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Production of Interleukin 2 in Human Peripheral Blood Lymphocytes: Optimal Condition for its Culture

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ABSTRACT

For the assay of the production of Interleukin 2 (IL-2) in human peripheral blood lymphocytes (human PBL), a study was made on the optimal condition for its culture.

1) The optimal condition for the production of IL-2 was considered to be incubation time of 24 hr, number of PBL of 1×10^6 cells/ml, and phytohemagglutinin-M (PHA-M) concentration of 1 — 5%

2) By one way mixed lymphocyte reaction (one way MLR), IL-2 activity similar in level to that by PHA-M stimulation could also be obtained. It was maximal at the 4th day of culture.

3) By PHA-P (0.06%) stimulation, IL-2 could also be produced similar to that by PHA-M stimulation but it decreased in the order of Con A, PWM and PPD and hardly any production of IL-2 could be observed by LPS.

In 1976 Morgan et al[17,20] have reported that by the addition to human bone marrow cells of the culture supernatant of lymphocytes stimulated by PHA, T cells can be selectively proliferated and be cultured in vitro for an extended term. In the following year of 1977, Gillis et al[10,11], using a similar method, were successful in making a long term culture *in vitro* of antigen specific cytotoxic T lymphocytes (CTL) and in their cloning by limiting dilution. The soluble factor which enables long term culture of T cells *in vitro* is called T cell growth factor (TCGF) and has attracted much interest as a means of cloning T cells. The soluble factor which has a similar biological activity had a number of names in addition to TCGF[6,7,19], but at the Second International Lymphokine Workshop held in 1979[1] the name of Interleukin was proposed and it was decided that factors of T cell origin such as TCGF were named Interleukin 2 (IL-2) and that factors of macrophage origin which had been called lymphocyte activating factor (LAF)[16] were named interleukin 1 (IL-1). As for the use of IL-2, it has made possible long term culture of normal T cells with their function maintained intact and has provided the methods for analyzing unknown functions of T cells at the clone level. Since the report of Gillis et al, the properties of IL-2 have been analyzed by many workers and have been published in many reports. In summary, IL-2 are primarily produced by helper T cells ($OKT4^+/Leu\text{-}3^+$ cells in man)[18,25] and in this production antigen or mitogen stimulation together with the presence of IL-1 are indispensable. It has been reported that human IL-2 is a glycoprotein composed of 133 amino acid residues and sugar chains with a molecular weight of 15,000 and an

isoelectric point of 6.5[12,23]. As for its biological activity, it brings rise to proliferation of NK cells as well as various effector T cells including antigen specific CTL together with helper T cells which produce IL-2[5,10,26,27] and furthermore it promotes the production of other lymphokines such as Interferon γ (IFN-γ)[9]. Thus, it is not an exaggeration to say that IL-2 possessing these various important biological activities serves as a nucleus in immune response. consequently, a great deal of interest has been directed toward the assay of IL-2 production of lymphocytes in individual humans and animals as well as toward the analysis of the properties of IL-2 itself and to date a good number of pertinent reports have been published[2,13,24]. The present report describes the assay method of IL-2 and the optimal condition for culture of human PBL.

MATERIALS AND METHODS

IL-2 production

As the samples of IL-2, the culture supernatant of human PBL stimulated by mitogen, antigen or MLR was employed. Human PBL was separated from heparinized blood collected from two normal volunteers (donor 1: 29 year old male and donor 2: 24 year old female) by Ficoll-Hypaque gradient separation. As for stimulator cells to be employed in one way MLR, an equal number of PBL obtained in a similar procedure from six normal volunteers were mixed and subjucted to cobalt radiation of 1250 rad.

The media employed in the culture was prepared by adding 2.5% fresh human AB serum (heat inactivated, 56°C for 30 min) collected from one normal volunteer to RPMI 1640 media (Gibco Laboratories, New York, USA) supplemented with 1% penicillin-streptomycin (Gibco), 4 mM HEPES (Boehringer Mannheim, West Germany) and 300 μg/ml L-glutamine. Cultures were maintained in 12 × 75 mm polystylen tubes (Falcon 2003) at 37°C, 5% CO_2. After incubation supernatant was obtained by centrifugation for 10 min at 240g and preserved at −80°C.

In order to determine the optimal condition, the culture conditions were varied with the incubation time from 6 to 96 hr, concentration of PHA-M (Difco Laboratories, Detroit, USA) from 0.01 to 10%, and the number of PBL from 1 × 10^4 to 1 × 10^7 cells/ml. In one way MLR, responder cells at 1 × 10^6 cells/ml were added to stimulator cells at 1 × 10^6 cells /ml and the incubation time was varied from 1 to 7 days. In order to observe the difference due to various types of mitogens and antigens, culture was made at incubation time of 24 hr and number of PBL of 1 × 10^6 cells/ml with PHA-M at 1, 2 and 5%, PHA-P (Difco) at 0.03, 0.06 and 0.12%, concanavalin A (Con A, Sigma Chemicals, St. Louis, USA) at 10, 20, and 40 μg/ml, pokeweed mitogen (PWM, Gibco) at 0.25, 0.5 and 1%, *Escherichia coli* lipopolysaccharide (LPS, Sigma) at 10, 20 and 40 μg/ml, and purified protein derivative (PPD, Japan BCG, Tokyo, Japan) at 5, 10 and 20 μg/ml. In order to compare these with those with MLR, responder cells at 1 × 10^6 cells/ml for one way MLR, while PBL at 5 × 10^5 cells/ml of donor 1 were added to PBL at 5 × 10^5 cells/ml of donor 2 for two way MLR, both of which were incubated for 4 days.

In the study of the optimal condition, in order to compare the production of IL-2 with the proliferation of PBL, the proliferation of PBL at cpm was determined with the PBL of the same donor by varying the the conditions in the same manner. Here, 96-well flat-bottom microtest plate (Nunc, Denmark) was used with culture media at 200 μl/well and four hours prior to termination of incubation 0.4 μCi ^{3}H-thymidine (^{3}H-TdR, New England Nuclear, Boston, USA (specific activity: 5 Ci/mmol)) was added. Upon completion of incubation, PBL were harvested on the glass fiber filter with an automatic cell harvester (Labo Science, Tokyo, Japan) and the radioactivity was determined by a liquid scintillation counter.

IL-2 assay (Fig. 1)

As targert cells of IL-2 assay, IL-2 dependent tumor specific mouse cytotoxic T cell line (CTLL-2) established by Gillis et al was emplyed and as culture medium, to Click's medium (9.8 mg/ml Hanks' solution (Nissui), 1.76 mg/ml essential amino acid and vitamin (Nissui), 50 μl/ml nonessential amino acid (Gibco), 25 μg/ml uridine, 25 μg/ml cytosine, 25 μg/ml guanosine, 25 μg/ml adenosine, 600 μg/ml L-glutamine and 275 μg/ml pyruvate) were added 2% heatinactivated fetal bovine serum (FBS, Flow Laboratories, McLean, USA), 10 mM HEPES, and 2.5 ×

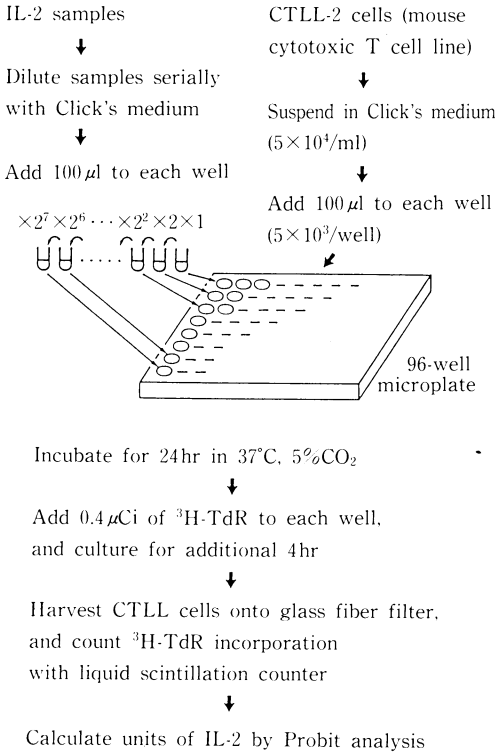


Fig. 1. Procedure of IL-2 assay

10^{-5}M 2-Mercaptoethanol (2-ME) to form complete Click's medium. As for the procedure of IL-2 assay, serial $\log_2$ dilution of eight stages was made of IL-2 samples with the use of complete Click 's medium and to each well of the 96-well microplate 100 μl of each dilution was added. To each well of this microplate, 100 μl of complete Click's medium in which were suspended CTLL-2 cells to make 5×10^4 cells/ml was added (5×10^3 cells/well). It was incubated for 24 hr at 37°C in 5% CO_2. Four hr prior to completion of incubation, 0.4 μCi of ^{3}H-TdR was added to each well and upon completion of incubation, CTLL-2 cells were harvested on the glass fiber filter with an automatic cell harvester. The radioactivity was determined with a liquid scintillation counter.

Determination of units of IL-2 activity (Fig. 2)

First, as the standard IL-2 for obtaining the units, 50% titer of the culture supernatant of PBL of 1×10^6 cells/ml obtained from one normal volunteer by stimulataion for 24 hr with 1% PHA-M was employed. This standard IL-2 was designated as 100% IL-2. As IL-2 samples to be assayed, 50%, 25%, 12.5%, 6.25% and 3.13% IL-2 were employed and the data of IL-2 assay are shown in Fig. 2A. When the percentage of each of these data as contrasted to maximum cpm of 100% IL-2 (Fig. 2A, ↙) was plotted to the probit axis, each of the points almost formed a straight line and the regression line could be obtained. If the dilution obtained from the X axis ($\log_2$ dilution) of the intercepts of these regression lines with the 50% line of the Y axis (probit axis) are assumed to be factor 50, then the value obtained by dividing factor 50 of the sample to be assayed by the factor 50 of the standard IL-2 will be the unit to be obtained. In obtaining the units of these five samples (3.13% — 50% IL-2), units which are almost proportional to concentration of IL-2 of the sample can be obtained.

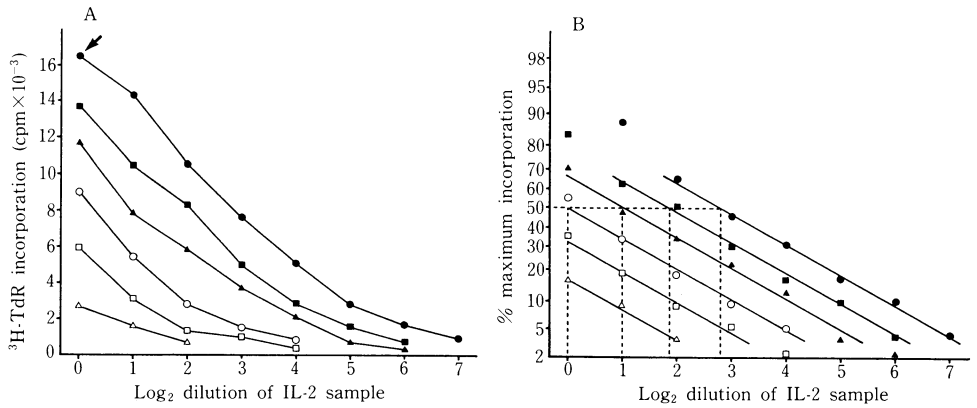
RESULTS

Influence of incubation time

Fig. 3 shows a comparison between IL-2 production and lymphocyte proliferation by incubation time. IL-2 activity in the culture supernatant can hardly be detected by six hr of incubation, but it suddenly increases by 12 hr of incubation to reach a peak in 24 hr and by 48 hr of incubation a decreasing trend can be observed to almost completely disappear by 96 hr of incubation. Even when the supernatant was exchanged with fresh media every 24 hr, the IL-2 activity in the culture supernatant increases to the maximum level in 0 — 24 hr of incubation, but noteworthily it shows a remarkable low value in 24 — 48 hr of incubation. In contrast to this, hardly any lymphocyte proliferation could be detected by 24 hr of incubation, but from 48 hr of incubation it gradually elevates to reach a peak in 96 hr of incubation.

Influence of concentration of PHA-M

Fig. 4 shows a comparison between IL-2 production and lymphocyte proliferation by the concentration of PHA-M in the culture media. When the concentration of PHA-M in the supernatant is less than 0.2%, hardly any IL-2 production can be detected, but at PHA-M concentration of 1% it suddenly elevates and between the PHA-M concentration of 1 — 5% a satisfactory value can be seen. In contrast to this, lymphocyte proliferation shows a rapid increase at PHA-M concentration of 0.2% with a peak being observed at PHA-M concentration of 1%.



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IL-2 Samples	X Axis Intercept at 50% Activity	Units of IL-2 Activity
100% IL-2 (●)	$2^{2.80}$ =7.0	7.0/7.0 =1.00
50% IL-2 (■)	$2^{1.87}$ =3.7	3.7/7.0 =0.53
25% IL-2 (▲)	$2^{1.00}$ =2.0	2.0/7.0 =0.29
12.5% IL-2 (○)	$2^{0.00}$ =1.0	1.0/7.0 =0.14
6.25% IL-2 (□)	$2^{-1.20}$=0.44	0.44/7.0=0.06
3.13% IL-2 (△)	$2^{-2.00}$=0.25	0.25/7.0=0.04

Fig. 2. Probit analysis of IL-2 assay. A, ^{3}H-TdR incorporation of CTLL-2 cells adding Log2 dilution of six IL-2 samples. 100% IL-2 (●); 50% IL-2 (■); 25% IL-2 (▲); 12.5% IL-2 (○); 6.25% IL-2 (□); 3.13% IL-2 (△). B, Percentage of each data to the maximum incorporation of 100% IL-2 (Fig. A↙) plotted to probit axis. C, Determination of units of IL-2 activity in each sample.

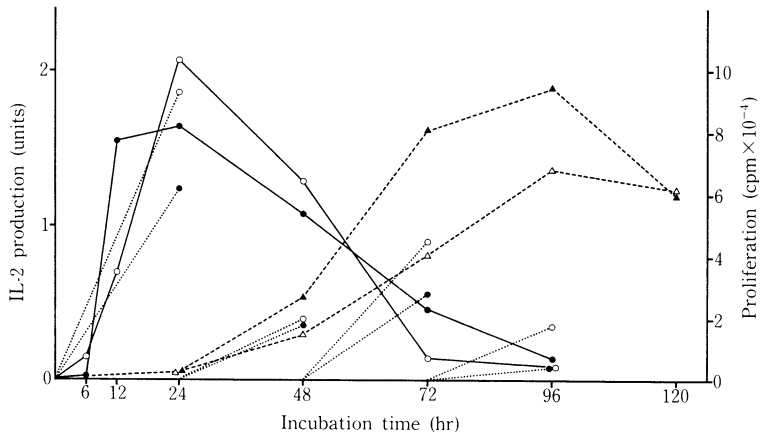

Fig. 3. Changes of IL-2 production and lymphocyte proliferation at different times. IL-2 production (1% PHA-M,PBL 1 × 10^6/ml); supernatants were decanted at different time (donor 1 ○—○ , donor 2 ●—●), supernatants were exchanged for fresh media every 24 hr (donor 1 ○···○ , donor 2 ●···●). Lymphocyte proliferation (1% PHA-M, PBL 1.25 × 10^5/well); ^{3}H-TdR incorporation at different times (donor 1 △···△ , donor 2 ▲···▲).

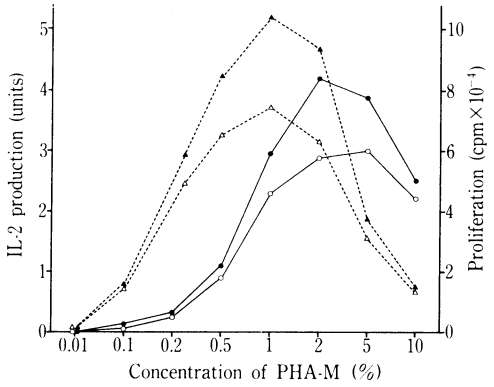

Fig. 4. Changes of IL-2 production and lymphocyte proliferation at different concentrations of PHA-M. IL-2 production (PBL 1 × 10^6/ml, 24 hr incubation); IL-2 activity at different concentrations (donor 1 ○—○ , donor 2 ●—●). Lymphocyte proliferation (PBL 1.25 × 10^5/well, 96 hr incubation); ^{3}H-TdR incorporation at different concentrations (donor 1 ▵···▵ , donor 2 ▲···▲).

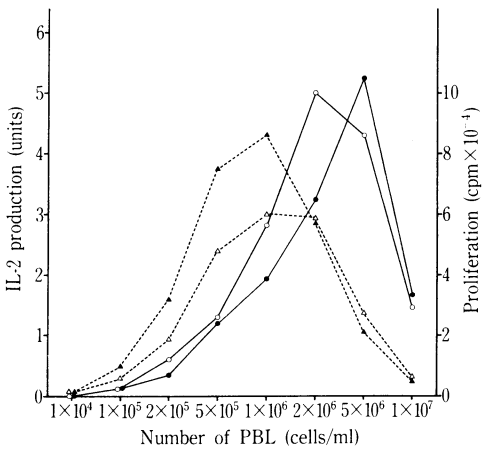

Fig. 5. Changes of IL-2 production and lymphocyte proliferation at different numbers of PBL. IL-2 production (1% PHA-M, 24 hr incubation); IL-2 activity at different numbers (donor 1 ○—○ , donor 2 ●—●). Lymphocyte proliferation (1% PHA-M, 96 hr incubation); ^{3}H-TdR incorporation at different number (donor 1 ▵···▵ , donor ▲···▲).

Influence of the number of PBL

A comparison between IL-2 production and lymphocyte proliferation is shown in Fig. 5 by the number of PBL. IL-2 activity in the supernatant is low when the number of PBL is 2 × 10^5 cells/ml, but at 0.5 — 1 × 10^6 cells/ml it suddenly increases to reach a peak at PBL number of 2 — 5 × 10^6 cells/ml. Though not shown in the figure, when converted to per lymphocyte, IL-2 activity reaches a peak at the PBL number of 0.5 — 1 × 10^6 cells/ml.

Table 1. IL-2 production stimulated by several mitogens and antigens.
Stimulation by mitogens and PPD (PBL 1×10^5/ml, 24hr incubation), by One Way MLR (respondor 1×10^6/ml, stimulator 1×10^6/ml) and by Two Way MLR (PBL of donor 1 : 5×10^5/ml, PBL of donor 2 : 5×10^5/ml).

Mitogen or Antigen		Units of IL-2	
		donor 1	donor 2
Medium control		0.00	0.00
PHA—M	5%	2.99	3.87
	2%	2.87	4.19
	1%	2.28	2.95
PHA—P	0.12%	2.59	3.83
	0.06%	3.17	4.11
	0.03%	2.05	2.88
Con A	40μg/ml	2.05	1.74
	20μg/ml	2.02	1.92
	10μg/ml	1.16	1.33
PWM	1%	1.18	1.30
	0.5%	1.33	1.11
	0.25%	0.64	0.89
LPS	40μg/ml	0.02	0.02
	20μg/ml	0.02	0.01
	10μg/ml	0.00	0.01
PPD	20μg/ml	0.11	0.24
	10μg/ml	0.11	0.25
	5μg/ml	0.12	0.19
MLR	One Way	2.78	2.99
	Two Way	2.33	

IL-2 production in one way MLR

Comparison between IL-2 production and lymphocyte proliferation in one way MLR is presented by incubation time in Fig. 6. IL-2 activity in the supernatant can hardly be detected by incubation for 1 day, but from the second day it suddenly increases to reach a peak on the fourth day of incubation and it remarkably drops on the seventh day. In contrast to this, hardly any lymphocyte proliferation can be detected for three days of incubation, but it gradually increases from the fourth day to reach a peak on the seventh day.

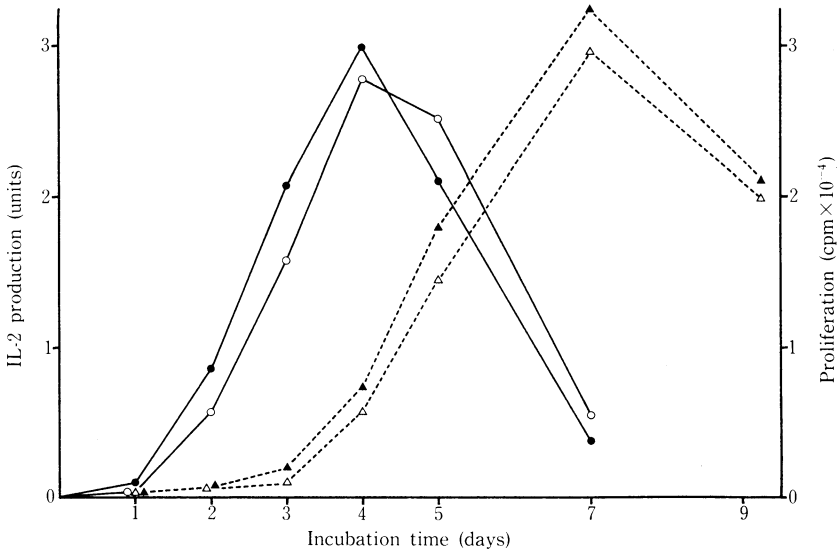

Fig. 6. Changes of IL-2 production and lymphocyte proliferation in one way MLR at different times. IL-2 production (respndor 1 × 10^6/ml, stimulator 1 × 10^6/ml); IL-2 activity at different times (donor 1 ○—○ , donor 2 ●—●). Lymphocyte proliferation (respondor 1.25 × 10^5/well, stimulator 1.25 × 10^5/well); ^{3}H-TdR incorporation at different times (donor 1 △···△ , donor 2 ▲···▲).

IL-2 production stimulated by several mitogens and antigens

Table 1 presents a comparison of IL-2 production by changing the type and concentration of mitogens and antigens. IL-2 activity in the supernatant shows an almost similar value by PHA-M stimulation and by PHA-P stimulation, but by Con A stimulation it is somewhat depressed and it is further depressed by PWM stimulation. By MLR stimulation, the value is similar by both one way and two way to that of PHA simulation, but it is low with PPD stimulation and almost 0 with LPS stimulation.

DISCUSSION

Cell line CTLL-2 reported by Gillis et al[10] in 1977 is a cytotoxic T cell line, which is derived from the spleen cells of C57BL/6 mouse immunized by the Friend leukemia virus-induced erythroleukemia cells (F4-5 cells) and stimulated by secondary mixed tumor-lymphocyte culture, is cultured by media containing IL-2, and is cloned by limiting dilution, and even after long term culture it maintains its CTL activity. The authors, after obtaining CTLL-2, have maintained CTLL-2 with the use of RPMI 1640 media containing 5% culture supernatant of rat spleen cells of 1 × 10^7 cells/ml stimulated for 48 hr by Con A of 5 μg/ml. The CTLL-2 maintained by the authors has a doubling time of 10 — 12 hr and when suspended in a media not containing IL-2, its proliferation is arrested within 2 hr and it almost completely perishes within 24 hr.

As the proliferation of CTLL-2 is dependent on IL-2 activity within the media[14], it can thus be employed as target cell in IL-2 assay, and at the same time it is of importance that human IL-2 possesses proliferative activity against CTLL-2. The specificity of IL-2 thus differs by species. Mouse IL-2 only possesses proliferative activity for mouse T cells, but human IL-2 possesses proliferative activity for not only human T cells but also T cells of monkey, horse, guinea pig, cat, rat and mouse[21].

As target cell in IL-2 assay, not only IL-2 dependent cell line such as CTLL-2 but also PBL[3], spleen cells [28] or thymocytes[7] stimulated by T cell mitogen (PHA or Con A) can be employed. These cells containing IL-2 non-dependent cells are inferior in their specificity and as a matter of course being affected by T cell mitogen, when IL-2 sample contains T cell mitogen, its influence must be taken into account. On the contrary, cloned IL-2 dependent cell line can be said to be a much superior tar-

get cell, being completely unresponsive to T cell mitogen.

In proliferation assay with the use of CTLL-2, the presence or absence of IL-2 can be demonstrated in ^{3}H-TdR incorporation thus obtained, but the difference in titer of the individual IL-2 samples cannot be necessarily shown. In order to compare IL-2 production of human PBL by individuals, its activity must be expressed as unit by some method. Gillis et al[14] by applying probit analysis employed by Jordan[15] in interferon plaque reduction assay in expressing the unit of IL-2 activity, made it possible to express the difference in titer between the individual samples. Of course, this method is not faultless, but at the present moment it can be considered to be the most reliable method in expressing the results of bioassay in unit.

Incubation time, number of PBL, concentration of mitogen, and type of mitogen can be considered to conditions involved in the IL-2 production of human PBL. As an almost unlimited number of combinations can be produced by changing each of these four parameters, making it impossible to determine the optimal condition, with the use of the basic condition of incubation time of 24 hr, number of PBL of 1×10^6 cells/ml and PHA-M concentration of 1% given in the reports of Gillis et al[13,14] and Alvarez et al[3], the authors proceeded to determine the optimal culture conditions by changing each of these three parameters. A study was also made on the difference due to the type of mitogens and antigens with detailed inquiry being made by one way MLR.

First with regard to incubation time, it was evident as shown in Fig. 3 that the optimal incubation time was 24 hr.

It is considered that the activity of IL-2 is expressed by absorption to the receptor located on the membrane of its target cell[4,22] and in fact IL-2 activity in the culture supernatant is expended by the target cell. Thus, the equation "IL-2 titer in the culture supernatant = Produced IL-2 activity — Expended IL-2 activity" is obtained. Therefore, in order to attain "IL-2" titer in the culture supernatant = Produced IL-2 titer" a condition must be selected where the IL-2 consumption by PBL themselves is the minimal. As evident in Fig. 3, there is an apparent lymphocyte proliferation after 24 hr of incubation and when fresh media were exchanged every 24 hr, a remarkable depression was observed between 24 — 48 hr of incubation, suggesting indirectly that a large amount of IL-2 was expended during this period. The optimal incubation time of 24 hr in man differs from the optimal incubation time of 48 hr in mouse[14].

As apparent from Fig. 4, there is no great difference in optimal concentration of PHA-M between 1% and 5%. It is therefore considered that the condition of 1% PHA-M employed by Gillis et al[13,14] and Alvarez et al[3] is satisfactory.

As for the number of lymphocytes, it is evident from Fig. 5 that IL-2 titer in the culture supernatant presented satisfactory values at $1 \times 10^6 - 5 \times 10^6$ cells/ml. However, this titer showed the highest value at $5 \times 10^5 - 1 \times 10^6$ cells/ml when converted to per lymphocyte. It is considered that the culture condition in which the maximum IL-2 production can be attained per lymphocyte is superior to the culture condition in the maximum IL-2 production can be attained by all the lymphocytes in culture, but at the same time the titer itself of the culture supernatant should also be adequate. It was therefore assumed that the optimal condition is 1×10^6 cells/ml, the value common to both conditions.

In the culture supernatant of one way MLR, IL-2 activity equivalent to that by PHA-M stimulation was observed. However, in the case of PHA-M stimulation, the peak was reached on the first day of culture (24 hr.), whereas in the case of one way MLR, the peak was observed on the fourth day of incubation (Fig. 3 and Fig. 6.) In both cases, lymhocyte proliferation reached its peak three days after the peak of IL-2 activity. This finding indicates indirectly that the produced IL-2 induces lymphocyte proliferation and further the delay in lymphocyte proliferation observed in one way MLR in comparison with that by PHA-M stimulation is considered to be attributable to delay in IL-2 production. It is assumed that the difference in response pattern between mitogen (lectin) and antigen is related to this.

As for the mitogens which induce production of IL-2 in human PBL, PHA-M and PHA-P have almost equivalent activity as can be seen in Table 1, but Con A which is a T cell mitogen simi-

lar to PHA has an activity slightly lower in level than PHA. The same can be said with regard to lymphocyte proliferation. However, in the case of mouse, this relationship between PHA and Con A becomes reversed[14]. In comparison with these T cell mitogens, IL-2 production induced by PWM which is a stimulator for both T cells and B cells is still lower in level, while IL-2 is hardly produced at all by LPS which is a stimulator of both B cells and macrophages. Despite the fact that PPD skin test was positive for both donor 1 and donor 2, IL-2 production by PPD stimulation was unexpectedly low in level. This may be attributalble to the fact that the action of PPD itself is weaker as an antigen than that of MLR and to the fact that the incubation time of 24 hr might have been too short in view of the culture condition of MLR.

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