RPMI. The cells were adjusted to $7.2x10^7$ cells/ml with 5% FCS-RPMI. IL1 samples were assayed in triplicate by the addition of 200 μl of various IL1 dilutions per thymocyte culture ($1.2x10^6$ thymocytes) in a 96-well flat-bottom Linbro plate (Linbro Co, New Haven, CN, U.S.A.). Fifty microlitres of medium containing 0.1 μg of phytohaemagglutinin (PHA-P, Difco) were added into each well. The plate was incubated for 48 hours at 37°C in a 5% CO_2 incubator. Thymidine incorporation into DNA was measured by the addition of 0.2 μCi of tritiated thymidine per well in 50 μl of 5% FCS-RPMI. After 12-16 hours of incubation, the cultures were harvested and washed onto glass-fibre filters with an automatic cell harvester. Filters were washed with distilled water and then air-dried. The radioactivity was counted by a liquid scintillation spectrophotometry system (Beckman LS7000 Liquid Scintillation, Beckman Instruments, Inc., Fullerton, CA, U.S.A.). Results were expressed as mean counts per minute ± SEM of triplicate samples.

Helper or suppressor activity assay by lymphocyte transformation test

Varying percentages of mitomycin C-treated Con A-stimulated PBML or T lymphocytes were added to normal human PBML allogeneic cells ($1x10^5$/well). After adding 1.25 μg of PHA-P to 10 μl of the liquid, the cell mixtures were incubated for 48 hours at 37°C in 5% CO_2, then ^{3}H-thymidine was added and the cultures were harvested after 16-18 hours using an automatic cell harvester. The harvested cells were counted in a liquid scintillation counter.

Helper or suppressor activity assay by IL1-CMI test

Two hundred microlitres of appropriate dilutions of IL1 were added to $1.2x10^6$ mouse thymocytes in each well of a 96-well Linbro plate. Various concentrations of purified human T lymphocytes or PBML with or without Con A stimulation were added to this mixture of thymocytes and IL1, and 0.1 μg of PHA-P in 50 μl of RPMI was added to each well. The cell cultures were incubated for 48 hours at 37°C in a 5% CO_2 incubator and the thymidine incor-

porated into DNA was measured as in IL1 activity assay. Calculation of per cent help or suppression was as follows:

$$\% \text{ suppression} = \left(1 - \frac{\text{CPM of thymocytes with human T cells}}{\text{CPM of thymocytes without human T cells}}\right) \times 100$$

RESULTS

Three pools of supernatants with high IL1 activity were used; each pool was obtained from monocytes of 4 healthy normal human volunteers. The highest activity of two of these pools was at a dilution of 1:3 while for one pool it was the undiluted supernate (Fig. 1).

The effects of different percentages on the standard lymphocyte transformation test of Con A- and non-Con A-stimulated PBML and T lymphocytes are shown in Figures 2 and 3 respectively. The per cent of suppression gradually increased with the increasing percentage of either Con A-treated PBML or T lymphocytes. These findings were more clearly and emphatically demonstrated when our newly developed IL1-CMI test was used to evaluate the activity of the similar cells, as shown in Figures 4 and 5. Figure 4 shows the gradually increasing suppressive activity of various concentrations of Con A-treated PBML. Figure 5 shows the same pattern of result when Con A-treated T lymphocytes were tested. Therefore, both Con A-treated PBML and T lymphocytes showed suppressor activity gradually increasing with the number of cells added. However, the non-Con A-treated cells showed very low or no suppressor activity.

A study of freshly isolated T lymphocytes from three selected active pulmonary tuberculosis patients showed enhancing (helper) activity by this IL1-CMI technique (Fig. 6).

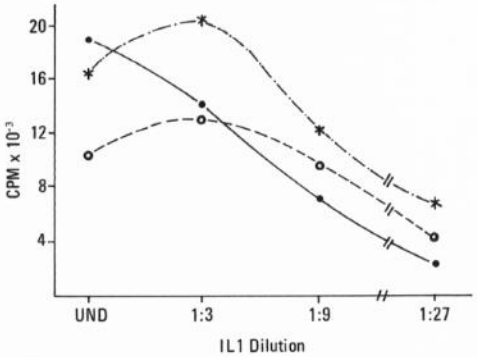

Fig. 1 Production and titration of IL1 activity in three pools of normal human monocyte culture supernatants.

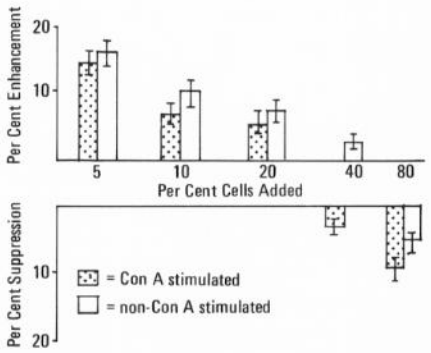

Fig. 2 Percentage of enhancement or suppression by various concentrations of PBML from healthy volunteers in the lymphocyte transformation tests.

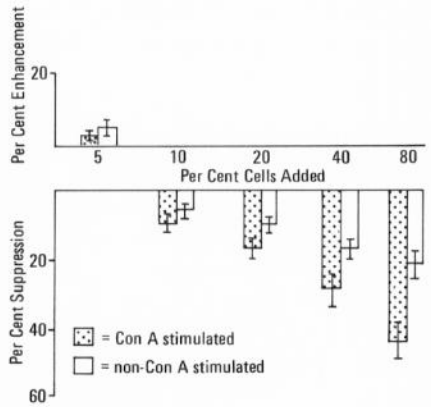

Fig. 3 Percentage of enhancement or suppression by various concentrations of T lymphocytes from healthy volunteers in the lymphocyte transformation test.

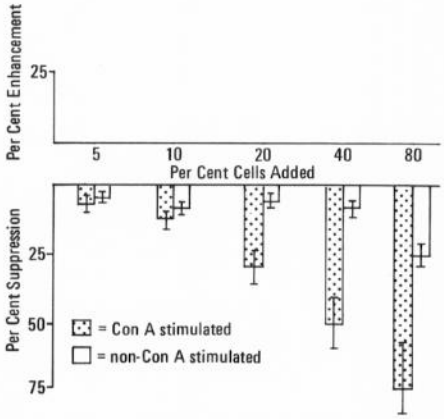

Fig. 4 Percentage of enhancement or suppression by various concentrations of PBML from healthy volunteers in the IL1-CMI test.

DISCUSSION

Human IL1 is known to activate mouse thymocytes to proliferate very actively in the presence of PHA.[9-11] This technique has been used to assay the presence of IL1 in the supernatant fluid of activated

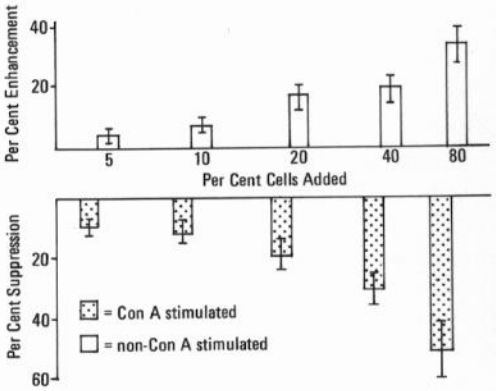

Fig. 5 Percentage of enhancement or suppression by various concentrations of T lymphocytes from healthy volunteers in the IL1-CMI test.

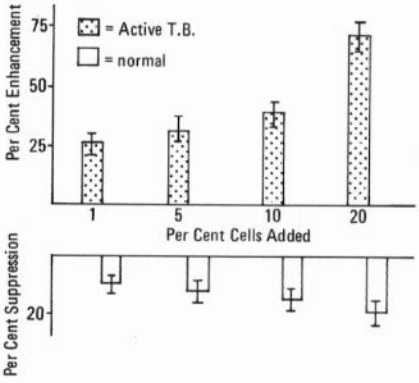

Fig. 6 Percentage of enhancement or suppression in the IL1-CMI test by various concentrations of T lymphocytes from three selected patients with active tuberculosis.

monocytes or macrophages.[12,13] However, this technique can be modified and applied to be used for the evaluation of CMI in various immune disorders. Therefore, an assay has been developed to evaluate the helper or suppressor activity of human T lymphocytes for CMI *in vitro*. The principle of this IL1-CMI test is quite straight-forward and can be summarised diagrammatically as follows:

IL1 + mouse thymocytes + PHA + N (Th/Ts) ⟶ normal CPM

IL1 + mouse thymocytes + PHA + ↑ (Th/Ts) ⟶ increase CPM

IL1 + mouse thymocytes + PHA + ↓ (Th/Ts) ⟶ decrease CPM

When mouse thymocytes are mixed with human IL1 and PHA, they proliferate. If normal T lymphocytes (mitomycin treated), which had T helper (Th) and T suppressor (Ts) activity in normal proportion, were added to this assay system, there was no or little change in the level of thymidine uptake. When T lymphocytes having a high Th:Ts ratio were added to this assay system, there was a high level of thymidine uptake. However, with T lymphocytes having a low Th:Ts ratio, there was a low level of thymidine uptake, observed in this assay system.

In this study, three pools of supernatants with high IL1 activity from LPS-stimulated monocyte cultures were used. The highest level of activity of two of the three pools occurred at a dilution of 1:3; the third pool was the undiluted supernate. When various concentrations of Con A-stimulated PBML from eight normal healthy volunteers were tested by lymphocyte transformation test (LTT), the lower per cent of PBML cells added gave some enhancement while the higher per cent cells added gave some suppression (Fig. 2). When T lymphocytes from normal healthy volunteers were studied by LTT, there was a gradually increasing per cent of suppression with the increasing per cent of Con A-treated T lymphocytes. However, when the same PBML or T lymphocytes were studied by IL1-CMI assay, one could see more clearly the gradual increase in suppression. When freshly isolated T lymphocytes from three selected active pulmonary tuberculosis patients were tested by IL1-CMI assay, the thymidine uptake increased significantly compared with that of four normal healthy volunteers. This IL1-CMI technique *in vitro* should be used widely for the evaluation of CMI status in various immune disorders.

ACKNOWLEDGEMENTS

The authors are most grateful to Dr. David M. Scollard for his cogent criticism of the manuscript.

Research supported by Re-Entry Grant 800209 from the World Health Organisation and USPHS Grants 5 P01 AI 16308 from NIAID to the Chiang Mai/Illinois Leprosy Research Project.

REFERENCES

1. Weir DM, ed, Handbook of experimental immunology. Vol. 2. Oxford: Blackwell Sci Pub, 1978.
2. Stites DP. Immunologic laboratory tests. In: Stites DP, Stobo JD, Fudenberg HH, Wells JV. Basic and clinical immunology. 4th ed. California: Lange Med pub, 1982.
3. Bloom BR, David JR, eds, *In vitro* methods in cell mediated and tumor immunity. New York: Academic Press, 1976.
4. Bullock WE, Watson S, Nelson KE, Schauf V, Makonkawkeyoon S, Jacobson RR. Aberrant immunoregulatory control of B lymphocyte function in lepromatous leprosy. Clin Exp Immunol 1982; 49: 105-14.
5. Mshana RN, Haregewoin A, Belehu A. Thymus-dependent lymphocytes in leprosy. II. Effect of chemotherapy on T-lymphocyte subpopulations. J Clin Immunol 1982; 2:69-74.
6. Venkataraman M, Levin RD, Westerman MP. Subpopulations of human helper and suppressor T lymphocytes. Human Immunol 1983; 7:163-75.
7. Boyum A. Isolation of mononuclear cells and granulocytes from human blood. Scand J Clin Lab Invest 1968; 21 (suppl 97):77-89.
8. Antel JP, Weinrich M, Arnason BGW. Circulating suppressor cells in man as a function of age. Clin Immunol Immunopathol 1978; 9:134-41.
9. Bladen G, Handschumacher RE. Purification and properties of human lymphocyte activating factor (LAF). J Immunol 1977; 118:1631-8.
10. Diamantstein T, Handschumacher RE, Oppenheim JJ, *et al.* Nonspecific "lymphocyte activating" factors produced by macrophages. J Immunol 1979; 122: 2633-5.
11. Katz SP, Shimamura T, Dessaint JP, Braverman D, Waksman BH. Mechanisms of action of "lymphocyte-activating factor" (LAF): IV. Differential stimulation of T lymphocytes by induced macrophage enzymes (Catheptic carboxypeptidase B and serine proteases). Cell Immunol 1980; 56:68-79.
12. Masuzawa Y, Sawada S, Sawada J, Osawa T. Lymphocyte-potentiating factor from human peripheral blood adherent cells. Int Arch Allergy Appl Immunol 1978; 57: 451-61.
13. Oppenheim JJ, Togawa A, Chedid L, Mizel S. Components of mycobacteria and muramyl dipeptide with adjuvant activity induce lymphocyte activating factor. Cell Immunol 1980; 50:71-81.