Immunology 1986 **59** 57–61

Synergy between interleukin-2 and a second factor in the long-term growth of human T cells

BONNIE J. MILLS,* J. E. SHIVELY,* PEGGIE S. MITSKY,* D. H. HAWKE,* S. J. FORMAN,† CHRISTINE L. WRIGHT† & C. W. TODD* **Division of Immunology, Beckman Research Institute of the City of Hope, and †Department of Hematology and Bone Marrow Transplantation, City of Hope National Medical Center, Duarte, California, U.S.A.*

Accepted for publication 7 May 1986

SUMMARY

It has recently been shown that factors in addition to interleukin-2 (IL-2) are required for the proliferation or differentiation of at least some murine T-cell lines. We have previously shown that conditioned medium from human mononuclear cells stimulated with phorbol ester and staphylococcal enterotoxin A is superior to commercial sources of IL-2 for the long-term growth of human T cells. We have identified in these supernatants a non-IL-2 factor (synergistic factor, SF) which synergizes with JURKAT IL-2 in the long-term growth of human T cells. [^{3}H]TdR incorporation by IL-2-dependent human T cells after growth in IL-2 or SF alone for 14 days was slight, but significant. By contrast, growth in a combination of SF and IL-2 for 14 days stimulated [^{3}H]TdR incorporation 10–20-fold higher, generally equal to the high incorporation measured when cells were grown in the presence of the conditioned medium from which SF was obtained. In a standard 2-day IL-2 assay, there was no correlation between activity and long-term growth-promoting ability. These results suggest that the 14-day assay better discerns the growth-promoting activity of various factors or combinations of factors. The mechanism of this interaction between SF and IL-2 remains to be elucidated. It is clear, however, that T-cell growth factor activity, when assessed by the long-term growth of human T cells, is not due to interleukin-2 alone.

INTRODUCTION

The discovery of T-cell growth factor (TCGF, interleukin-2, IL-2) a few years ago made it possible for the first time to maintain long-term cultures of non-leukaemic T cells, and further, to obtain antigen-specific helper or cytotoxic T-cell lines (Gillis *et al.*, 1978a; Glasebrook & Fitch, 1980; Lamb *et al.*, 1982). The problems encountered in purifying many lymphokines have necessitated the use of crude supernatants as the primary source of these for experimental use, making it difficult to assign specific function to specific molecules. It has, in fact, recently been shown that additional factors are required for the induction of alloreactive cytolytic T cells (Garman & Fan, 1983) and long-term culture of murine natural killer cells (Olabuenaga *et al.*, 1983). The differing ability of IL-2 preparations to support long-term T-cell growth, even though all may induce maximal proliferation in an IL-2 assay, suggests that there are either differences in the IL-2 molecule from different sources, or that additional factors may be involved. We have previously described a method for the preparation of conditioned medium that is superior to commercial IL-2 sources for the support of long-term growth of human T cells. These studies suggested the presence of either an altered, more potent IL-2 or an additional T-cell growth factor(s) in these supernatants. We show here evidence for the synergistic interaction of at least two factors to produce optimal growth of IL-2-dependent human T cells without cycles of antigen or mitogen restimulation. We have designated as synergistic factor (SF) the substance(s) present in our conditioned medium, which is able to synergize with IL-2 to allow optimal long-term growth of human IL-2-dependent T cells.

Abbreviations: CM-AT, acid-treated conditioned media; IL-2, interleukin-2; SEA, staphylococcal enterotoxin A; SF, synergistic factor; TCGF, T-cell growth factor; cTCGF, commercial TCGF; TdR, thymidine; TPA, 12-0-tetradecanoyl-phorbol-13-acetate.

Correspondence: Dr Bonnie J. Mills, Division of Immunology, Beckman Research Institute of the City of Hope, 1450 E. Duarte Road, Duarte, CA 91010, U.S.A.

MATERIALS AND METHODS

Reagents

Staphylococcal enterotoxin A (SEA) was kindly provided by Dr G. K. Murthy, FDA, Cincinatti, OH. 12-0-tetradecanoyl-phorbol-13-acetate (TPA) was purchased from Sigma Chemical Co., St Louis, MO. Antibody to the IL-2 receptor was purchased from Becton-Dickinson (Mountain View, CA).

T-cell lines and conditioned medium

Human peripheral blood mononuclear cells obtained by lymphocytophoresis were preincubated at 37° for 1–2 hr (10^6 cells/ml) in RPMI-1640 containing 1X antibiotics (Gibco, Grand Island, NY), 10% fetal calf serum and 5 ng/ml TPA. SEA, 0·1 μg/ml, was then added, and culture was continued at 37° in 5% CO_2 for 40 hr. Supernatants were then harvested, acid treated as previously described (Mills, Mitsky & Todd, 1984), sterile filtered, and used for SF purification or as an IL-2 source for our T-cell cultures. Most T-cell cultures were established from 4-day TPA/SEA-stimulated human mononuclear cells, which were subsequently maintained in complete medium (RPMI-1640 supplemented with 10% fetal calf serum, 1% antibiotics, 10^{-5} M mercaptoethanol, 50 mg/l vitamin C, 110 mg/l sodium pyruvate) with 20–25% acid-treated conditioned medium (CM-AT) from TPA/SEA-induced cells. More recently, we have been able to establish cultures from human mononuclear cells initially stimulated for 5–7 days in culture with human cytomegalovirus antigens as described by Forman *et al.* (1985). Commercially produced IL-2 (cTCGF) was purchased from Associated Biomedics (Buffalo, NY), partially purified JURKAT IL-2 was a gift from Dr Carl Ware (University of California, Riverside, CA), and recombinant IL-2 was purchased from Amgen (Thousand Oaks, CA).

Partially purified SF

The synergistic factor was partially purified by sequential reverse-phase HPLC steps. Briefly, large volumes (up to 2 l) of supernatant were run over a large-capacity HPLC column containing either Microbondapak phenyl (Waters Associates, Milford, MA) or Microbondapak ODS (Waters Associates). Elution was performed with a stepwise CH_3CN gradient in 0·1% trifluoroacetic acid. Active fractions (54–64% CH_3CN) were pooled and subjected sequentially to Microbondapak phenyl, Vydac C4 (The Separations Group, Hesperia, CA) and Microbondapak phenyl HPLC steps using the same elution conditions. Activity was eluted at 60–78%, 66–76% and 62–64% CN_3CN, respectively. Activity from the last step corresponded to the major protein peak (Fig. 1). However, this material still contained three to four major protein bands, all between 12,000 and 30,000 molecular weight (MW), as determined by SDS–polyacrylamide gel electrophoresis. For long-term assays, fraction no. 44 (Fig. 1) of partially purified SF was used at a dilution of 1/10,000, the highest dilution that usually retained maximal activity in the short-term assay.

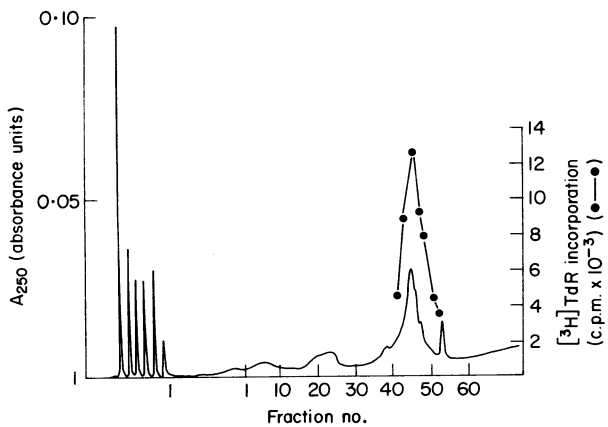

Figure 1. Protein (solid line) and activity (●——●) profiles of partially purified SF from the final RP-HPLC step (Microbondapak phenyl) from which Fraction 44 was obtained. Activity shown is that detected in a short-term IL-2 assay. Elution was with a CH_3CN gradient in 0·1% TFA. Multiple peaks early in the profile are related to multiple sample injections. The bar below the last injection peak indicates the start of the CH_3CN gradient.

IL-2 assay

IL-2 activity in cell supernatants and column fractions was determined in a short-term IL-2 microassay (Gillis *et al.*, 1978b) using a single lot of commerical TCGF (Associated Biomedics), hereafter referred to as cTCGF, as a standard. Briefly, IL-2-dependent human T cells (10^5 cells/100 μl/well) were incubated in complete medium together with appropriate dilutions (100 μl/well) of control or test samples for 24 hr prior to the addition of 0·5 μCi[^{3}H]TdR/10 μl/well (6·7 Ci/mmol). After an additional 6–8 hr of incubation, the labelled cells were harvested onto glass fibre filters, and [^{3}H]TdR incorporation was determined. In the absence of an IL-2 source, these cells do not incorporate appreciable [^{3}H]TdR.

Because of subtle differences in the ability of various IL-2 preparations to maintain long-term T-cell growth, some preparations or column fractions were also tested in long-term assays, which make these differences more obvious. Long-term IL-2 assays were set up in a fashion identical to the short-term IL-2 assay described above. Nutrients were replenished every 3 days by the removal of 100 μl of medium/well and the addition of 100 μl of medium containing the test sample of IL-2 at the appropriate concentration. The source and concentration of Il-2 supplied to any single well was consistent thoughout the experiment. After 13 days of culture in 5% CO_2 at 37°, 0·5 μCi/10 μl/well [^{3}H]TdR was added. Following an additional 24 hr of incubation, the plates were harvested and the [^{3}H]TdR incorporation was determined. Each value represents an average of three determinations.

Characterization of cell lines

Cell surface markers were determined by indirect immunofluorescence with monoclonal antibodies from Ortho (Raritan, NJ) or Becton-Dickinson (anti-IL-2 receptor) according to published methods (Forman *et al.*, 1982). In some instances, fluorescence was evaluated using a FACS IV cell sorter (Becton-Dickinson).

RESULTS

Relative activity of growth factors

Figure 2 illustrates the relative activities of various sources of T-cell growth factor, as determined in a short-term assay. It is interesting to note that although standard calculations would indicate much greater activity in the SF preparation and slightly more in CM-AT when compared to cTCGF, neither stimulated [^{3}H]TdR incorporation to the same level as cTCGF, even at optimal concentrations (Fig. 2a). Thus, IL-2 units may be a somewhat misleading description of the growth-stimulating activity of a particular preparation. Arrows in Fig. 2 indicate the concentration of each growth-factor source that was tested in the long-term assay. For cTCGF, CM-AT, Amgen and SF, this was a dilution that usually produced maximal or near maximal

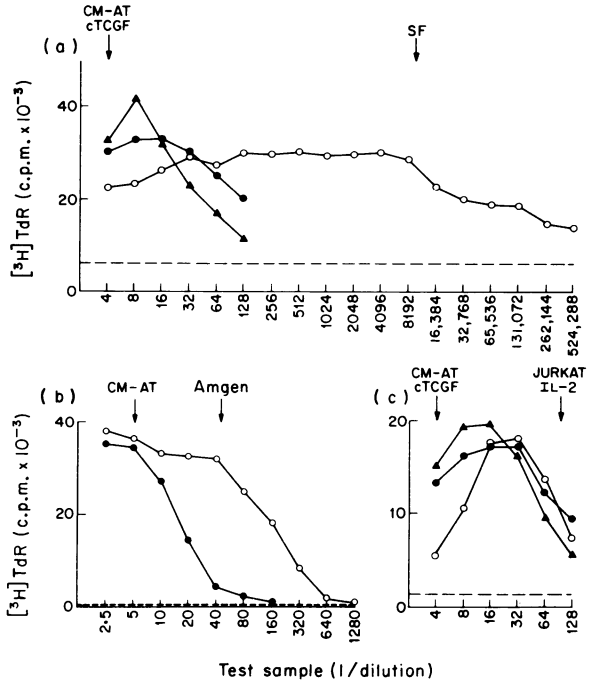

Figure 2. Relative abilities of T-cell growth factor sources to stimulate [^{3}H]TdR incorporation by IL-2-dependent T cells in a short-term assay. (a) Relative activities of CM-AT (●——●), partially purified SF (○——○) and cTCGF (▲——▲). (b) Relative activities of recombinant (Amgen) IL-2 (○——○) and CM-AT (●——●). (c) Relative activities of CM-AT (●——●), partially purified JURKAT IL-2 (○——○) and cTCGF (▲——▲). Arrows indicate the concentration of each used for long-term assays. Dashed lines indicate [^{3}H]TdR incorporation in the absence of a growth-factor source.

stimulation (Fig. 2a and b). The concentration of JURKAT IL-2 used in the long-term assay (1/100) was based on recommendations of others who had used the same preparation, and was in fact less than optimal (Fig. 2c), but still sufficient to stimulate significant [^{3}H]TdR incorporation.

SF, IL-2-dependent cell lines

Using SF-containing CM-AT, we have been able to maintain mitogen- or antigen-stimulated human T cells in continuous culture for up to 1 year without restimulation. After some time in culture (>6 weeks) the uncloned cells become relatively homogeneous. Preliminary characterization suggests a range of cell phenotypes, all of T-cell origin, that develop under our culture conditions (Table 1). These conditions appear to favour the establishment of T8+cell lines, as the majority have this phenotype. All require both IL-2 and SF for maintenance of proliferation. Staining with antibody to the IL-2 receptor was performed on cell lines maintained in 20–25% CM-AT for >6 weeks. All cell lines showed weak or no detectable staining with this antibody in spite of their continued ability to respond to IL-2.

Although partially purified SF no longer contains the mitogen SEA, as determined by its distant elution position from the reverse-phase columns used to purify SF, our cell lines are maintained in 20–25% CM-AT, which still contains low levels of

Table 1. Cell surface markers, as determined by indirect immunofluorescence, of representative cell lines maintained >6 weeks in 20–25% CM-AT

		Cell surface markers					
Cell line	Inducer	T3	T4	T8	T10	Ia	Tac
830	TPA/SEA	+	+	−	NT*	NT	NT
426	TPA/SEA	+	+	±	+	+	NT
86	TPA/SEA	+	+	−	+	+	NT
JM 1211	TPA/SEA	+	−	+	NT	NT	−
CMV	CMV	+	−	+	+	+	NT
GP 64	GP 64	+	−	+	+	+	NT
MG-CMV	CMV	+	−	+	NT	NT	−
MG-GP 64	GP 64	+	−	+	NT	NT	−

* NT, not tested.

the inducers SEA and TPA. We were concerned that, although SF works well in a 2-week assay, SEA and/or TPA may be responsible for some of the very long-term TCGF activity of CM-AT. In order to test this, cells from a single individual, initially stimulated with cytomegalovirus antigens, were maintained for 46 days in 20% CM-AT; on Day 47 they were split into three flasks and grown in medium containing 20% CM-AT, comparable levels of SEA and TPA plus recombinant IL-2, or recombinant IL-2 only. The concentration of recombinant IL-2 used was that which produces optimal or near optimal [^{3}H]TdR incorporation in a short-term assay (Fig. 2b). Cell counts and viability were determined once or twice weekly. Media were changed, and cells readjusted to 10^6 cells/ml at this time. In addition, aliquots of each culture were removed and pulsed for 6 hr with [^{3}H]TdR to determine proliferation.

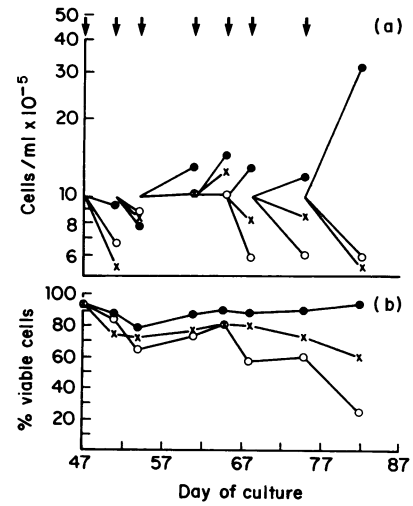

Figure 3. Cell counts (a) and viability (b) of a T-cell line maintained in CM-AT; TPA, SEA and IL-2; or IL-2 only. Cells that were initially stimulated with cytomegalovirus antigens were cultured in 20% CM-AT for 46 days. On Day 47 they were seeded at 10^6 cells/ml into 10 ml of media containing 20% CM-AT (●——●), SEA and TPA concentrations equivalent to 20% CM-AT plus 5U/ml IL-2 (Amgen) (×——×), or 5 U/ml IL-2 only (○——○). Media were changed and cells readjusted to 10^6 cells/ml once or twice weekly (indicated by arrows).

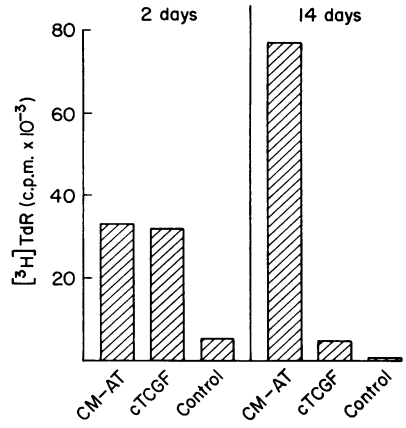

Figure 4. [^{3}H]TdR incorporation by IL-2-dependent human T cells in the presence of CM-AT (1/4) or cTCGF (1/4) as determined in a short-term (2 days) or long-term (14 days) assay. The control contains no IL-2 source.

As shown in Fig. 3, after an initial lag, all cultures picked up for at least a brief time. The number of viable cells in the SEA, TPA and IL-2, or IL-2 only cultures eventually dropped so low that the cultures could no longer be maintained. The cells in 20–25% CM-AT continued to proliferate and are now at Day 90 of culture. [^{3}H]TdR incorporation paralleled cell growth (data not shown).

Synergistic activity of SF and IL-2

Although cTCGF and CM-AT showed similar IL-2 activity titres in the short-term assay, when equivalent concentrations were tested in a long-term assay, only the CM-AT was able to support the long-term growth of IL-2-dependent T cells (Fig. 4). Because CM-AT was generally a better source of growth factor(s) for T-cell cultures than commercial IL-2 sources, and

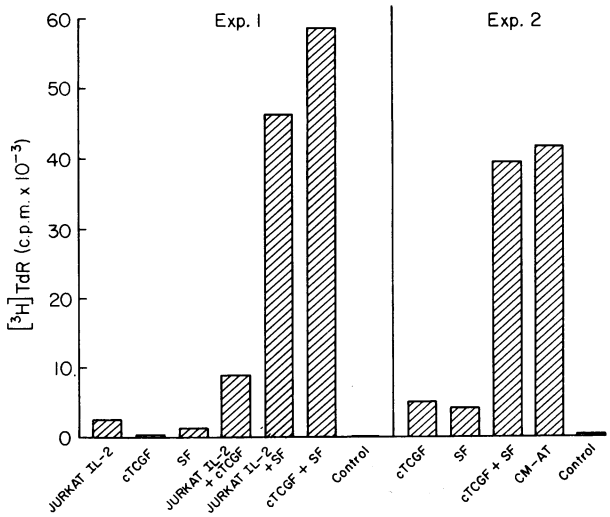

Figure 5. Stimulation of [^{3}H]TdR incorporation by IL-2-dependent human T cells in the presence of CM-AT (1/4), JURKAT IL-2 (1/100) or partially purified SF (1/10,000) alone or in combination, as measured in the long-term assay. The control contains no IL-2 or SF source.

because each is induced from different cells and/or with different inducers, we reasoned that IL-2 activity from CM-AT may be due to a related but more active IL-2 molecule, a non-IL-2 growth factor, or interaction between several factors. The partially purified SF from CM-AT in the absence of IL-2 did not support growth in a long-term assay (Fig. 5). Because of the observation that mixing sources of IL-2 sometimes improved their ability to support long-term growth (unpublished observation), various combinations of IL-2 sources, including partially purified SF, were tested in a long-term assay. As illustrated in Fig. 5, when SF was used in combination with cTCGF or with IL-2 from JURKAT cells, an impressive synergism was observed, thus the name synergistic factor. In some instances, the ability to support long-term growth was restored to that of CM-AT. By contrast, a combination of JURKAT IL-2 and cTCGF usually stimulated [^{3}H]TdR incorporation roughly equal to the total incorporation produced by either alone, and never greater than that shown in Experiment 1 (Fig. 5). Only SF was able to synergize consistently and convincingly with either JURKAT IL-2 or cTCGF, suggesting similarity between IL-2 activity from these two sources.

DISCUSSION

The definition of interleukin-2 as a lymphokine capable of supporting the long-term growth of stimulated normal T lymphocytes was based on its activity as part of a complex mixture of lymphokines in a short-term assay. Even when pure human IL-2 became available, activity was based on a short-term assay. It has been shown that CTLL-2, a commonly used murine target cell, may be unique in its requirement for only IL-2 for long-term growth (Olabuenaga *et al.*, 1983). Some other murine IL-2-dependent cell lines apparently require additional growth factors. Several groups have indicated the necessity for, or the interaction of, additional non-IL-2 factors in the proliferation or differentiation of murine cytotoxic T-cell (Lutz, Glasebrook & Fitch, 1981; Raulet & Bevan, 1982; Finke *et al.*, 1983), and helper T-cell-like lines (Hapel *et al.*, 1981).

We have previously described a method for the preparation of conditioned medium from human mononuclear cells (CM-AT) which has T-cell growth-promoting activity superior to other IL-2 sources. Using CM-AT, we have been able to maintain human T cells in continuous culture for up to 1 year without mitogen or antigen restimulation. All cell lines were initiated from mitogen- or antigen-stimulated fresh human mononuclear cells. The cell surface markers, which have been determined for most of the cell lines at several time-points, indicate that the cells become relatively homogeneous after 6–8 weeks of culture, that all are of T-cell origin, and that they lose high level expression of IL-2 receptors.

Although part of the improved activity of CM-AT was attributed to the elimination of toxic substances by acid treatment (Mills *et al.*, 1984), comparable treatment of commercial TCGF did not similarly improve their ability to support long-term (> 6 weeks) T-cell growth. In order to determine the reason for this improved activity, we attempted to isolate IL-2 from CM-AT for comparison with JURKAT IL-2. We obtained a partially purified preparation from CM-AT with high activity in a standard IL-2 assay. The data presented here suggest, however, that this material contains a factor distinct from IL-2. We have named this factor synergistic factor (SF)

because of its ability to synergize with IL-2 in the long-term culture of human T cells. The strong synergism seen by mixing partially purified SF with JURKAT IL-2 or cTCGF suggests that these factors are different from SF, and that both (all) of these together facilitate T-cell growth far better than a single growth factor alone. The possibility that SF is a product of the induced mononuclear cells is indicated by the inability of non-conditioned media containing SEA and TPA and reconstituted with IL-2 to support long-term T-cell growth as well as CM-AT. It is clearly the 'conditioning' that makes CM-AT so effective. It is likely that the CM-AT contains all of the necessary factors since growth is optimal using this as an IL-2 source. However, since partially purified SF is active in the short-term IL-2 assay, the relative amounts of SF and IL-2 in any supernatant cannot be determined.

The increased incorporation stimulated by the addition of SF to JURKAT IL-2 could be explained simply by an increase in available IL-2, since the JURKAT IL-2 was used at a suboptimal concentration. If this were true, however, cTCGF should be equally effective in boosting JURKAT IL-2 since it is present at above optimal concentration. Likewise, cTCGF at optimal concentration should not require additional IL-2. It was, however, significantly boosted by the addition of SF. It is unlikely that the cells run out of IL-2 in the long-term assay since the media is replenished every 3 days, and since a comparable amount of CM-AT supports the cells exceptionally well.

It has been shown that restimulation of cell lines allows continued growth by reinduction of receptors for IL-2 (Andrew & Braciale, 1984). Absence of high IL-2 receptor expression on most cell lines maintained in CM-AT suggests that SF does not work by maintaining receptor expression. Others have described a 'threshold' type of mechanism for IL-2 response (Cantrell & Smith, 1984). Conceivably, SF allows cells to respond to IL-2 at below threshold level of receptor expression either by altering the receptor or the IL-2 molecule itself. This type of mechanism implies that highly purified SF would not have IL-2 activity. If, in fact, SF associates with IL-2, it is not unreasonable that at least small amounts may copurify with SF. In the presence of SF, such small amounts may show considerable activity in a short-term assay even though insufficient for long-term growth support. If so, it is this fortuitous association that has allowed us to partially purify SF by virtue of its IL-2 activity. Studies are in progress to obtain highly purified SF which should allow its mechanism to be elucidated.

Our data indicate that the long-term assay would improve the predictability of the long-term growth-promoting activity of various factors, as there is good correlation between the activity of IL-2 sources in this assay and their ability to maintain long-term T-cell cultures. Thus, CM-AT has been an excellent source of growth factors for IL-2-dependent cells in contrast to most commercial preparations. A primary goal of future studies is the development of an easier, less lengthy assay for IL-2 synergistic growth factors, as the 2-week assay is tedious, difficult, and subject to many variables, and therefore impractical to use at each purification step. It is now clear that IL-2 and T-cell growth factor are not equivalent. At least one, and possibly more, additional factors are required for the long-term growth of at least some human IL-2-dependent T-cell lines.

ACKNOWLEDGMENTS

The authors thank Dr Carl Ware for JURKAT IL-2, Mr Jim Bolen for FACS analysis, and Marla Capalbo and Anita Milliken for secretarial assistance.

This research was made possible in part by the Chuck Buttner Research Fund.

REFERENCES

ANDREW M.E. & BRACIALE T.J. (1984) Regulation of interleukin 2 receptor expression on murine cytotoxic T lymphocyte clones. *J. Immunol.* **132,** 839.

CANTRELL D.A. & SMITH K.A. (1984) The interleukin-2 T cell system: a new cell growth model. *Science*, **224,** 1312.

FINKE J.H., SCOTT J., GILLIS S. & HILFIKER M.L. (1983) Generation of alloreactive cytotoxic T lymphocytes: evidence for a differentiation factor distinct from IL-2. *J. Immunol.* **130,** 763.

FORMAN S.J., NOCKER P., GALLAGHER M., ZAIA J., WRIGHT C., BOLEN J., MILLS B., HECHT J. & BLUME K. (1982) Pattern of T cell reconstitution following allogeneic bone marrow transplantation for acute hematological malignancy. *Transplantation*, **34,** 96.

FORMAN S.J., ZAIA J.A., CLARK B.R., WRIGHT C.L., MILLS B.J., POTTATHIL R., RACKLIN B.C., GALLAGHER M.T., WELTE K. & BLUME K.G. (1985) A 64,000 dalton matrix protein of human cytomegalovirus induces *in vitro* immune responses similar to those of whole viral antigen. *J. Immunol.* **134,** 3391.

GARMAN R.D. & FAN D.P. (1983) Characterization of helper factors distinct from interleukin 2 necessary for the generation of allospecific cytolytic T lymphocytes. *J. Immunol.* **130,** 756.

GILLIS S., BAKER P.E., RUSCETTI F.W. & SMITH K.A. (1978a) Long-term culture of human antigen-specific cytotoxic T cell lines. *J. exp. Med.* **148,** 1093.

GILLIS S., FERM M.M., OU W. & SMITH K.A. (1978b) T cell growth factor: parameters of production and a quantitative microassay for activity. *J. Immunol.* **120,** 2027.

GLASEBROOK A.L. & FITCH F.W. (1980) Alloreactive cloned T cell lines. I. Interactions between cloned amplifier and cytolytic T cell lines. *J. exp. Med.* **151,** 876.

HAPEL A.J., LEE J.C., FARRAR W.L. & IHLE J.N. (1981) Establishment of continuous cultures of Thy 1.2^+, Lyt $1^+,2^-$ T cells with purified interleukin 3. *Cell*, **25,** 179.

LAMB J.R., ECKELS D.D., LAKE P., JOHNSON A.H., HARTZMAN R.J. & WOODY J.N. (1982) Antigen-specific human T lymphocyte clones: induction, antigen specificity, and MHC restriction of influenza virus-immune clones. *J. Immunol.* **128,** 233.

LUTZ C.T., GLASEBROOK A.L. & FITCH F.W. (1981) Alloreactive cloned T cell lines. III. Accessory cell requirements for the growth of cloned cytolytic T lymphocytes. *J. Immunol.* **126,** 1404.

MILLS B.J., MITSKY P.S. & TODD C.W. (1984) Acid treatment enhances IL-2 activity of conditioned medium. *J. immun. Meth.* **67,** 73.

OLABUENAGA S.E., BROOKS C.G., GILLIS S. & HENNEY C.S. (1983) Interleukin 2 is not sufficient for the continuous growth of cloned NK-like cytotoxic cell lines. *J. Immunol.* **131,** 2386.

RAULET D.H. & BEVAN M.J. (1982) The differentiation factor required for the expression of cytotoxic T cell function. *Nature (Lond.)*, **296,** 754.