

BIOCHEMICAL AND BIOLOGICAL CHARACTERIZATION OF LYMPHOCYTE REGULATORY MOLECULES

I. Purification of a Class of Murine Lymphokines*

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The ease with which it is possible to detect lymphokine-like activities in the supernates of cultured murine cells has led to the description of a large number of ill-defined factors which may or may not be of biological significance. In recent years, the situation has become aggravated by the increasing number of *in vitro* assays for putative lymphokines with insufficient attention directed to the association of defined biological activities with purified material.

An excellent source of murine lymphokines is the supernate from cultures of concanavalin A (Con A)¹-stimulated mouse spleen cells (1). Investigations in our laboratories have demonstrated that such lymphokines can enhance a variety of B- and T-cell responses (2-12).² The assays we use appear to detect both proliferation and differentiation inducing activities in these culture supernates. In this report we describe work in which the biological activity of lymphokines in Con A supernates has been purified by successive gel filtration, ion-exchange chromatography, and isoelectric focusing (IEF) in an attempt to separate molecules with different activities from each other. In the course of these separative procedures, the biological activities measured included: (a) T-cell growth factor (TCGF) (3), (b) the promotion of Con A-induced mitogenesis in thymocyte cultures (10), (c) helper T-cell-replacing factor (TRF) activity in athymic spleen cell cultures (9, 10), and (d) the induction of

* We use the term lymphokine to refer to molecules that exert biological activities on lymphocytes. We do not imply that the cellular origin of these molecules is known.

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¹ *Abbreviations used in this paper:* Con A, concanavalin A; CTL, cytotoxic lymphocytes; FCS, fetal calf serum; [³H]TdR, tritiated thymidine; IEF, isoelectric focusing; LAF, lymphocyte activating factor; LPS, *Escherichia coli* lipopolysaccharide; MLC, mixed lymphocyte culture; MTLC, mixed tumor-lymphocyte culture; pI, isoelectric point; SRBC, GRBC, sheep and goat erythrocytes; TCGF, T-cell growth factor; TMF, thymocyte mitogenesis factor; TRF, T-cell-replacing factor.

² Thoman, M., and J. Watson. Molecular weight determination and subunit composition of a helper T cell-replacing factor. Submitted for publication.

cytotoxic lymphocytes (CTL) in both thymocyte (6, 10) and nude mouse spleen cell cultures (5).

When considering the broad range of activities tested it was not expected that a single class of molecules of a particular size and charge could be found to be responsible for biological activity in all assays. The striking molecular homogeneity of the apparently different activities present in this lymphokine preparation has led to several restrictions in considering the mode of action of such molecules. In following papers, we describe the purification of molecules with similar biological activities from rat³ and human⁴ sources.

Materials and Methods

Mice. Female BALB/c and C57BL/6J mice were obtained from The Jackson Laboratory, Bar Harbor, Maine. Athymic (nude) C57BL/6J and BALB/c mice were from our breeding colony, University of California, Irvine, Calif. (9). NIH nude mice were obtained from Harlan Industries, Indianapolis, Ind.

Biochemical Purification of Lymphokines

SOURCE OF BIOLOGIC ACTIVITY. Spleen cells from normal BALB/c or C57BL/6J mice were cultured in the presence of Con A (2 μ g/ml) at a density of 5×10^6 cells/ml in RPMI-1640 (Grand Island Biological Company, Grand Island, N. Y.) supplemented with 5×10^{-5} M 2-mercaptoethanol, 1 mM glutamine, 50 U/ml penicillin, and 50 μ g/ml streptomycin. The cells were cultured in Falcon 3024 tissue culture flasks (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) in vol of 50 ml. The cultures were incubated at 37°C in a gas mixture of 7% oxygen, 10% carbon dioxide, 83% nitrogen. After 16–18 h of culture, cells were removed by two sequential centrifugations (2,000 *g* for 10 min and 10,000 *g* for 20 min). The supernate was then concentrated by vacuum dialysis.

CHROMATOGRAPHIC PROCEDURES. All chromatography was performed at 4°C using sterile buffers.

(a) Sephadex G-100 was obtained from Pharmacia Fine Chemicals, Inc., Uppsala, Sweden, and equilibrated in 0.9% saline. 10-ml vol of concentrated conditioned medium were layered on Sephadex G-100 columns (2 \times 90 cm) and fractions eluted with 0.9% saline. The protein content of the 40–60 8-ml fractions collected was monitored with the aid of an LKB Uvicord II calibrated for absorbance at 280 nm (LKB Produkter, Bromma, Sweden). Sephadex G-100 columns were calibrated with the following molecular weight standards: bovine serum albumin (mol wt 69,000), ovalbumin (mol wt 44,000), and cytochrome *c* (mol wt 12,500).

(b) DEAE cellulose (DE-52) was obtained from Whatman, Inc., Springfield Mill, England. DEAE columns (1.5 \times 8 cm) were equilibrated in 0.05 M sodium phosphate buffer (pH 7.6). Elution was performed using a gradient from 0.05–0.5 M sodium phosphate buffer (pH 7.6). The ionic strength of the fractions collected (10 ml) was determined using a conductivity meter (type CDM, The London Company, Cleveland, Ohio).

(c) IEF. Flat bed IEF of Con A supernate was performed in horizontal layers of Sephadex (13). Samples were prepared in a 100-ml solution containing 1% glycine and 2% Ampholines (pH 3–10, LKB Produkter). The sample was mixed with 5 gm Ultrodex (specially treated Sephadex G-75; LKB Produkter) and the gel suspension spread in a gel tray. The tray was transferred to a cooling plate (5°C) and electrophoresed for 26–30 h under a constant current of 8 mA. During electrophoresis the voltage was allowed to increase from 200–1,000 V. After electrophoresis, the gel was sectioned into 30 portions and the pH in each compartment determined directly with a surface glass electrode. The gel content of each compartment was then transferred to a small column (1 \times 5 cm) and eluted with 5–10 ml sterile water. Each sample was dialyzed against 0.9% saline containing 1 μ g/ml polyethylene glycol 6000 before

³ Gillis, S., K. A. Smith, and J. Watson. Biochemical and biological characterization of lymphocyte regulatory molecules. II. Purification of a class of rat lymphokines. Submitted for publication.

⁴ Gillis, S., K. A. Smith, and J. Watson. Biochemical and biological characterization of lymphocyte regulatory molecules. III. Purification of a class of human lymphokines. Submitted for publication.

assay for biologic activity. The protein content of samples was measured as described elsewhere (14).

Biological Assays for Lymphokine Activities

ASSAYS FOR THE PRESENCE OF TCGF ACTIVITY WERE PERFORMED USING TWO T-CELL LINES. (a) CTLL-2 cells, a continuous TCGF-dependent line of cytotoxic T cells (2), or (b) HTL-1, a continuous TCGF-dependent line of helper T cells,⁵ using a procedure described elsewhere (3). Briefly, T cells were cultured in replicate 200- μ l cultures in flat-bottom microplate wells (3596; Costar, Data Packaging, Cambridge, Mass.) in Click's medium (Altick Associates, Hudson, Wisc.), supplemented with 10% heat-inactivated (56°C for 30 min) fetal calf serum (FCS), 300 μ g/ml L-glutamine, 25 mM Hepes buffer, 16 mM sodium bicarbonate, 50 U/ml penicillin, and 50 μ g/ml gentamycin. Each well contained 4,000 cells/well together with a limiting concentration of putative TCGF-containing sample. After a 24-h incubation at 37°C in the gas mixture described above, microplate wells were pulsed with 0.5 μ Ci of tritiated thymidine ($[^3\text{H}]\text{TdR}$, Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y., sp act 1.9 Ci/mM). Cultures were harvested 4 h later on to glass fiber strips and $[^3\text{H}]\text{TdR}$ incorporation determined using a liquid scintillation counter (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). Results were quantified by probit analysis (3, 15). TCGF activity was expressed in units by comparing experimental probit data with that obtained using a standard TCGF sample assigned a value of 1 U/ml. T cells for use in TCGF microassays were maintained in continuous culture in a solution containing 50% crude rat TCGF (2) and 50% Click's medium supplemented with 2% FCS. T cells were routinely subcultured to 1×10^4 cells/ml after reaching a saturation density of $1-3 \times 10^5$ cells/ml.

INDUCTION OF THYMOCYTE MITOGENIC RESPONSES TO CON A. Thymocytes were resuspended in RPMI-1640 supplemented with 5% FCS, to a density of 10^6 cells/ml. 100- μ l aliquots (10^5 cells) were dispensed to individual microculture wells (Microtest II plates, Falcon Labware). Factor preparations were then added in 10- μ l aliquots. The cultures were incubated for 72 h at 37°C in the above-detailed gas mixture and then pulsed for 6 h with 0.25 μ Ci of $[^3\text{H}]\text{TdR}$. Cultures were collected on glass fiber filters with a multiple automated sample harvester, and $[^3\text{H}]\text{TdR}$ incorporation determined in a liquid scintillation system (10).

TRF ACTIVITY AND ANTIBODY SYNTHESIS. Antibody synthesis was measured in the microculture system described by Lefkovits (16, 17) as detailed elsewhere (9). Spleen cells from BALB/c nu/nu mice were resuspended in RPMI-1640 (supplemented with 20% FCS) to a density of 10^7 cells/ml and distributed into microculture trays (No. 3034, Falcon Labware) in 10- μ l aliquots yielding cell concentrations of 1.0×10^5 cells/well. These wells also contained 0.05% sheep or goat erythrocytes (SRBC, GRBC) as antigen, and the concentrations of Con A supernate detailed in the text (Results). In all assays, 120 microcultures (two microculture trays) were regularly employed to test each factor concentration. The cultures were incubated for 5 d, (37°C) and the supernates from each microculture were assayed using a lytic spot test (17). Results are expressed in terms of the fraction of responding cultures present in the 120 microcultures tested (9).

FACTOR-DEPENDENT GENERATION OF CTL. Three separate mixed lymphocyte/mixed tumor-lymphocyte (MLC/MTLC) culture systems were used: (a) C57BL/6J (H-2^b) thymocytes were cultured in 200- μ l vol (2×10^6 cells) in V-bottom microplate wells (15-MVC-96-TC, Linbro Chemical Company, Hamden, Conn.), with x-irradiated (5,000 rad) P815 (H-2^d) tumor cells (3×10^4 cells/well); (b) spleen cells from NIH athymic (nude) mice were cultured in 200- μ l vol (1×10^6 cells/well) with 1.5×10^4 x-irradiated (5,000 rad) P815 tumor cells; and (c) spleen cells from NIH nude mice were cultured in 200- μ l vol (1×10^6 cells/well) with an equal number of x-irradiated (1,500 rad) BALB/c thymocytes. All irradiations were performed using a Philips-Maximar x-ray unit (Philips Electronic Instruments, Mahwah, N. J.).

Samples assayed for their ability to induce cytolytic reactivity were tested at concentrations ranging from 20 to 50%. Methods for the preparation of responder spleen and thymocyte populations have been previously described (5, 7). After 5 d of culture at 37°C in the gas mixture described above, microcultures were assayed for cytolytic reactivity against ^{51}Cr -labeled P815 (H-2^d) tumor cells as detailed elsewhere (5, 7). The culture conditions necessary for the growth of P815 tumor cells have been described elsewhere (5, 7).

⁵ Watson, J. The establishment of continuous cultures of helper T cells. Submitted for publication.

The percent specific lysis was determined by the following equation:

$$\text{percent specific lysis} = 100 \times \frac{\text{experimental counts per minute} - \text{medium control counts per minute}}{\text{maximum release counts per minute} - \text{medium control counts per minute}}$$

Results

Biological Activities in Con A Supernates. Spleen cells from BALB/c mice were cultured at a density of 5×10^6 cells in serum-free RPMI-1640 medium containing 2 $\mu\text{g/ml}$ Con A for 20 h. Cells were removed by centrifugation (10,000 g for 20 min), and the supernates concentrated by vacuum dialysis. In this manner, 2–5 liters of supernate was rapidly concentrated to vol of 20–30 ml.

Similar crude supernates have been shown to contain factors that stimulate a variety of lymphocyte responses (1–12).² In an attempt to determine whether specific factor activities could be associated with biochemically distinct material, we subjected concentrated serum-free conditioned media to a variety of biochemical separation techniques. After each step in the purification scheme fractions were tested in four different assay systems: (a) the exponential growth of differentiated effector T lymphocytes TCGF microassay (3), (b) the promotion of thymocyte proliferation in response to Con A (mitogenic response or thymocyte mitogenesis factor [TMF] assay) (12, 18–20), (c) the in vitro generation of cytotoxic cells in MLC/MTLC where the responding populations were limiting for mature T cells (thymocytes and nude spleen cells) (5), and (d) the in vitro stimulation of plaque-forming responses against erythrocyte antigens under culture conditions which were limiting for helper T cells (TRF assay) (9).

Gel Filtration. Concentrated Con A supernates were fractionated by gel filtration using Sephadex G-100 column chromatography. Column fractions were collected as detailed in Materials and Methods, and assayed for activity in each of the above culture systems. The data presented in Fig. 1 summarizes the results obtained. In Fig. 1 A, the TCGF activity as tested on CTLL-2 cells (2) is shown to elute from Sephadex G-100 in the size range of 30,000 daltons. Similarly, the data in Fig. 1 B shows that the factor activity stimulating Con A responses in thymocyte cultures elutes as a sharp peak also in the molecular size range of 30,000 daltons. Both the TRF activity of the column fractions assayed by stimulation of antibody responses to SRBC antigens (Fig. 1 C) and factors stimulating the generation of cytotoxic responses (Fig. 1 D) elute in a broad peak in the region of 30,000–40,000 daltons. Furthermore, the factor activities responsible for the generation of cytotoxic responses in either thymocytes or nude spleen cells appeared to be inseparable (Fig. 1 D).

Two aspects of these gel filtration studies should be emphasized: (a) when Con A supernates are prepared and concentrated as described, the only biological activity detected in the assay systems described is found in the 30- to 40,000-dalton region, and (b) rechromatography of the peak fractions does not alter their elution profiles.

Ion-Exchange Chromatography. In a previous report it was shown that the biological activity tested in three of the four assay systems used here co-eluted following an ion-exchange chromatography (10). These three activities tested were the stimulation of antibody responses in T-cell-depleted spleen cultures, the generation of alloantigen-reactive cytotoxic thymocytes and the promotion of thymocyte mitogenic responses to Con A (10).

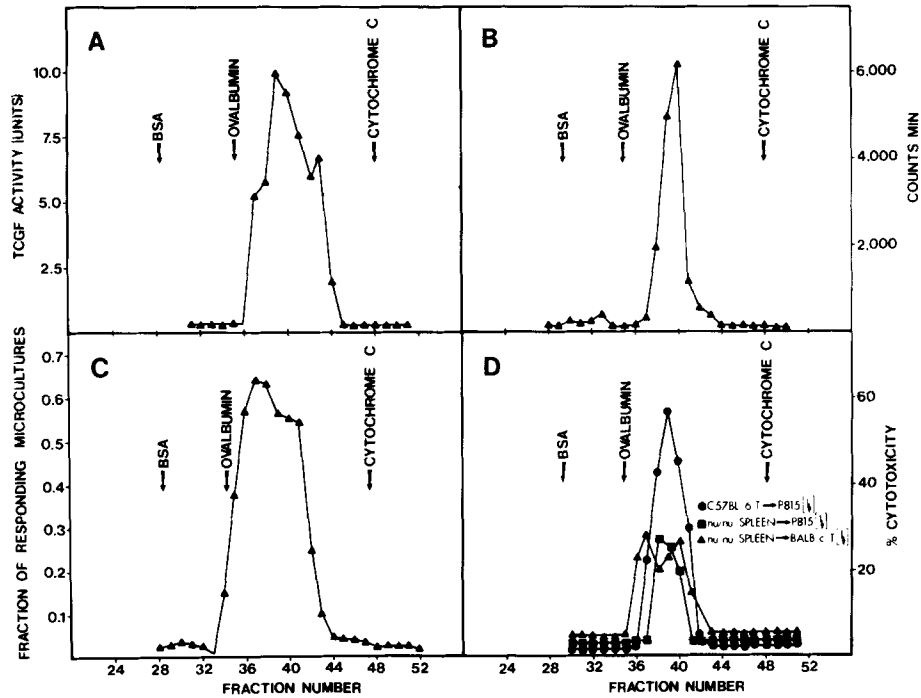


FIG. 1. Lymphokine activities assayed after Sephadex G-100 gel exclusion chromatography of concentrated medium conditioned by Con A-stimulated mouse spleen cells. (A) TCGF activity. (B) Ability to promote thymocyte mitogenesis. (C) Ability to promote anti-erythrocyte antibody production in nude mouse spleen cell cultures. (D) Ability to induce CTL responses in C57BL/6 thymocyte P815 MTL (●), NIH nude spleen by P815 MTL (■), and NIH nude spleen by BALB/c thymocyte MTL (▲).

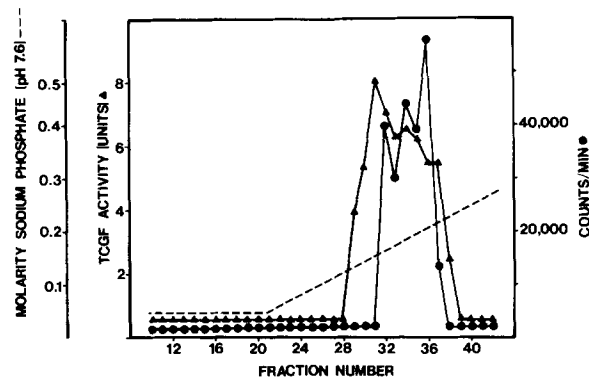


FIG. 2. TCGF activity (▲) and thymocyte mitogenesis factor activity (●) obtained after DEAE ion-exchange chromatography of active fractions pooled after Sephadex G-100 gel exclusion chromatography.

We show in Fig. 2 that TCGF activity assayed on established functional T-cell lines co-chromatographed with the factor promoting thymocyte mitogenic responses to Con A. These activities eluted from DEAE ion-exchange resin between 0.15 and 0.2 M sodium phosphate buffer at pH 7.6 (Fig. 2).

IEF. When factor activity in column fractions obtained by Sepharose G-100 gel

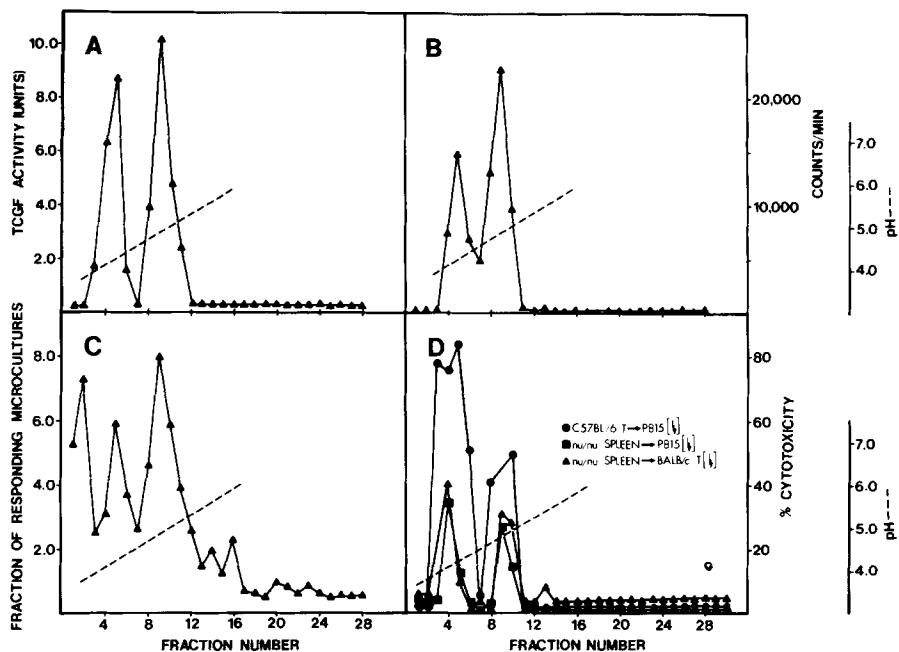


FIG. 3. Lymphokine activities assayed after flat bed IEF (pH 3-10) of active fractions pooled after Sephadex G-100 gel exclusion chromatography. (A) TCGF activity. (B) Ability to promote thymocyte mitogenesis. (C) Ability to promote anti-erythrocyte antibody production in nude mouse spleen cell cultures. (D) Ability to induce CTL responses in C57BL/6 thymocytes P815 MTL (●), NIH nude spleen P815 (■), and NIH nude spleen by BALB/c thymocyte MLC (▲).

filtration (Fig. 1) or by DEAE ion-exchange chromatography (Fig. 2) are separately pooled and examined by IEF, the following results were consistently obtained:

(a) Factor activity electrophoresed over a wide range pH gradient (2.5-10) separated into two major peaks of activity (peaks I and II). The factors that allowed the growth of T cells in culture, stimulated mitogenic responses to Con A in thymocyte cultures, or enhanced the generation of CTL in either thymocyte or nude spleen cultures, appeared to be identical (Fig. 3 A, B, and D). The isoelectric point of peak I was 4.3 and for peak II was 4.9.

(b) When helper TRF activity was assayed on nude spleen cell cultures, the pH range of activity was found to be considerably more diverse. It was clear that both peak I (isoelectric point [pI] 4.3) and peak II (pI 4.9) material, which was active in all other assay systems, also stimulated immune responses to SRBC (Fig. 3). However, there existed additional material which stimulated antibody responses to SRBC which fractionated in the pH range of 3.0-4.0.

To further examine and compare these biological activities following isoelectric separation, focusing was performed over a narrower pH range, 2.0-6.0 (Fig. 4). The resulting fractions were assayed for the stimulation of antibody responses to two erythrocyte antigens (GRBC and SRBC), in athymic nude spleen cell cultures, and also for TCGF activity. By TCGF microassay analysis there was once again a characteristic clear separation of two active components (pI 4.3 and 4.9). Both components stimulated antibody responses to GRBC and SRBC antigens (Fig. 4). However, again there was additional material in the pH range from 3.0-4.0 which

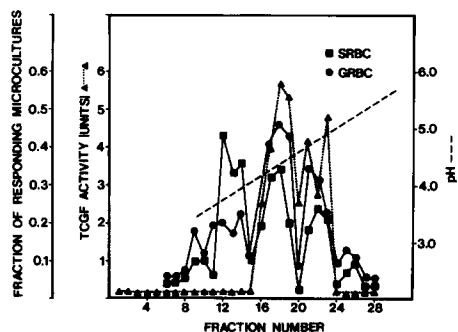


FIG. 4. Lymphokine activities assayed after IEF over a narrow pH range (pH 3-6). TCGF activity (\blacktriangle), ability to promote anti-erythrocyte (SRBC \blacksquare , GRBC, \bullet) antibody responses in nude mouse spleen cell cultures.

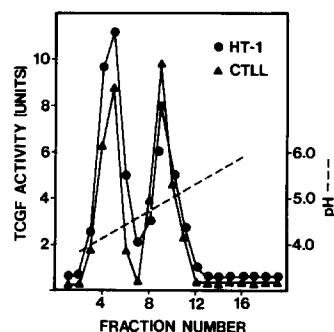


FIG. 5. IEF, assayed using either cultured cytotoxic T cells (\blacktriangle) or cultured helper T cells (\bullet) as the TCGF microassay indicator cell population.

stimulated antibody responses, but possessed no T-cell growth activity. This material stimulated antibody responses to both GRBC and SRBC antigens.

TCGF Activity Assayed on Two Classes of Effector T Cells. As a result of the introduction of culture methodology allowing for the maintenance of continuous T-cell lines, it has become important to determine whether molecules that promote the growth of cytotoxic T cells also promote the growth of other T-effector-type cells. Using established cytotoxic (2) and helper⁵ T-cell lines, we analyzed the TCGF activity present in Con A supernate which had been subjected to sequential gel filtration chromatography and flat bed IEF. The data presented in Fig. 5 demonstrates that the growth promoting activities for both cell lines appeared to be identical. Both the pI 4.3 (peak I) and 4.9 (peak II) components stimulated the growth of cytotoxic and helper T-cell lines.

Discussion

Molecular Properties of Lymphokines. There are many reports which show that antigen- or mitogen-stimulated murine macrophages and T cells release factors that enhance lymphocyte proliferation, differentiation, and function (1-12; footnotes 2-4; 18-40). Such factors we term here lymphokines. The diversity of activities and assays, as well as the limited data concerning molecular properties of lymphokines has not allowed the emergence of an integrated description of lymphokine activities. In this work a range of different assays has been applied at each stage of the isolation and

characterization of lymphokines from supernates of Con A-activated mouse spleen cells.

The main result from experiments presented here is that there exists one class of lymphokine capable of affecting lymphocyte proliferation and differentiation as measured by its ability to promote both T- and B-cell immune responses. After fractionation by gel filtration, ion-exchange chromatography and IEF (Figs. 1-5), the various assay systems revealed a class of molecules with an apparent molecular size of ~30,000 daltons. This class was separable into two components which differed in charge having pI of 4.3 and 4.9 (Figs. 1-4). Both components supported the continuous proliferation of cytotoxic and helper T cells in culture (Fig. 5), promoted the proliferation of Con A-treated thymocytes, and induced the generation of antibody forming cells in T-lymphocyte-depleted cultures and cytotoxic cells in either thymocyte or nude mouse spleen cell cultures.

In addition, within the factor(s) in the 30,000-dalton fraction, another subclass of molecules was detected by its ability to induce antibody synthesis to heterologous erythrocyte antigens in cultures of nude mouse spleen cells. This material showed considerable heterogeneity in charge ranging from a pI of 3.0 to 4.2 (Fig. 4). No other lymphokine activity was detected when these molecules were tested in the other assay systems.

Relationship to Other Lymphokines. TCGF was originally described as the activity present in the supernates of Con A-activated murine spleen cells which allowed the continuous proliferation of preselected antigen-reactive cytotoxic T cells (2-8). It was later found that TCGF could be produced by either T-cell mitogen or antigen stimulation (3, 4) but not following *Escherichia coli* lipopolysaccharide (LPS) activation (3). The production of TCGF required the presence of both T cells and adherent cells (presumably macrophages). Thymocytes (predominantly immature T cells) as well as nude spleen cells produced very little, if any, TCGF when stimulated with Con A; although, significant quantities were produced by Con A-activated (cortisol) resistant thymocytes (mature T cells [6]). The target cell for TCGF appears to be an activated T cell. The repeated observation that mitogen- or antigen-activated T lymphocytes absorb TCGF and respond after absorption by proliferation has led to the suggestion that TCGF alone functions as the T-cell proliferation-inducing signal in both T-cell mitogenesis and antigen stimulation. In support of this hypothesis are the observations that neither nonactivated mouse spleen cells nor LPS-treated spleen cells absorb TCGF or proliferate in response to prolonged culture with TCGF. Wagner and Rollinghoff (21) have recently confirmed and extended these observations by discovering that Ly1+ T cells appear to fulfil the T-cell requirement for TCGF production and that Ly2,3+ cells respond to the factor as assayed by the in vitro generation of CTL. It is important to distinguish that although this compartmentalization of TCGF producer and responder T cells on the basis of Ly-antigen surface phenotype, appears to hold for the generation CTL, the same dissociation may not apply to all of the immune response assay systems detailed in this report.

On the simplest level of consideration, it is possible to discuss two basic types of factors capable of influencing immune reactivity, those having some degree of specificity and those which are nonspecific with respect to the immune response being investigated. If TCGF is a T-cell proliferative factor with some of the characteristics of a hormone-like material, it should be compared with other nonspecific lymphokines.

Farrar et al. (22), Farrar and Koopman (23), and Simon et al. (24) have shown that T-cell mitogenic factor (TMF) stimulates thymocyte proliferation and provides helper activity for both antibody responses and cytotoxic T-cell responses in culture. Both TCGF as described in this communication and TMF (22) appear to be indistinguishable from the thymocyte-stimulated factor described by Chen and DiSabato (25), the cytotoxic helper factor of Shaw et al. (19, 20), and several other nonspecific helper factors (26-30). The lymphocyte-activating factors (LAF) secreted by macrophages, or tumor cell lines that retain macrophage-like functions, appear to be a separate class of factors (31-34) as they are associated with molecules having a mol wt of 15,000. Under the conditions described here for the production of TCGF in serum-free spleen cell cultures, no activity was detected in fractions having the characteristics of LAF (31-34).

Not only is there an apparent lack of antigenic specificity with TCGF but there is a lack of species specificity. TCGF prepared from stimulated rat or human lymphocytes is capable of supporting the proliferation of mouse T cells (footnotes 3 and 4). It is, however, wise to distinguish between true nonspecific factors and the appearance of broad specificity perhaps due to the presence of many specific (monoclonal?) factors produced following mitogen stimulation (11). Factors may have specificity with respect to the antigen used in their production (35, 36) or for the type of lymphocyte (in the above cited cases, a B cell) which is responding to the antigen (37-40). For this reason, it must be stressed that there may be a major difference between the activity (detected in all assays) of the two molecular species of TCGF (pI 4.3 and 4.9) and the class of molecules which are detected only in the assay involving the stimulation of antibody responses to heterologous antigens (Fig. 4). We have yet to establish the relationship between this latter class of factors and the specific factors described by others (35, 36).

Mode of Action. The striking co-purification of all four lymphokine activities to a single mol wt class of 30,000 forces some conclusions to be drawn concerning the mode of action of the lymphokine. Although the main activity could be separated by charge into two components with pI of 4.3 and 4.9, no difference in biological activity was detected between these components, so perhaps they should be considered as variants of the same molecule. Both components stimulate the proliferation of established cytotoxic and helper T-cell lines (Fig. 5). As a result it can be stated that TCGF appears to act equally well on functionally different classes of effector T cells (2-8, footnote 5). Together with the remainder of the systems tested, there is strong evidence that the two species of lymphokine (IEF peaks I and II) act in an identical fashion by promoting or enhancing immune reactivity via the clonal expansion of activated T cells. As a result of previous studies ascribing this activity to TCGF (2-8), it seems most appropriate that both IEF species be called TCGF.⁶ The following considerations of each assay support this contention:

(a) For the growth of effector T-cell lines in culture, there is an absolute requirement for the presence of TCGF (5, 6, 8, footnote 5). In that the T-cell lines have been

⁶ At the Second International Workshop, Ermatingen, Switzerland, a number of participants developed a system of nomenclature based on the ability of factors to act as communication signals between different populations of leukocytes. The term "Interleukin" (between leukocytes) will be used for two different factors. Lymphocyte activating factors (31) will be termed Interleukin I (IL-1), the molecule described in this paper as T-cell growth factor (TCGF) will be termed Interleukin II (IL-2).

cloned (8, footnote 5), it is safe to assume that TCGF exerts its effect directly on the T cell. This conclusion is further supported by the observation that activated T cells absorb TCGF whereas nonactivated cells or activated B cells do not. It furthermore seems plausible that activation of T cells by antigen or mitogen results in the appearance of receptors for TCGF (6).

(b) Both antigen and TCGF are required to generate cytotoxic effector T cells from nude mouse splenocytes. By definition, pre-T cells are present in the spleens of nude mice. In that T-cell antigen or mitogen alone cannot initiate the proliferation of nude mouse lymphocytes (nude mice being incapable of producing TCGF, [6]), it follows that addition of TCGF to responder pre-T-cell populations promotes proliferation to the point where meaningful cytotoxic reactivity can be witnessed. Although it cannot be concluded that TCGF is the normal stimulus that drives pre-T cells to maturity, it opens the possibility that the limiting step in the differentiation of pre-T cells to mature effector T cells may be solely the presence or absence of a proliferative signal.

(c) There may be antigen reactive pre-T cells other than those able to become cytotoxic cells in nude spleens. In the presence of TCGF and antigen, therefore, specific T helper cells may be generated which are able to subsequently contribute to antibody responses. In support of such a hypothesis are the reported observations that (i) exposure to TCGF promotes the rapid proliferation of small numbers of antigen-activated lymphocytes (4, 6), and (ii) that TCGF does not interact directly with B cells (12). In consideration of such a scheme of events, it is interesting that both TCGF production and the stimulation of nude spleen cells to produce antibody, require macrophages (6, 12).

(d) Finally with regard to the generation of thymocyte reactivity, helper T cells (Ly1+) and adherent cells have been shown to be required for both the polyclonal sensitization of murine T cells by Con A or phytohemagglutinin and the generation of cytotoxic T cells from thymocyte cultures (41-43). The T cells stimulated to proliferate and mature to effector cells are in fact, antigen-sensitive T cells from killer, suppressor, and helper subclasses. The data in this work compounded with the previously cited studies by Wagner and Rollinghoff (21) suggest that Con A activation may have two roles; the first being to mimic the primary signal normally provided by an antigen and the second being to stimulate the production of proliferative factor, which we have designated TCGF (8).

Until the precise cellular origin of TCGF has been established, it is difficult to ascribe a definitive physiological role to this class of lymphokines. The simplest suggestion based on available data is that the main TCGF fraction may constitute a class of hormones which induces proliferation of suitably activated target T cells.

Further studies will be required to define the exact molecular characteristics of the TRF which has no apparent activity on T cells. One might predict that activated B cells would absorb TRF in much the same manner that activated T cells absorbed TCGF activity. Nonetheless, the results of the experimentation described herein suggest that at least two distinct molecular entities exist that have similar molecular sizes, yet differ in charge and biologic activity. Further studies of the nature of the target cells of these factors, as well as their *in vitro* and *in vivo* immunobiological function, should provide for an era of a hormonal approach to our understanding of the regulation of the immune response.

Summary

Murine spleen cells activated by concanavalin A (Con A) in culture produce a class of lymphokine molecules which possess biological activity in a number of lymphocyte response assays. Lymphokines with a mol wt of 30,000, as estimated from gel filtration studies, can be resolved into two components which differ by charge, with isoelectric point (pI) values of 4.3 and 4.9, respectively. Both components stimulate (a) the growth of established T-cell lines in culture, (b) the proliferation of thymocytes in the presence of Con A under culture conditions where Con A alone is nonmitogenic, (c) the induction of antibody responses to heterologous erythrocyte antigens in athymic (nude) spleen cultures, (d) the generation of cytotoxic T lymphocytes (CTL) in thymocyte cultures, and (e) the generation of CTL in nude spleen cultures. In each of these culture systems we suggest that the assays are detecting a single class of lymphokine which acts directly on activated T cells. Nonactivated T cells must be stimulated by either antigen or mitogen before becoming responsive to lymphokine, but do not require antigen or mitogen for continued growth with lymphokine. The two molecular species, separable by isoelectric focusing are referred to as the T-cell growth factor (TCGF). A lymphokine, similar in size (30,000 daltons) to TCGF but heterogeneous in charge (pI 3.0–4.0), stimulates immune responses to erythrocyte antigens in T-cell-depleted spleen cultures but has no stimulatory activity in the other lymphocyte assay systems described. The data have been interpreted as showing the two molecular forms of murine TCGF (pI 4.3 and 4.9) are responsible for many of the lymphokine activities described elsewhere as thymocyte mitogenic factor, nonspecific T-cell-replacing factor and killer helper factor or costimulator. The other lymphokine, separable from TCGF by charge, appears to have true T-cell-replacing activity.

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