Eur. Cytokine Netw., Vol. 3 n°2, *March-April 1992*, pp. 97-102

A comparison of assays for the response of primary human T-cells upon stimulation with interleukin-2, interleukin-4 and interleukin-7

Albert Duschl, Ute Jahn, Claudia Bertling and Walter Sebald

Theodor-Boveri-Institüt fur Biowissenschaften der Universität Würzburg, Physiologische Chemie, Am Hubland, 8700 Wurzburg, FRG

Correspondence: Dr. Albert Duschl, Theodor-Boveri-Institüt fur Biowissenschaften der Universität Würzburg, Physiologische Chemie, Am Hubland, 8700 Wurzburg, FRG. Tel : (49) 931-888-4117 Fax : (49) 931-888-4113

ABSTRACT. The most commonly used assay to quantitate the response of peripheral T-cells upon stimulation with growth factors is determination of incorporated [^{3}H]TdR. We compared this test to three other methods: 1. direct counting of cells with a Coulter type counter as reference assay, 2. a colorimetric assay using the tetrazolium dye 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium (MTT), which is a cheap and increasingly popular non-radioactive method and 3. incorporation of the thymidine analog 5-bromo-2'-deoxyuridine detection with a monoclonal antibody on cytospins. Primary human PHA-blasts from >30 healthy individuals were stimulated with IL-2, IL-4 and IL-7 and assayed with up to four different methods. We discuss the advantages and disadvantages of the assays used and the effects of differences between cell preparations. We observed no significant variations between individuals for the dose dependence, but the relative efficiency of IL-4 compared to IL-2 and IL-7 was variable. This was probably due to the slower response observed upon stimulation with this factor.

Keywords: T-cells, proliferation assays, IL-2, IL-4, IL-7.

INTRODUCTION

Interleukins (IL) induce numerous effects in lymphocytes. Ion movements, protein phosphorylation, gene activation, expression of surface markers and other events associated with differentiation are observed upon stimulation with appropriate IL's [1 - 8]. Most frequently, however, induction of DNA synthesis is used to monitor cellular response to IL. We have compared the widely used [^{3}H]TdR-assay with three other methods that can detect post-stimulation events in T-cells. Counting of cells by means of a Coulter-type counter, incorporation of the thymidine analog 5-bromo-2'deoxyuridine (BrdU) and detection with a monoclonal antibody on cytospins, and cleavage of the tetrazolium dye 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial dehydrogenases.

Human PBMC enriched in T-cells were preactivated for 3 d with PHA and subsequently stimulated with IL-2, IL-4 or IL-7. All of these factors induce proliferation of primed T-cell populations independent of other growth factors [9 - 11], but have quite different properties otherwise. IL-2 is produced by activated T-cells and promotes the proliferation and differentiation of T-cells, B-cells and NK-cells, among others [9]. IL-4 is produced by a small percentage of activated T-cells (most recently estimated to be ~ 0.1% for BALB/c mice [12]) and by mast cells, as first described in the mouse system [13]. It was described as a differentiation factor for hematopoietic stem cells, thymocytes, T-cells and B-cells, with a particular relevance for allergic responses [14, 15]. IL-7 is produced by stromal cells in bone marrow, spleen and thymus, and is best known for its role in thymocyte development and hematopoiesis [16, 17].

We used T-cell preparations from over 30 different individuals to compare the maximal proliferation induced, the dose-dependence and the relative efficiency of the three factors. Our goal was to establish the effect of the assay used and of differences between cell preparations and individual donors on the parameters measured. The results of this study allow considerations about the selection of an appropriate assay in experimental situations and the minimal number of cell batches that should be tested for quantitative estimations.

MATERIALS AND METHODS

Cells and growth factors. Lymphocyte concentrates from healthy blood donors were obtained from two local institutions (Institut für Transfusionsmedizin der Universität and Blutbank des Roten Kreuzes). For experiments involving repeated testing of samples from the same individuum, 30-35 ml of whole blood was collected from volunteers within the laboratory. In all cases, PBMC were enriched by Ficoll-centrifugation, washed three times in HANKS's buffered salt solution and cultured in RPMI 1640 with an addition of 10% FCS, 100 U/ml Penicillin and

100 µg/ml Streptomycin (all media components from Biochrom, Berlin, FRG) at 37°C and 5% CO_2. Cells were prestimulated for 3 days with 9 µg/ml PHA (HA15; Wellcome, Dartford, England), washed twice with RPMI 1640 and incubated with complete medium in a 96 well mikrotitre tray with different concentrations of interleukin.

Human IL-2, IL-4 and IL-7 were expressed in *Escherichia coli* and purified as described [18, 19]. All three factors were prepared in the laboratory.

MTT-test. The colorimetric assay for cleavage of the dye MTT was performed as described by Mosmann [20]. Briefly, 3 d PHA-prestimulated cells were suspended in 96 well roundbottom microtitre trays with different concentrations of interleukin at 2.5 x 10^5 cells/ml and 100 µl volume. For the assay, 10 µl of a 5 mg/ml solution of MTT (Sigma) was added and the tray was incubated at 37°C for 4 hours 100 µl isopropanol/0.04 N HCl was added and the dye crystals formed were suspended with an 8-tip-pipet. The optical density of each well was determined at 550 nm against a reference of 630 nm with an automatic plate reader (MR 600; Dynatech, Billingshurst, England). All experiments were performed in duplicate.

[^{3}H]TdR incorporation. Cells were incubated in duplicate with or without interleukins at a volume of 200 µl and a concentration of 5 x 10^5/ml. [^{3}H]TdR (Amersham Buchler, Braunschweig, FRG) was added at a concentration of 100 nM and a specific activity of 0.5 µCi/well. After incubation at 37°C for 4 hours, cells were harvested with a Cell Harvester (Skatron Instruments, Lier, Norway) on original Skatron filters, which were dried and counted in a radio-TLC-analyser (RITA-90; Raytek Scientific Ltd., Sheffield, England).

BrdU-test. For incorporation and detection of 5-bromo-2'-deoxyuridine [21, 22], the BrdU/alkaline phosphatase-kit (RPM 20) from Amersham Buchler was used according to the manufacturers directions. Incubation time with BrdU was varied between 1 hour and 4 days. Labelled cells were centrifuged onto slides in a cytospin, airdried and the label was developed. The cells were counterstained with the cytoplasmic dye Eosin Y (Serva).

Cell counting and analysis. PHA-prestimulated cells were cultured at a volume of 200 µl and a density of 1-5 x 10^5 cells/ml in microtitre trays with IL-2, IL-7 (both at 50 ng/ml), IL-4 (250 ng/ml) or in medium alone. After the incubation times indicated, duplicate 25-100 µl aliquots of cells were resuspended in 10 ml PBS and immediately counted in an automatic cell counter (CASY 1; Schärfe System, Reutlingen, FRG). Analysis of surface markers was performed with a Becton Dickinson (Mountain View, USA) FACScan. PE-coupled anti-CD3-antibody was from Becton Dickinson, FITC-coupled PHA-E was from E-Y Labs (San Mateo, USA).

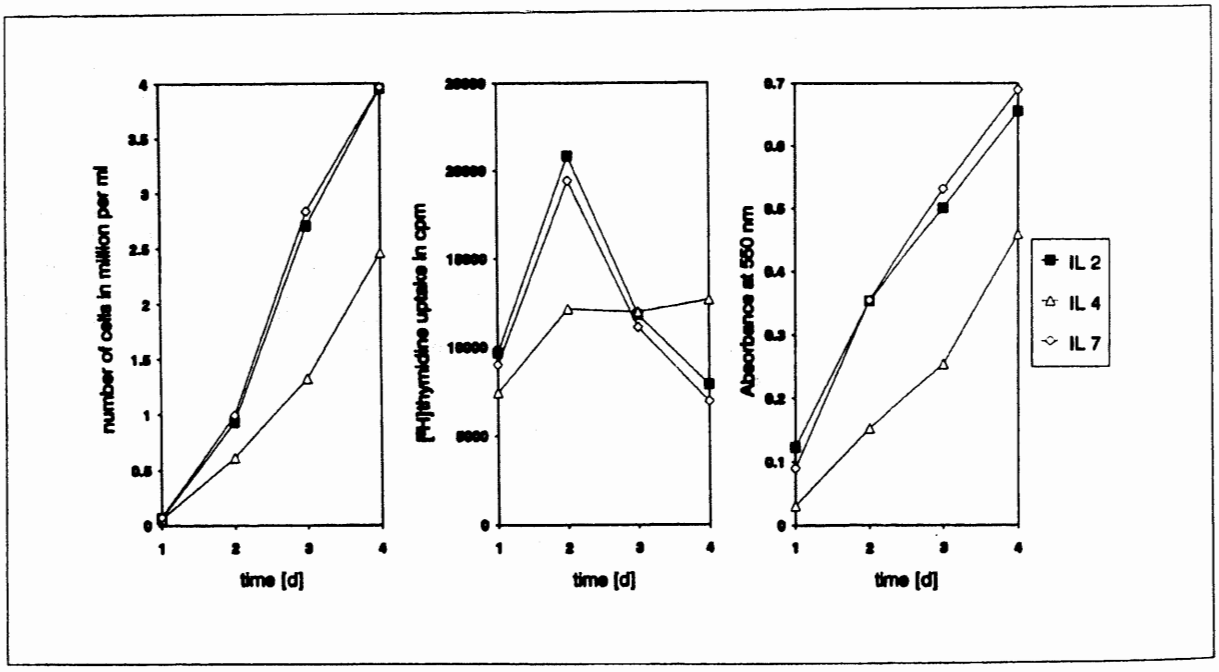

Figure 1.
Cells were preactivated for 3 days with PHA, stimulated with 50 ng/ml of IL-2 or IL-7, or 250 ng/ml of IL-4 and assayed with cell counter (a), [^{3}H]TdR-incorporation (b) and MTT-test (c). Values for unstimulated control cells were subtracted. The results shown give the average of three separate experiments, where the same cell preparation was tested with each of the three methods.

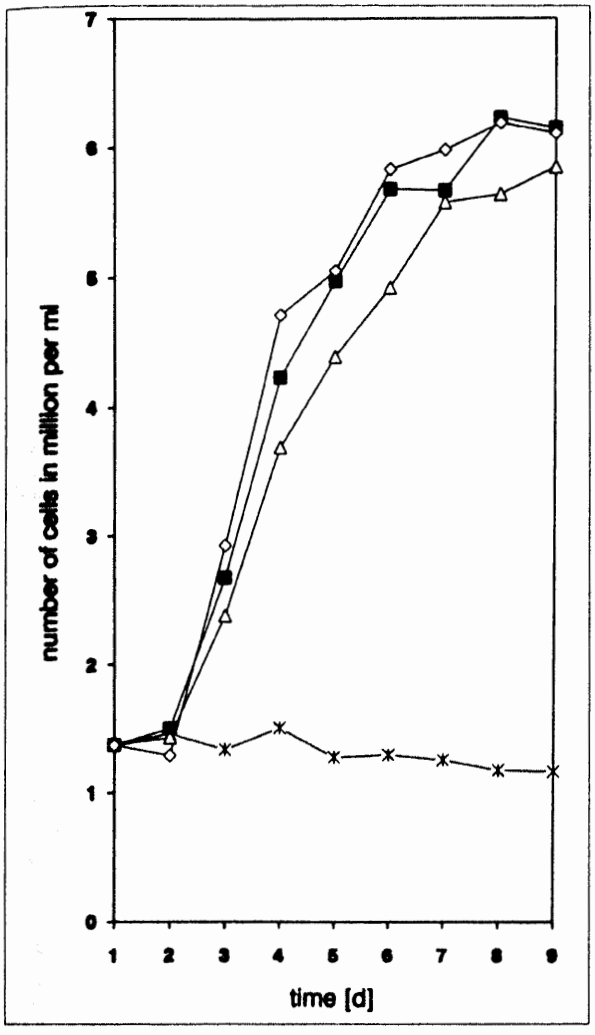

Figure 2.
Cell numbers counted upon stimulation of PHA-preactivated cells with saturating concentrations of IL-2, IL-7 (both 50 ng/ml) or IL-4 (250 ng/ml). Data given are the average of three independent experiments performed in duplicate. Control cells were kept in medium without stimulus.

RESULTS AND DISCUSSION

Pretreatment of cells. Interleukin-dependent proliferation of fresh lymphocytes reached a peak after 3-4 days of prestimulation with saturating concentrations of PHA, regardless of the cytokine tested. Flow cytometry showed that 80% (± 6%) of cells were CD3+ at that time and >95% bound PHA. However, a contribution of contaminating B-cells to the observed effects can not be excluded. Throughout this study, cells were stimulated with IL after 3 days of prestimulation with PHA. The first cell division started 16 hours after addition of IL-2, IL-4 or IL-7, but afterwards no synchronization was apparent (results not shown).

Cell counting with a Coulter type counter. Actual counting of cells is the only method that really determines proliferation, i.e. increase in cell number by cell division. The method is reliable and particularly well suited if the kinetics of cell proliferation have to be measured (figures 1a and 2). Cell counting is a necessary standard if the validity of a proliferation assay is tested (see below). The main drawback of the method is the limited number of samples that can be processed.

Incorporation of [^{3}H]TdR. The determination of radioactive labelled thymidine is a simple and straightforward assay to measure DNA-synthesis in a cell population. It can be seen from figure 1b that the timing of the assay is important, if factors are compared that induce DNA synthesis with different kinetics. IL-2 and IL-7 stimulated PHA-blasts in an identical fashion, while IL-4 acted slower even at the saturating concentration applied [23]. Measuring [^{3}H]TdR uptake at single time points to compare the efficiency of the three growth factors would lead to different results depending on the time of the assay. In fact, cell counting showed that IL-4 induced the same number of cell divisions in PHA blasts as IL-2 and IL-7, but with slower kinetics (figure 2). Cells might also give variable results if they grow partially synchronized. This can be overcome to some extent by using longer incubation times with the radiocative analog. An important advantage of the [^{3}H]TdR test is that it can usually be applied to different cell types without modifications, in contrast to the MTT-method.

MTT-assay. Data obtained from MTT-assays reasonably well reflected the cell number determined by automatic counting (figures 1a and 1c). It has been claimed that this method can not be applied to peripheral lymphocytes, due to some physiologic property of these cells, because cells stimulated 3 days with PHA failed to give a signal in the MTT assay, while producing strong [^{3}H]TdR-incorporation [24]. We have found the same effect, but cell counting showed that the number of cells after 3 days stimulation with PHA had recovered after a initial drop to approximately the same number as at the start of the experiment. For this reason no increase in MTT-cleavage could be measured, but as the cells proliferated vigorously, [^{3}H]TdR was incorporated (not shown). We therefore confirm that the MTT-assay is a good method to estimate cell numbers of lymphocytes [25]. For longer incubation times (3-4 days) the MTT-test indicated a lower value than the real cell number (figures 1a and 1c). This may have been due to a declining mitochondrial activity [20, 25]. However, after 3 days, our standard incubation

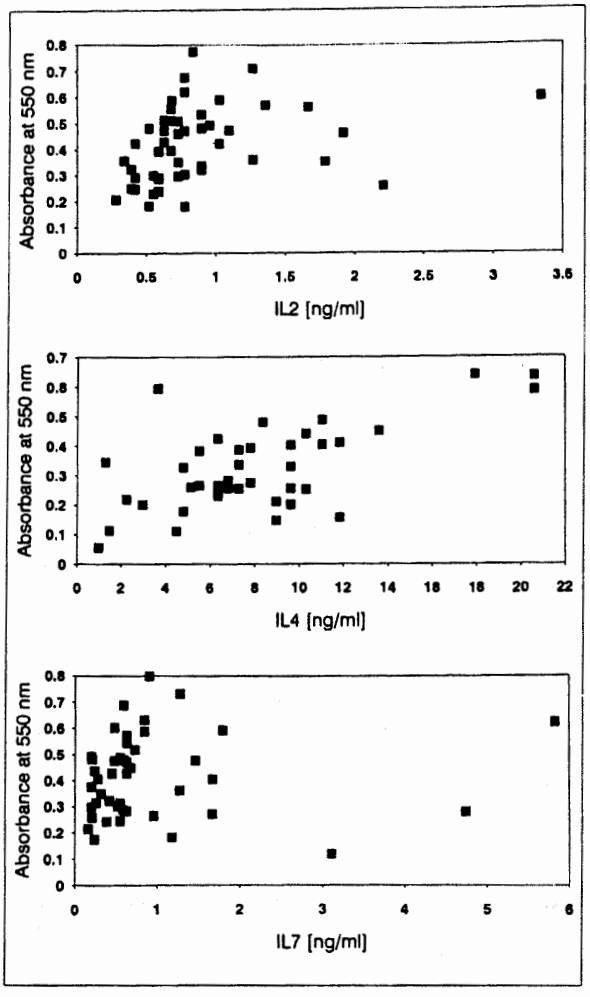

Figure 3.
Proliferation induced by IL-2, IL-4 or IL-7 in 3d PHA-blasts. The maximal signal obtained in the MTT-assay and the IL-concentration required for half-maximal stimulation are indicated. Values of the IL-dilution necessary for half-maximal stimulation followed a Gaussian distribution with a standard deviation of 0.7 dilution steps for IL-2, 1.0 for IL-4 and 1.2 for IL-7. The mean concentration required for half-maximal response was 780 pg/ml for IL-2, 6.8 ng/ml for IL-4 and 630 pg/ml for IL-7. The number of data points is larger here than in **figure 4**, because some experiments are included in which not all interleukins were tested.

time, the values were still reasonable. The MTT-method is much cheaper than any radioactive assay, but the compound is cancerogenic.

BrdU-test. Incubation with BrdU and detection with alkaline phosphatase-coupled antibodies stained up to 50% of the cells for each factor. While this allowed visualisation of individual responding cells, the method was in our experience not suitable as quantitative assay. Cells stained unequally on the slides and massive cell death occured after incubation with BrdU for one day or more.

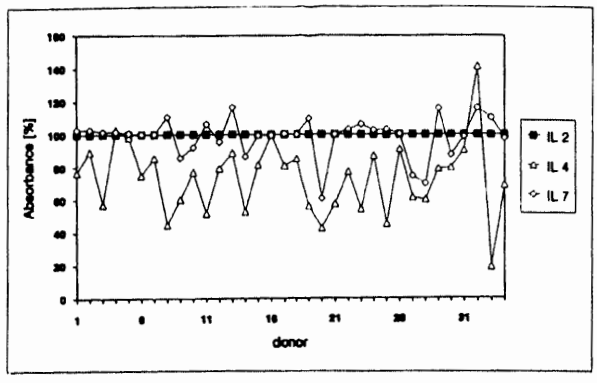

Figure 4.
Relative maximal proliferation induced by IL-2, IL-4 or IL-7 in PHA-blasts from 34 different donors. IL-2-induced proliferation is set as 100% and the relative response obtained with the other two interleukins is indicated.

Variations between cell preparations. All cell batches tested proliferated upon treatment with IL-2, IL-4 and IL-7 (figure 3). The concentrations required for half-maximal stimulation were distributed around a single peak with average values of 780 pg/ml (50 pM) for IL-2, 6.8 ng/ml (440 pM) for IL-4 and 630 pg/ml (37 pM) for IL-7.

Cell numbers obtained upon stimulation varied at least fivefold between different experiments for all factors tested. Maximal proliferation and dose requirement were independent of each other (figure 3). We estimate that about ten cell batches should be tested to quantitate the maximal proliferative response for a particular interleukin, while fewer batches may be sufficient to give a reasonable dose response value.

The proliferation induced by IL-4 was not correlated with the response to IL-2 or IL-7 (figure 4). IL-2 and IL-7 were comparably efficient as proliferation factors for PHA-blasts, as reported by others [26, 27], while IL-4 had not only to be applied in tenfold higher amounts, but even at saturating concentrations gave responses ranging from around 40% to >100% of the proliferation induced by IL-2 in the same test (figure 4). The large variations found for IL-4 were due to the time of assay (figure 2), which illustrates the importance of the protocol used for the determination of cellular responses.

Variations between individual donors. To find out how individual variations contribute to differences between cell preparations, we have repeatedly tested lymphocytes from four individual donors over a period of two month. The maximal proliferation induced varied by about a factor of two between different experiments with cells from the same donor,

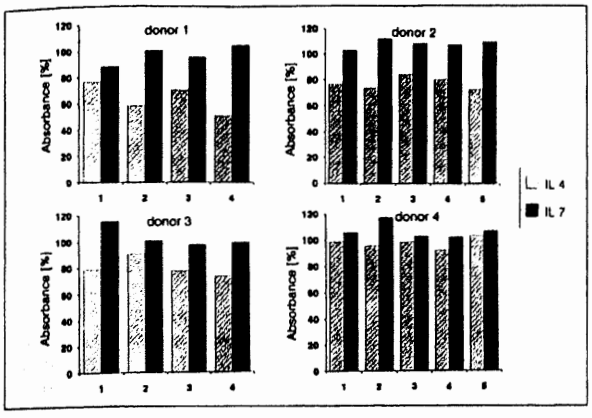

Figure 5.
Maximal proliferation induced by IL-2, IL-4 and IL-7 determined by MTT-assays in subsequent experiments over a period of two months for PHA-blasts from four individual donors. IL-2-induced proliferation is set as 100% and the relative response obtained with the other two interleukins is indicated. Grey column: IL-4, black column: IL-7.

while the relative response towards the three factors when compared to each other was rather reproducible (figure 5). In particular, low values for IL-4-induced proliferation were found in cells from donor 1, while high values were determined for donor 4 and intermediate ones for donors 2 and 3 (figure 5). The main variation between individuals seemed to be in the time that IL-4-stimulated cells needed to proliferate as vigorously as cells treated with IL-2 or IL-7. We conclude that differences during the preparations and in the composition of cell batches can not account for all variations observed. Therefore, cell samples from a number of individuals should be used whenever possible.

ACKNOWLEDGMENTS. We thank Drs. A. Simm and J. Hoppe for their help with the cell counter, and Dr. W. Pette and M. Goebel for flow cytometry. Technical assistance of C. Harmening and M. Schwartz is acknowledged.

REFERENCES

1. Farrar W L, Cleveland J L, Beckner S K, Bonvini E and Evans S W. 1986. Biochemical and molecular events associated with interleukin-2 regulation of lymphocyte proliferation. *Immunol. Rev.* 92:49.

2. Winkelstein A, Weaver L D, Salva N and Machen L L. 1990. Interleukin-2-induced lymphoproliferative responses. *Cancer Immunol. Immunother.* 32:110.

3. Turner B, Rapp U, App H, Greene M, Dobashi K and Reed J. 1991. Interleukin-2 induces tyrosine phosphorylation and activation of p72-74 Raf-1 kinase in a T-cell line. *Proc. Natl. Acad. Sci. USA.* 88:1227.

4. Augustine J A, Schlager J W and Abraham R T. 1990. Differential effects of interleukin-2 and interleukin-4 on protein tyrosine phosphorylation in factor-dependent murine T-cells. *Biochim. Biophys. Acta.* 1052:313.

5. Mills G B, Cragoe E J, Gelfand E W and Grinstein S. 1985. Interleukin-2 induces a rapid increase in intracellular pH through activation of a Na^+/H^+ antiport. Cytoplasmic alkalinization is not required for lymphocyte proliferation. *J. Biol. Chem.* 260:12500.

6. Ashida T, Kubo K I, Kawabata I, Katagiri M, Ogimoto M and Yakura H. 1990. Signal transduction mechanisms of Ia induction in B cells by interleukin-4 and immunoglobulin receptors. *Cell. Immunol.* 126:233.

7. Smyth M J, Norihisa Y, Gerard J R, Young H A and Ortaldo J R. 1991. IL-7 regulation of cytotoxic lymphocytes: pore-forming protein gene expression, interferon-γ production, and cytotoxicity of human peripheral blood lymphocyte subsets. *Cell. Immunol.* 138:390.

8. Uckun F M, Tuel-Ahlgren L, Obuz V, Smith R, Dibirdik I, Hanson M, Chandan-Langlie M and Ledbetter J A. 1991. Interleukin-7 receptor engagement stimulates tyrosine phosphorylation, inositol phospholipid turnover, proliferation, and selective differentiation to the CD4 lineage by human fetal thymocytes. *Proc. Natl. Acad. Sci. USA.* 88:6323.

9. Smith K A. 1988. Interleukin-2: inception, impact and implications. *Science.* 240: 1169.

10. Hu-Li J, Shevach E M, Mizuguchi J, Ohara J, Mosmann T and Paul W E. 1987. B cell stimulatory factor 1 (interleukin-4) is a potent costimulant for normal resting T lymphocytes. *J. Exp. Med.* 165:157.

11. Chazen G D, Pereira G M B, Le Gros G, Gillis S and Shevach E M. 1989. Interleukin-7 is a T-cell growth factor. *Proc. Natl. Acad. Sci. USA.* 86:5923.

12. Seder R A, Le Gros G, Ben-Sasson S Z, Urban J, Finkelman F D and Paul W E. 1991. Increased frequency of interleukin-4-producing T cells as a result of polyclonal priming. Use of a single-cell assay to detect interleukin-4-producing cells. *Eur. J. Immunol.* 21:1241.

13. Brown M A, Pierce J H, Watson C J, Falco J, Ihle J N and Paul W E. 1987. B cell stimulatory factor-1/interleukin-4 mRNA is expressed by normal and transformed mast cells. *Cell.* 50:809.

14. Jansen J H, Fibbe W E, Willemze R and Kluin-Nelemans J C. 1990. Interleukin-4. A regulatory protein. *Blut.* 60:269.

15. Paul W E. 1991. Interleukin-4: A prototypic immunoregulatory lymphokine. *Blood.* 77:1859.

16. Okazaki H, Ito M, Sudo T, Hattori M, Kano S, Katsura Y and Minato N. 1989. IL-7 promotes thymocyte proliferation and maintains immunocompetent thymocytes bearing αβ or γδ T-cell receptors *in vitro*: synergism with IL-2. *J. Immunol.* 143:2917.

17. Henney C S. 1989. Interleukin-7: effects on early events in lymphopoiesis. *Immunol. Today.* 10:170.

18. Weigel U, Meyer M and Sebald W. 1989. Mutant proteins of human interleukin-2. Renaturation yield, proliferative activity and receptor binding. *Eur. J. Biochem.* 180:295.

19. Kruse N, Lehrnbecher T and Sebald W. 1991. Site-directed mutagenesis reveals the importance of disulfide bridges and

aromatic residues for structure and proliferative activity of human interleukin-4. *FEBS Lett.* 286:58.

20. Mosmann T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods.* 65:55.

21. Gratzner H G, Leif R C, Ingram D J and Castro A. 1975. The use of antibody specific for bromodeoxyuridine for the immunofluorescent determination of DNA replication in single cells and chromosomes. *Exp. Cell Res.* 95:88.

22. Gratzner H G. 1982. Monoclonal antibody to 5-bromo- and 5-iododeoxyuridine: A new reagent for detection of DNA replication. *Science.* 218:474.

23. Lorre K, Van Damme J and Ceuppens J L. 1990. A bidirectional network involving IL-2 and IL-4 in the alternative CD2 pathway of T cell activation. *Eur. J. Immunol.* 20:1569.

24. Chen C H, Campbell P A and Newman L S. 1990. MTT colorimetric assay detects mitogen responses of spleen but not blood lymphocytes. *Int. Arch. Allergy Appl. Immunol.* 93:249.

25. Denizot F and Lang R. 1986. Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J. Immunol. Methods.* 89:271.

26. Armitage R J, Namen A E, Sassenfeld H M and Grabstein K H. 1990. Regulation of human T cell proliferation by IL-7. *J. Immunol.* 144:938.

27. Gately M K, Desai B B, Wolitzky A G, Quinn P M, Dwyer C M, Podlaski F J, Familletti P C, Sinigaglia F, Chizonnite R, Gubler U and Stern A S. 1991. Regulation of human lymphocyte proliferation by a heterodimeric cytokine, IL-12 (cytotoxic lymphocyte maturation factor). *J. Immunol.* 147:874.

Received : February 25, 1992 ; Accepted : March 31, 1992