The Journal of Immunology

RESEARCH ARTICLE | DECEMBER 01 1980

Thymoma production of T cell growth factor (Interleukin 2). FREE

J J Farrar; ... et. al

J Immunol (1980) 125 (6): 2555–2558.
https://doi.org/10.4049/jimmunol.125.6.2555

0022-1767/80/1256-2555$02.00/0
THE JOURNAL OF IMMUNOLOGY

Vol. 125, No. 6, December 1980
Printed in U.S.A.

THYMOMA PRODUCTION OF T CELL GROWTH FACTOR (INTERLEUKIN 2)

JOHN J. FARRAR,[1] JANET FULLER-FARRAR, PHILIP L. SIMON, MARY L. HILFIKER, BEDA M. STADLER, AND WILLIAM L. FARRAR

From The Laboratory of Microbiology and Immunology, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland 20205, and, The Department of Pathology, The Johns Hopkins School of Medicine, Baltimore, Maryland 21205

Phorbol-12-myristate-13-acetate stimulates a subline of mouse EL-4 thymoma cells to produce, *in vitro*, in very high titer, T cell growth factor (Interleukin 2, IL 2). The EL-4-derived IL 2 has the same m.w. (30,000) and isoelectric point heterogeneity (pI 3.8-4.4) as the IL 2 produced by Con A-stimulated spleen cells. In addition, the thymoma-derived IL 2 exhibits the same spectrum of biologic activities as has been reported for spleen cell-derived IL 2.

Stimulated mouse spleen cells produce in culture a 30,000 m.w. protein (pI 4.0–5.0) that has been designated Interleukin 2 (IL 2)[2, 3]. IL 2 exhibits a broad spectrum of immunoenhancing activities on mouse lymphocytes *in vitro* including augmentation of antibody synthesis (1, 2), induction of allospecific cytotoxic T cells (1–4), enhancement of mitogen-induced T cell proliferation (1–3, 5), and maintenance of T cell line growth (6). Factors with similar biologic activities are also produced in rat as well as human lymphocyte cultures (7). Recently, partially purified preparations of murine IL 2 have been shown to be efficacious in restoring immunologic deficiencies *in vivo* (8, 9). We recently reported that the tumor promotor phorbol-12-myristate-13-acetate (PMA) enhanced the production of IL 2 by concanavalin A (Con A)-stimulated mouse spleen cells (10) and replaced the requirement for macrophages during the production of IL 2 by purified splenic T cells (11). The purification of lymphokines in general and IL 2 in particular has been hindered by the inability to generate large quantities of factor with high specific activity. In this report, we demonstrate that PMA stimulates the EL-4 thymoma to produce, in high titer, an IL 2 that is biochemically and biologically indistinguishable from normal spleen cell-derived IL 2.

MATERIALS AND METHODS

EL-4 thymoma. EL-4 (H-2^b) thymoma cells were grown in RPMI 1640 medium containing gentamicin (50 μg/ml) and 10% fetal calf serum (FCS) to a concentration of approximately 1 × 10^6 cells/ml. In some experiments, EL-4 cells were obtained after passage of the tumor cells in the ascitic form in C57BL/6 mice.

Culture supernatants. EL-4 cells were harvested from either culture or the ascitic fluid, washed 3 times in serum-free RPMI 1640 medium, and resuspended to a cell concentration of 1 × 10^6/ml in medium with or without 1% FCS. The cells were cultured in tissue culture flasks for 40 hr in the presence or absence of 10 ng/ml PMA. At the end of the incubation period, the culture supernatants were harvested by centrifugation, sterilized by filtration, and assayed for biologic activity as described below. A standard Con A-induced mouse spleen cell culture supernatant was also prepared as previously described (1). Briefly, 10^7 spleen cells from C57BL/6 mice were stimulated in 1.0 ml cultures containing 1% FCS with 2.0 μg/ml Con A. The culture supernatants were harvested after 40 hr incubation at 37°C. This standard culture supernatant was dialyzed against tissue culture medium (RPMI 1640) to remove inhibitory substances and assayed for T cell growth activity as described below.

Gel filtration chromatography. Supernatants from PMA-stimulated EL-4 thymoma cultures were concentrated on Diaflow UM-10 membranes and applied to a calibrated 1.5 × 90 cm AcA-54 (LKB) column that had been equilibrated in 0.05 M Tris, 0.1 M NaCl buffer (pH 7.5, 4°C). The column was eluted at 4°C by using reverse flow at a flow rate of approximately 10 ml/h. Each fraction was assayed for biologic activity as described below.

Electrofocusing. Gel filtration fractions containing biologic activity (see below) were pooled and dialyzed against 0.01% polyethylene glycol (PEG) (m.w. 6000) and incorporated into a 0 to 40% sucrose gradient containing pH 3–10 ampholines (final concentration 1%) and 0.01% PEG. The electrofocusing was carried out in an LKB 8102 column at 6 watts with periodical adjustment to 1000 volts over the first 24 hr and run for a total of 66 hr after which the fractions were collected and assayed for biologic activity as described below.

Biological Assays for Interleukin 2

T cell growth activity. Culture supernatants and gel filtration and electrofocusing fractions were assayed for T cell growth activity as previously described (11). Briefly, the samples were tested for activity in cultures containing 10^4 CT-6 (IL 2-dependent T cell line) cells in a 24-hr assay followed by a 6-hr pulse with 0.5 μCi of ^{3}H-TdR (specific activity 2 Ci/mM). The amount of IL 2 was quantitated by the dilution analysis method described previously (11). Briefly, the standard Con A-induced mouse spleen cell culture supernatant was arbitrarily designated 100 units/ml and titrated in each experiment to deter-

Received for publication June 2, 1980.
Accepted for publication August 28, 1980.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Address reprint requests: Dr. John J. Farrar, Bldg 30, Rm. 326, NIDR, NIH, Bethesda, MD 20205.

[2] See letter to the editor by Aarden *et al.* in the December, 1979 issue of the Journal of Immunology on revised nomenclature for T cell proliferation and helper factors.

[3] Abbreviations used in this paper: IL 2, Interleukin 2; PMA, phorbol-12-myristate-13-acetate; PEG, polyethylene glycol; ^{3}H-TdR, tritiated thymidine; PFC, hemolytic plaque-forming cell; TPA, 12-O-tetradecanoyl-phorbol-13-acetate.

mine maximum cpm and from the regression line, the dilution giving a response equal to 50% of the maximum cpm response. Each unknown sample was also titrated and the dilution corresponding to the $cpm_{50\%\ max}$ similarly determined from the regression lines. The ratio of dilutions of sample divided by standard multiplied times 100 units per ml equals the number of units of T cell growth activity (IL 2) per ml.

Helper activity for antibody synthesis. Electrofocusing fractions were assayed for their ability to enhance the sheep red blood cell (SRBC)-specific IgM plaque-forming cell (PFC) response of 1×10^6 C57BL/10 ScN nude mouse spleen cells as previously described (1). The results are expressed as anti-SRC PFC/culture.

Helper activity for cytotoxic lymphocyte induction. Electrofocusing fractions were assayed for their ability to enhance the alloantigen-specific cytotoxic T cell response of 1×10^5 C57BL/6 thymocytes stimulated with 5×10^5 BDF_1 (C57BL/6 × DBA-2) spleen cells as described previously (12). The results are expressed as percent specific isotope release from ^{51}Cr-labeled P-815 ($H\text{-}2^d$) cells.

RESULTS AND DISCUSSION

Figure 1 shows the results of a dilution response experiment comparing the T cell growth activity of a standard Con A-stimulated spleen cell culture supernatant with a PMA-stimulated EL-4 thymoma culture supernatant. This EL-4 culture supernatant contained 5462.4 units of IL 2 activity per ml. Unstimulated EL-4 cells failed to produce detectable T cell growth activity (<0.01 units IL 2/ml). We have previously shown (11) and confirmed in these experiments that PMA, itself, is neither active in the T cell growth assay nor does it synergize with or augment the activity of sub-optimal amounts of IL 2. Subsequent experiments demonstrated that EL-4 thymoma cells harvested from an ascites tumor and cultured in the absence of serum produced comparable amounts of IL 2 (T cell growth assay) when stimulated with PMA (data not shown). In a number of experiments with three other sublines of EL-4 cells, we found that two sublines failed to produce IL 2 and one subline produced approximately 20% as much factor as the subline shown here. Although the reason for these differences is unknown, we found that continuous passage of the tumor as an ascites without intermittent *in vitro* culturing of the active subline resulted in reduced factor production subsequent to stimulation with PMA.

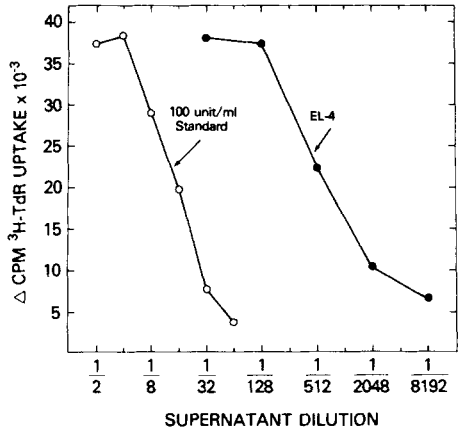

Figure 1. T cell growth activity of EL-4 thymoma-derived IL 2. EL-4 cells were cultured in the presence of 1% FCS, stimulated with PMA or left unstimulated (not shown) and the culture supernatants collected after 40 hr incubation. The T cell growth activity of the EL-4 thymoma-derived supernatant (●——●) was compared with that of a standard Con A-induced spleen cell culture supernatant (○——○) which was prepared as described previously (1). This EL-4 supernatant contained 5462.4 units of IL 2 activity per milliliter as determined by the dilution analysis method of quantitation as described previously (11). Background was 453 cpm.

Figure 2 presents the elution profile of IL 2 after gel filtration of a PMA-stimulated EL-4 thymoma culture supernatant. The T cell growth activity of the thymoma-derived IL 2 eluted as a single peak with a suggestion of a shoulder of activity on the low m.w. side. As can be seen by the inset, the m.w. of the EL-4-derived IL 2 is approximately 30,000. This estimate is in very close agreement with the m.w. of mouse spleen cell-derived IL 2 as reported by a number of laboratories (1–5). Experiments with radiolabeled PMA have shown that even though the PMA has a very low m.w., it elutes at the exclusion volume during gel filtration and is thereby clearly separated from IL 2.

Gel filtration fractions containing the IL 2 activity were pooled as indicated by the bracket shown above the activity peak in Figure 2 and fractionated further by preparative electrofocusing (Fig. 3). The data in panel A of Figure 3 show that the EL-4-derived IL 2, as measured by the T cell growth assay, exhibits charge heterogeneity with at least two peaks of activity between pH 3.8 and 4.4. The inset in panel A demonstrates that normal splenic IL 2 exhibits a similar charge heterogeneity in the same pH range. Thus, the isoelectric point heterogeneity of thymoma-derived and spleen cell-derived IL 2 are essentially the same. The data in the lower two panels of Figure 3 demonstrate that the peaks of IL 2 activity also exhibit helper activity for antibody synthesis in nude mouse spleen cell cultures stimulated with sheep erythrocytes (panel B) and helper activity for the induction of allospecific cytotoxic T cells in thymocyte cultures stimulated with semi-allogeneic spleen cells (panel C). Both of these latter helper activities have previously been ascribed to spleen cell-derived IL 2 (1–4).

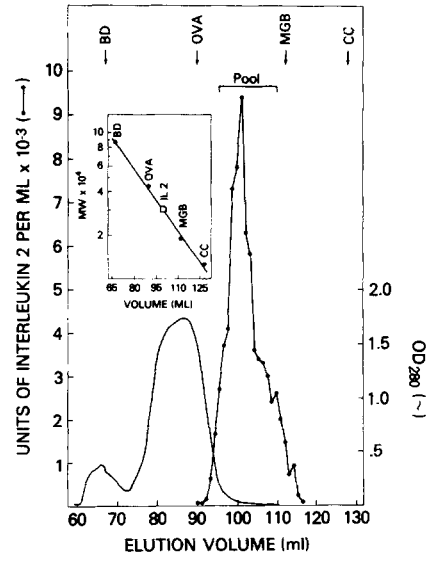

Figure 2. Gel filtration chromatography of EL-4 thymoma-derived IL 2. A PMA-stimulated EL-4 thymoma culture supernatant was concentrated 88-fold and 0.48 ml of the concentrate was fractionated by gel filtration on an AcA-54 column that had been calibrated with blue dextran (BD), ovalbumin (OVA), myoglobin (MGB), and cytochrome C (CC). The fraction volume was 1.098 ml. Aliquots of each fraction were diluted in tissue culture medium containing 1% FCS and assayed for T cell growth activity (●——●). The inset shows the m.w. of the EL-4 thymoma-derived IL 2 to be approximately 30,000.

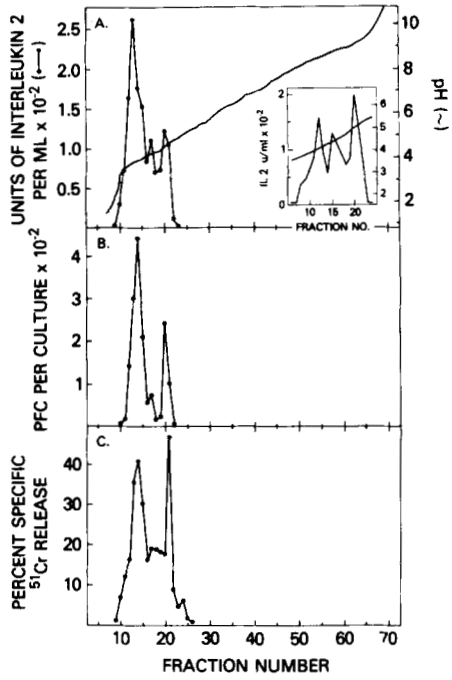

Figure 3. Electrofocusing of EL-4 thymoma-derived IL 2. The gel filtration fractions containing the T cell growth activity of IL 2 were pooled as shown by the brackets above the activity peak in Figure 2. The fraction pool (14.3 ml) was electrofocused, and the column eluted. The fraction volume was 5.5 ml. The pH of each fraction was measured after which FCS was added to a final concentration of 1% and each fraction was dialyzed against RPMI-1640. Each fraction was then assayed for T cell growth activity (*Panel A*), helper activity for antibody synthesis (*Panel B*), and helper activity for cytotoxic T cell generation (*Panel C*). Background values for *Panels B* and *C* were 4 PFC per culture and 7% ^{51}Cr release and have been subtracted.

The phorbol ester tumor promoters PMA and TPA (12-0-tetradecanoyl-phorbol-13-acetate) induce a wide variety of biologic and biochemical responses from a variety of cell types *in vitro*. In lymphocyte cultures, the phorbol esters are either mitogenic (13) or co-mitogenic (11, 14, 15) for T cells. A possible explanation for the enhancing effect of PMA on T cell proliferation comes from the observation that PMA enhances the production by Con A-stimulated splenic T cells of the T cell proliferative factor (IL 2) (10, 11). In these studies PMA was, by itself, unable to induce IL 2 production. In contrast, the results in this report demonstrate the PMA, by itself, does induce the EL-4 cells to produce IL 2. The reason for this discrepancy between the effects of PMA in the splenic T cell and EL-4 thymoma systems is unknown.

The mechanism by which PMA stimulates IL 2 production by the EL-4 cells is unknown. A recent report demonstrated that TPA caused myeloid and myelomonocytic leukemia cells to accumulate in G1 phase of the cell cycle and to undergo terminal differentiation into cells that expressed functional macrophage properties (16). Similarly, Stadler *et al.* (17) have demonstrated that PMA caused Con A-stimulated human peripheral blood lymphocytes to accumulate in G1 phase and to exhibit enhanced IL 2 production. Hydroxyurea, which totally prevented entry of the cells into S phase, also enhanced factor production (17). Recent data have shown that PMA has a similar effect on the cell cycle of the EL-4 cells and that the degree to which different phorbol esters delay entry of the EL-4 cells into S-phase is directly proportional to the amount of IL 2 produced (Stadler and Hilfiker, unpublished observations). Thus G1 may represent the phase of the cell cycle during which factor production is maximal (17). Another set of data relevant to the mechanism-of-action of PMA is the observation that proliferating EL-4 cells have been shown to have receptors for IL 2 (capacity to reversibly bind factor at 4°C) and that PMA inhibits the binding of the IL 2 to the receptor (Hilfiker, unpublished observations). All of these data are consistent with the proposal that the EL-4 thymoma produces constitutively (in G1 phase) and utilizes (in S phase) its own growth factor. Thus, we would propose that significant accumulation of the growth factor in the EL-4 culture supernatant occurs only after PMA has enhanced production by 1) maintaining the cells in G1 phase and 2) inhibiting loss of the factor either by preventing binding of the factor to the receptor as has been seen with epidermal growth factor (18, 19) or by preventing expression of the receptor. Certainly there is precedence for the fact that tumor cells are capable of synthesizing and secreting their own growth factors (20).

In the past, IL 2 has been generated with cells from freshly explanted spleens or lymph nodes. These cell cultures have routinely produced culture supernatants with biologic activity comparable to the 100 unit/ml standard shown in Figure 1. As a result, despite the large number of reports on the biochemical characterization of IL 2, all attempts to purify the factor to homogeneity have been unsuccessful. Therefore, the discovery of a T cell line such as the EL-4 that produces IL 2 in very high titer presents us with the opportunity for large scale production that should make possible the final purification of the factor. The use of the EL-4 thymoma seems particularly suitable toward this end because 1) very large numbers of cells can be obtained inexpensively by growing the tumor in ascitic form (10^9 tumor cells per mouse), 2) the tumor cells can be induced to produce IL 2 with a nonprotein stimulant (PMA) under serum-free culture conditions, all of which reduces significantly the protein contamination of the culture supernatant, and finally 3) the IL 2 produced is biochemically and biologically indistinguishable from normal spleen cell-derived IL 2 (Figs. 2 and 3).

The purification of IL 2 will allow more precise investigations into the nature of the interaction between this factor and its receptor and ultimately to the purification of the receptor itself. In addition, anti-IL 2 monoclonal antibodies could be produced that would permit the comparison of this mediator with other cellular-derived biologically active factors. Finally, the large amounts of factor obtainable from the EL-4 thymoma should allow *in vivo* studies on possible therapeutic uses of IL 2.

Acknowledgments. The authors thank Drs. Robert Moore, Ann Sandberg, and Sharon Wahl for their critical reviews of this manuscript.

REFERENCES

1. Farrar, J. J., P. L. Simon, W. J. Koopman, and J. Fuller-Bonar. 1978. Biochemical relationship of thymocyte mitogenic factor and factors enhancing humoral and cell-mediated immune responses. J. Immunol. 121:1353.
2. Watson, J., L. A. Aarden, J. Shaw, and V. Paetkau. 1979. Molecular and quantitative analysis of helper T cell replacing factors on the induction of antigen-sensitive B and T lymphocytes. J. Immunol. 122:1633.
3. Shaw, J., V. Monticone, G. Mills, and V. Paetkau. 1978. Effects of costimulator on immune responses *in vitro*. J. Immunol. 120:1974.
4. Wagner, H., and M. Rollinghoff. 1978. T-T-cell interactions during *in vitro* cytotoxic allograft responses. I Soluble products from activated Ly^{1+} T cells trigger autonomously antigen-primed Ly^{23+}

T cells to cell proliferation and cytolytic activity. J. Exp. Med. 148: 1523.
5. Chen, D. M., and G. DiSabato. 1976. Further studies on the thymocyte stimulating factor. Cell. Immunol. 22:211.
6. Watson J., S. Gillis, J. Marbrook, D. Mochizuki, and K. A. Smith. 1979. Biochemical and biological characteristics of lymphocyte regulatory molecules. I. Purification of a class of murine lymphokines. J. Exp. Med. 150:849.
7. Gillis, S., K. A. Smith, and J. Watson. 1980. Biochemical characterization of lymphocyte regulatory molecules. II. Purification of a class of rat and human lymphokines. J. Immunol. 124:1954.
8. Wagner, H., C. Hardt, K. Heeg, M. Rollinghoff, and K. Pfizenmaier. 1980. T-cell-derived helper factor allows *in vivo* induction of cytotoxic T cells in nu/nu mice. Nature 284:278.
9. Baker, P. E. and K. A. Smith. 1980. The potential therapeutic utility of T-cell growth factor. Fed. Proc. 39:803.
10. Fuller-Farrar, J., M. L. Hilfiker, W. L. Farrar, and J. J. Farrar. 1980. PMA enhances the production of interleukin 2. Cell. Immunol. In press.
11. Farrar, J. J., S. B. Mizel, J. Fuller-Farrar, W. L. Farrar, and M. L. Hilfiker. 1980. Macrophage-independent activation of helper T cells. I. Production of interleukin 2. J. Immunol. 125:793.
12. Simon, P. L., J. J. Farrar, and P. D. Kind. 1977. The xenogeneic effect. III. Induction of cell-mediated cytotoxicity by alloantigen-stimulated thymocytes in the presence of xenogeneic reconstitution factor. J. Immunol. 118:1129.
13. Touraine, J-L., J. W. Hadden, F. Touraine, E. M. Hadden, R. Estensen, and R. A. Good. 1977. Phorbol myristate acetate: a mitogen selective for a T-lymphocyte subpopulation. J. Exp. Med. 145:460.
14. Mastro, A. M., and G. C. Mueller. 1974. Synergistic action of phorbol esters in mitogen-activated bovine lymphocytes. Exp. Cell Res. 88:40.
15. Rosenstreich, D. L., and S. B. Mizel. 1979. Signal requirements for T lymphocyte activation. I. Replacement of macrophage function with phorbol myristic acetate. J. Immunol. 123:1749.
16. Rovera, G., N. Olashaw, and P. Meo. 1980. Terminal differentiation in human promyelocytic leukaemic cells in the absence of DNA synthesis. Nature 284:69.
17. Stadler, B. M., J. J. Farrar, and J. J. Oppenheim. 1980. Cell cycle dependent production of human interleukin-2. Behring Inst. Res. Comm. In press.
18. Lee, L-S. and I. B. Weinstein, 1978. Tumor-promoting phorbol esters inhibit binding of epidermal growth factor to cellular receptors. Science 202:313.
19. Brown, K. D., P. Dicker, and E. Rozengurt. 1979. Inhibition of epidermal growth factors binding to surface receptors by tumor promoters. Biochem. Biophys. Res. Commun. 86:1037.
20. Todaro, G. J., J. E. De Larco, H. Marquardt, M. L. Bryant, S. A. Sherwin, and A H. Sliski. 1979. Polypeptide growth factors produced by tumor cells and virus-transformed cells: a possible growth advantage for the producer cells. *In* Hormones and Cell Culture. Edited by G. H. Sata and R. Ross. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.