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Production and characterization of a murine lymphocyte activating factor

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PRODUCTION AND CHARACTERIZATION OF A MURINE LYMPHOCYTE ACTIVATING FACTOR

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DEDICATION

To my wonderful parents

ABSTRACT

Murine lymphocyte activating factor (LAF), a leukocyte product which enhances DNA synthesis in thymocytes, was studied. *In vivo* stimulation with intraperitoneal injections of *E. coli* lipopolysaccharide (LPS) was employed, and spleen and peritoneal cells were found to produce LAF within three hours. The kinetics and optimal conditions for production of peritoneal and splenic LAF were explored. Enhancement of peritoneal LAF production after LPS challenge was attained by pre-treating the animals with the methanol extraction residue of the tubercle bacillus (MER).

The synergistic effect of murine LAF with several mitogens, including phytohemagglutinin-P, Concanavalin A and the supernatant fraction from human peripheral blood leukocytes was demonstrated. It was also shown that dialysis of crude LAF preparations apparently removed an inhibitory substance.

Preliminary data suggested that the molecular weight of murine LAF is 55,000 daltons and the isoelectric point is 5.0. Both these values are considerably different from those previously reported for human leukocyte LAF, but are comparable to values determined for a murine cell line.

LITERATURE REVIEW

The Role of Macrophages in the Immune Response

Macrophages or adherent mononuclear phagocytes have an important accessory role in cell-mediated immunity. Both the adherent and non-adherent populations of murine spleen cells are required to produce antibody to sheep red blood cells.[1] Macrophages appear to be responsible for the uptake and processing of antigens.[2] Antigens bound to macrophages are highly immunogenic. Live peritoneal exudate macrophages, when transferred from an animal with exposure to a specific antigen to an animal which has not been exposed to that antigen will provoke a specific immune response.[3, 4] Albumin bound to live macrophages is approximately one thousand-fold more immunogenic than a comparable amount of free antigen.[5]

The macrophage, however, does not appear capable of actually producing antibody. If stimulated macrophages are transferred into X-irradiated hosts[6] or recipients immunologically tolerant to the antigen,[5] there will be almost no antibody production.

The specificity of the immune response resides with the committed lymphocytes.[7] The macrophages can interact with many antigens. However, if a specific clone of lymphocytes in a population of spleen cells is rendered non-functional by a

highly radioactive antigen, the immune response to that antigen is lost when the spleen cells are transferred to X-irradiated mice.[8]

The question to be evaluated in this thesis is whether in a non-specific immune response (mitogenesis of thymocytes in the absence of antigen) the macrophage from a mouse exposed to antigen can be replaced by a soluble leukocyte-derived factor. As demonstrated below, evidence for the existence of such a factor has already been presented in the literature. The object of these studies is to determine the kinetics of its production *in vivo* and to partially purify and characterize the factor.

Soluble Leukocyte-Derived Specific Lymphocyte Stimulators

The earliest evidence that a soluble factor released by macrophages could be substituted for intact macrophages in certain immune responses came from systems which tested the ability of lymphocytes to respond to certain specific antigens. In 1970 Dutton *et al.*[9] demonstrated that adherent mouse spleen cells restored the responsiveness of non-adherent spleen cells to sheep erythrocytes. They also demonstrated that conditioned medium manufactured by incubated adherent cells could restore response to this antigen. Of interest was the fact that the supernatant fraction of cultured macrophages was effective even when the macrophages were not incubated in the presence of sheep erythrocytes. However, since the fetal bovine serum used in the medium contained

antigens which cross reacted with sheep erythrocytes, it was not possible to determine from these data whether the factor produced by the macrophages was non-specific. Bach et al.[10] studied the response of purified lymphocytes to allogeneic lymphocytes and demonstrated the need for a soluble macrophage factor, which he designated CMRF (conditioned medium reconstituting factor). The response was to a specific antigen and the macrophages were incubated in 20% plasma. Cross reactivity could not be ruled out.

A Non-Specific Lymphocyte Activating Factor

Gery, Gershon and Waksman[11, 12] were the first to demonstrate that a macrophage-derived factor could stimulate thymus-processed (T-) lymphocytes in the absence of antigen. Mouse thymocytes cultured without any stimulant or in the presence of phytohemagglutinin-P (PHA) incorporated only a small amount of tritiated thymidine. However, when as few as 6,000 human peripheral blood leukocytes were added in addition to PHA, a definite increase in thymidine incorporation occurred. When the supernatant fraction of human leukocyte cultures incubated with PHA or lipopolysaccharide (LPS) was added to the thymocyte cultures, it resulted in a greater than one hundred-fold rise in the incorporation of thymidine by the thymocytes, both in the presence and absence of PHA. Syngeneic spleen cells stimulated with PHA, LPS or Concanavalin A (CON A) also produced supernatant fractions which increased mouse thymocyte DNA synthesis.[13] No specific

antigens had to be added to the thymocyte cultures.

Additional experiments by Gery and Waksman[13] revealed that this factor, which they designated lymphocyte activating factor (LAF), was produced primarily, but not exclusively, by macrophages. They designated those cells which were adherent to plastic and nylon columns as macrophages, although no morphological data was presented. The adherent population of mouse spleen cells produced a conditioned medium with more activity than that produced by unpurified spleen cells. The adherent human leukocytes produced more potentiating factor per cell than the total leukocyte population. Although the adherent cell population consists primarily of macrophages, contamination with other cells cannot be excluded. It is possible that the cooperation of other cells is necessary for the production of LAF.

Experiments by Yoshinaga *et al.*[14] demonstrated that peritoneal exudate cells of mice stimulated intraperitoneally with caseinate or the supernatant fraction derived from peritoneal exudate fluid will induce DNA synthesis in mouse thymocytes. The peritoneal exudate cells obtained three hours after caseinate injection consisted of 93% polymorphonuclear leukocytes and only 2% macrophages. At 96 hours there were 73% macrophages and 26% lymphocytes. Supernatant fractions from cultures of both the 3-hour and 96-hour exudate cells enhanced thymocyte DNA synthesis. The number of macrophages contained in the 3-hour peritoneal exudate cells (6.4×10^2 cells/cc) was insufficient, by itself, to produce a supernatant fraction

capable of inducing mitogenesis. Although adherent cells definitely appear to produce LAF, other leukocytes, particularly polymorphonuclear leukocytes, also appear to produce this substance.

T-cell cooperation may be an important element in LAF production. Gery and Waksman[13] compared the production of LAF by cultures of mouse spleen cells with cultures of spleen cells from irradiated mice reconstituted with either syngeneic bone marrow cells or bone marrow plus thymus cells. Supernatant fractions from the PHA or CON A stimulated cultures obtained from the animals reconstituted with both thymus and bone marrow cells produced greater thymocyte response than the supernatant fractions from the cultures obtained from animals reconstituted with marrow cells alone. It would appear that there is a positive feedback mechanism involved in LAF production, in which the production of LAF by adherent cells is enhanced by T cells.[15] This was confirmed by Unanue *et al.*[16], who enhanced LAF production by peritoneal exudate cells from *Listeria* infected mice by adding *in vitro* the activated T-lymphocytes from the peritoneum of similarly infected animals.

LAF is produced *in vitro* by cells from many species, including guinea pig macrophages, dog leukocytes and spleen and lung macrophages, as well as monkey leukocytes. However, under very similar conditions thymocyte stimulation was not produced by conditioned medium from cultures of L1210, L5178Y,

vero, BHK, HeLa or L-fibroblasts, despite the addition of LPS to the cell cultures.[17, 18]

LAF has been produced *in vivo* after both acute and prolonged stimulation. Supernatant fractions obtained from 1-hour cultures of peritoneal exudate cells harvested 3 and 96 hours after casein injection displayed LAF activity. Cell-free peritoneal exudate harvested at 3 and 9 hours also enhanced thymocyte mitogenesis.[14] Mitchell *et al.* found LAF activity in the conditioned medium of 24 hour cultures of spleen cells from mice given the BCG strain of tubercle bacillus intravenously 7 to 14 days previously.[19] Gery *et al.* demonstrated enhanced production of LAF by peritoneal cells in animals which 7 days previously had received an intraperitoneal injection of the methanol extraction residue of BCG.

LAF production can be stimulated by a variety of substances. Supernatant fractions with LAF activity have been obtained from human leukocytes incubated for 24 hours with LPS. Mitogens such as PHA and CON A promoted the release of LAF,[13] as did cyclic GMP.[21] Irradiation *in vivo* increased the production of LAF by peritoneal exudate cells.[22] Other investigators[16, 18] have added latex particles, antibody-coated red blood cells, *Listeria* organisms and beryllium salt *in vitro* to peritoneal exudate cell cultures and found that these substances increase the production of a factor capable of interacting with macrophages and producing enzymatic and morphological changes. However, Unanue's group did not find

a direct relationship between morphologic changes in the macrophage and the secretion of lymphostimulatory material. Gery *et al.*[24] found that LAF production by peritoneal cells activated either *in vitro* or *in vivo* did not necessarily correlate with the secretion of lysosomal enzymes.

There is evidence for the existence of macrophage products which both stimulate and inhibit lymphocytes.[25] It is important to note that several authors have found a soluble macrophage product which inhibits thymidine incorporation by lymphocytes stimulated with PHA.[26-29] Opitz and his group[30] studied this suppressor and found that it merely reduced thymidine incorporation without inhibiting DNA synthesis as measured by cell proliferation. Inhibition was temporary and ceased when the substance was washed out. The suppressing factor was dialyzable and heat stable. The most attractive explanation was that the macrophages were releasing thymidine, which diluted the radioactively labelled thymidine. Macrophages cultured with T-lymphocytes present on the opposite side of a separation chamber did not produce the inhibitor of thymidine uptake, and Opitz *et al.* postulated that macrophages phagocytize and degrade dead lymphocytes and subsequently release degradation products, including thymidine, into the medium.

LAF obtained from human leukocytes and mouse peritoneal cell cultures stimulated with endotoxin has been purified and characterized.[18, 31-34] It was non-dialyzable, heat labile,

and it could be precipitated by ammonium sulfate. Sephadex chromatography localized activity in fractions containing molecules ranging in weight from 5,000 to 25,000 daltons with a peak of activity at 15,000.[32] Calderon et al. described a molecule with similar stimulatory activity and a molecular weight of between 15,000 and 21,000 daltons. The molecule was not sensitive to diisopropylfluorophosphate treatment, but was sensitive to chymotrypsin and pepsin. The "enhancing factor" discussed by Nakamura et al.[34] was prepared from polymorphonuclear leukocytes in the mouse peritoneal exudate. It was also capable of potentiating DNA synthesis by syngeneic thymocytes stimulated with PHA. This factor had a molecular weight of 19,000, was non-dialyzable, heat labile and stable in solutions with a pH ranging between 3 and 9. In addition, the LAF activity was associated with a proteolytic activity on ^{3}H-acetyl hemoglobin. Both the thymocyte stimulating effect and the proteolytic effect were abolished by Trasylol, a protease inhibitor. Since two leukocyte products could elute in the same Sephadex fraction, these findings do not prove that the molecules are identical. Lachman et al.[35] presented evidence that LAF from a murine peritoneal cell line P388-D1 has a much higher molecular weight (80,000 daltons).

Enhancement of Immune Responsiveness and Its Possible Role in Tumor Immunotherapy

Over one hundred years ago, Busch, a Viennese physician,

noted that some malignant tumors appeared to regress coincidentally with the occurrence of erysipelas.[36] Fifty years ago Coley treated cancer patients with bacterial culture fluids (Coley's toxin), which contained endotoxin among other cellular products. He achieved striking tumor regression in some of his patients.[37] Although the mechanism by which these agents might have acted is unclear, the results provided impetus for scientists to study immune enhancement as a means of cancer therapy.

BCG Immunotherapy

Recently a modified strain of the tubercle bacillus, (Bacillus Calmette-Guerin or BCG) has been investigated in humans as an adjuvant to surgery and conventional cancer chemotherapy.[38, 39] Many investigators have demonstrated a response to BCG in animal tumors, particularly those induced by carcinogens and a mouse lymphoid leukemia line.[40-44]

The mechanism of action of BCG, endotoxin and other immunotherapeutic agents is not completely understood, but clues have been emerging. Mitchell *et al.*[19] reported increased cell-mediated immunity in mice inoculated with BCG. Spleen cells from these animals had increased capacity to lyse tumor cells. Also the spleen cells from BCG-treated animals produced more of the lymphocyte activating factor, as demonstrated by the ability of supernatant fluid from the splenocytes to increase syngeneic thymocyte DNA incorporation. The adherent spleen cells from the treated

animals produced 3 to 3½ times the LAF activity of the normal mouse adherent spleen cells.

The human melanoma appears to be an ideal tumor for immunotherapy, since it is capable of causing an immune response. Nagel et al.[45] observed specifically sensitized lymphocytes in an individual who had recently undergone surgery for malignant melanoma. Bodurtha et al.[46] found a complement dependent cytotoxic antibody to autochthonous tumor cells in the serum of nine of ten patients with localized or regionalized melanoma and in only one of eleven patients with disseminated metastases.

Endotoxin, the Immune Response and Tumor Immunotherapy

Endotoxin or lipopolysaccharide (LPS) from gram negative bacteria has been utilized to stimulate an immune response and render macrophages cytotoxic. In certain situations endotoxin has been used successfully to cause tumor necrosis and increase mouse resistance to tumor.

The immune response to LPS appears to involve primarily B-lymphocytes and macrophages.[47] Both athymic (nude) mice and their normal littermates respond in the same primary and secondary fashion to LPS. This was not the case for the response to equine erythrocytes. The immune response to this agent was severely impaired in the athymic mice. Both an antibody response and a specific plaque forming cell response can be demonstrated after an intraperitoneal or intravenous injection of LPS. A primary effect can be demonstrated after

the injection of as little as 10^{-3} ug of LPS.

Alexander and Evans[48] demonstrated that endotoxin as well as double-stranded RNA could "activate" mouse macrophages and provide them with the capability of killing mouse lymphoma cells. A macrophage preparation obtained from peritoneal cells was unable to inhibit the growth of lymphoma cells unless the macrophages had been exposed to endotoxin for at least thirty minutes. These "activated" macrophages differed from "stimulated" peritoneal macrophages, which resulted from intraperitoneal injection of BCG or thioglycollate. Stimulated macrophages contained an increased number of lysosomes but were incapable of inhibiting the growth of lymphoma cells. Currie and Basham[49] reported that cellular apposition was not a prerequisite for macrophage toxicity and that a stable supernatant product was capable of lysing tumor cells.

Several derivatives of endotoxin appear to have anti-tumor activity. A group in Japan[50, 51] found a protein fraction of endotoxin from Pseudomonas aeruginosa was capable of inhibiting the growth of ascites sarcoma 180, while the intact LPS from these bacteria was ineffective. Nowotny, Golub and Key[52] demonstrated that the complete endotoxin, the non-toxic endotoxoid (prepared from endotoxin by deacylation with potassium methylate) and endotoxin glycolipids all were capable of producing hemorrhagic necrosis in tumors.

The literature is replete with demonstrations of the effectiveness of endotoxin in inhibiting tumor growth.

Several mechanisms appear to be operational: hemorrhagic necrosis, enhancement of tumor antigenicity by endotoxin coating of tumor cells, and generalized enhancement of the immune recognition system.

Parr, Wheeler and Alexander[53] studied several variables related to hemorrhagic necrosis. Treatment with endotoxin or double-stranded RNA was effective against subcutaneous or intradermal tumors, but had no effect on intraperitoneal tumors. Unless the tumor was "established" (had been implanted for seven days), endotoxin was not effective. The authors demonstrated that several host factors contributed to tumor regression. Vascular hemorrhage had to occur and this could be blocked by heparin. Immunosuppression by whole body irradiation or anti-lymphocyte serum also interfered with tumor regression, despite the fact that hemorrhage still occurred. Tumor antigenicity (the number of cells rejected after immunization with irradiated tumor cells) correlated with the magnitude of anti-tumor activity of systemically introduced endotoxin against specific tumors.[54] Based on these results, it would appear that both vascular damage, which permits the endotoxin and various cellular elements and products to gain access to the tumor, and activation of an immune response are required.

A serum factor has been isolated which appears to mediate hemorrhagic necrosis and mimics endotoxin in this effect. It can be produced by administering endotoxin intravenously to mice which had received BCG two weeks previously. The tumor

necrosis factor is not residual endotoxin. This glycoprotein has a molecular weight of 150,000 daltons and migrates with the α-globulins. The BCG appears to "activate" macrophages and produces an increase in lysosomes. The macrophages apparently become extremely sensitive to endotoxin, which releases the macrophage contents by disruption.[55, 56]

Two groups produced tumor rejection by administering tumor cells or modified tumor cells in the presence of endotoxin.[57, 58] The endotoxin had no apparent direct toxic effect on the tumor cells, but rather appeared to cause a localized stimulation of host resistance, perhaps by coating the tumor cells and making them more immunogenic. The animals were able to resist a lethal dose of tumor cells administered after the simultaneous administration of tumor and endotoxin. This response was not obtained when only modified tumor cells or only endotoxin was given prior to the lethal dose challenge.

Bober and her group[59] found intraperitoneal injection of small amounts (0.1 to 0.5 ug) of endotoxin protected mice against a challenge of a mineral oil induced plasma cell tumor. Endotoxin treatment before and after tumor injection provided the best protection and this effect was seen with both intraperitoneally and subcutaneously injected tumor.

Mice injected with the TA3-Ha mouse ascites cell line were able to suppress tumor growth when immunized with a small amount (10 ug) of endotoxin prior to tumor challenge.[60, 61] The TA3-Ha tumor line is one which lost strain specificity but

which retained major histocompatibility antigens. Sensitization of allogeneic mice with skin grafts from strain A mice in which the tumor originated caused the recipient mice to become relatively tumor resistant. Stimulation of the host immune system by the intraperitoneal injection of 10 ug of endotoxin 3 to 0 days prior to tumor inoculation was sufficient to enable the host to destroy a lethal tumor dose. The endotoxin did not have a direct cytotoxic effect on tumor cells. Protection against tumor could be adoptively transferred by normal spleen cells exposed *in vivo* and *in vitro* to endotoxin. These experiments support the use of generalized enhancement of the immune response and host resistance as a means of controlling tumor growth and suggest that this approach should be especially successful against tumors with a high degree of antigenicity.

MATERIALS AND METHODS

Animals

B6D2F1 mice obtained from Cumberland Farms were used for all experiments unless otherwise noted. Upon arrival the animals weighed 18 to 20 grams and were approximately 6 weeks old. The mice were given a one week course of streptomycin in the drinking water (40 ug/cc) and completed treatment at least one week prior to the experiments. The animals were fed Purina Lab Chow. At the time of the experiments the mice were $3\frac{1}{2}$ to 5 months of age and weighed 30 ± 3 grams. CD1 mice (Cumberland Farms, age 18 weeks, average weight 45 grams) and C3H mice (Charles River Laboratories, age 17 weeks, average weight 27 grams) were used in one experiment.

Thymus cells for the lymphocyte activating factor (LAF) assay (see below) were obtained from CD1 mice age 8 to 10 weeks.

Reagents

Lipopolysaccharide B from *Escherishia coli* strain 055:B5 (LPS, Difco Laboratories, Detroit, MI, Lot 611788) was used. Suspensions were prepared in 0.9% sterile saline, and the required dosage was contained in 0.2 cc of saline.

The methanol extraction residue of Bacillus Calmette-Guerin

(Phipps strain) or MER was a gift of the National Cancer Institute. The preparation used, Lot BV 73-240, contained MER in a concentration of 2 mg/cc, sodium chloride (9 mg/cc), sodium carboxymethyl cellulose (5 mg/cc) and polysorbate 80 (0.004 cc/cc) in sterile water. In some experiments the mice received 0.5 cc (1 mg) of MER intraperitoneally 5 days prior to receiving an intraperitoneal injection of 50 ug of LPS.

All injections were done intraperitoneally using a #26 gauge disposable needle and a 1 cc or 5 cc Plasti-pak syringe (Becton-Dickenson, Rutherford, NJ).

Recovery of ^{3}H-LPS from Peritoneal Washings

Tritiated LPS was prepared by New England Nuclear (0.166 mCi/mg, special order #841-269-2977) by exposing 190 mg of E. coli LPS to 3 curies of tritium at 24°C for 2 weeks and lypholyzing the product 4 times. Twelve mice received an intraperitoneal injection of a mixture containing 10 ug of tritiated LPS and 90 ug of non-radioactive LPS in 0.2 cc of normal saline. Immediately following injection, 6 animals were sacrificed and 6 animals were killed 4 hours after the injection. Three cc of Minimal Essential Medium with Earle's salts (MEM, Grand Island Biological Company, Grand Island, NY) supplemented with glutamine (2 mM), penicillin (1000 U/liter), streptomycin (1000 ug/liter) and 0.05 M HEPES (Calbiochem, La Jolla, CA) at pH 7.4 was injected intraperitoneally with a #26 gauge needle and the peritoneum was

gently agitated. (Note: Throughout the remainder of this report MEM will refer to the supplemented MEM just described.) Two cc (±10%) of fluid was aspirated back and centrifuged at 500 g for 15 minutes. The supernatant fraction was decanted, added to 2 cc of scintillation fluid (described below), and counted in a liquid scintillation counter for 1 minute.

Production of Lymphocyte Activating Factor (LAF)

Peritoneal washings that contained lymphocyte activating factor (LAF) were prepared by injecting B6D2F1, CD1 and C3H mice intraperitoneally with various dosages of lipopolysaccharide (LPS). At a specified time period after the LPS injection the mice were sacrificed by cervical dislocation. The skin and abdominal muscles were incised to expose the peritoneum. Three cc of MEM was injected intraperitoneally and the peritoneum was gently agitated. Approximately 2 cc of fluid was aspirated back using the same needle. The fluid was centrifuged at 500 g for 10 minutes and the supernatant fraction was sterile filtered through a 45 μ Millipore filter (Swinnex 0.45 μ, 13 mm; Millipore Corporation). Samples were either assayed immediately or frozen at -4°C following the addition of human serum (Grand Island Biological Company, Lot 4030068) equivalent to 5% of sample volume.

Splenic LAF was produced by injecting various strains of mice with LPS in the manner described above. The animals were sacrificed and the spleens were removed. The spleens

were placed in 5 cc of MEM and homogenized individually by hand with a Potter Elvejehm homogenizer with a Teflon pestle (McAllister Bicknell Co.). The spleen cells were then cultured at various concentrations and for various periods of time in 15 x 100 mm plastic Petri dishes (Falcon Plastics, #1029, Oxnard, CA). Plates were incubated at 37°C with 5% CO_2. After 0 to 28 hours the plates were removed from the incubator and the suspension was centrifuged at 500 g for 10 min. The supernatant fraction was decanted and sterile filtered. The filtrate was either assayed immediately or treated with serum (5% v/v) and frozen.

In some experiments various substances were added to the splenocyte cultures. These included LPS, human AB+ donor plasma (New York City Red Cross Blood Bank), and isogeneic thymocytes. The thymocytes were prepared by removing the thymuses from B6D2F1 mice and homogenizing them in 5 cc of MEM, as described previously. The thymocytes were counted in a hemocytometer and diluted with MEM to a concentration of 1×10^7 cells per cc. One cc of this suspension was then added to a suspension containing 5×10^6 splenocytes per cc. The final thymocyte concentration was 1×10^6 cells/cc.

The LAF Assay

The standard LAF assay initially developed by Gery, Gershon and Waksman[11] and modified by Blyden and Handschumacher[18] was used. Essentially, this assay measured the ability of a

substance to promote thymidine incorporation in a population of thymus cells. Thymuses were removed from 8 to 10 week CD1 mice and homogenized individually in MEM with a Potter Elvejehm homogenizer with a Teflon pestle. The thymocytes were pooled and their concentration determined in a hemocytometer. The concentration was adjusted to 5×10^6 cells per cc by dilution with MEM. Human serum was added to this suspension to a final concentration of 5% (v/v), and 2-mercaptoethanol was added to a final concentration of 0.05 mM. One cc aliquots of this suspension were pipetted into the cylindrical wells on a plastic plate (Linbro Scientific Co., Hamden, CT, catalog #FB16-24TC). Into each well was added 0.1 cc of the solution being tested for LAF activity. Into selected wells either PHA (Phytohemagglutinin-P, Difco Laboratories; final concentration of 50 ug/cc), CON A (Concanavalin A, Calbiochem, 2.5 ug/cc) or 0.1 cc of the supernatant fraction from LPS-stimulated cultured human peripheral blood leukocytes was added. The plates were incubated at 37°C with 5% CO_2. After 48 hours 1 uCi of tritiated thymidine (1.9 uCi/mM; New England Nuclear) in 0.1 cc of normal saline was added. The plates were then incubated for an additional 24 hours. The contents of the wells were harvested on a 24 sample manifold using glass fiber gilter paper (Whatman, Inc., Clifton, NJ; catalog #934AH) soaked in 1 mM thymidine. The filter paper was then washed with saline and 5% trichloroacetic acid and allowed to air dry. The filter paper discs were placed in vials

containing 2 cc of scintillation fluid (prepared from 3.8 l. toluene, 19 gm 2, 5-diphenyloxazole, 0.950 gm p-bis [2-(5 phenyloxazolyl benzene]]) and counted for 1 minute in a liquid scintillation counter.

All samples were assayed in triplicate, and the results reported are the means ± standard error.

Purification and Characterization of LAF

LAF was partially purified by eliminating certain dialyzable inhibitory products. Two to three cc of crude LAF preparation was placed in a length of dialysis tubing and allowed to stand overnight (12 to 18 hours) in 2 l. of pH 7.4 HEPES-saline solution (0.05 M HEPES + 0.9% saline). The non-dialyzable fraction was sterile filtered with a 45 u Millipore filter prior to assay.

LAF was concentrated using an Amicon ultrafiltration unit (Model 52) with a UM-10 membrane (nominal retention ≅ 10,000 daltons). Forty cc. of crude splenic or peritoneal LAF was concentrated to 10 cc. These concentrates were used in the molecular weight and isoelectric point determinations outlined below.

Sephadex G-150 gel (Pharmacia) was prepared by boiling for 5 hours in 0.04 M HEPES-0.15 M sodium chloride buffer, pH 7.5. The gel was poured into 25 x 0.5 cm columns and equilibrated with the same buffer. Columns were standardized with globulin, albumin and cytochrome C. The four-fold concentrated samples (1.25 cc aliquots) were placed on top of the columns and eluted

with the HEPES-chloride buffer. One cc samples were collected for assay.

Isoelectric focusing was performed on an LKB Model 8100 apparatus (Produkter, Sweden) with a 110 cc column containing Ampholines with pH ranging from 3 to 10. Eight cc of concentrated LAF preparation was added to the column, and after 36 hours of focusing, 4 cc fractions were collected and the pH of each sample was measured. The samples were dialyzed against two changes of the HEPES-chloride buffer and sterile filtered prior to assay.

RESULTS

Production of LAF in the Peritoneum

Within the peritoneum, the response to intraperitoneally injected lipopolysaccharide (LPS) is relatively rapid. Analysis of the supernatant fraction of peritoneal washings revealed a peak of mitogenic activity at 3 hours in the absence of additional mitogens in the assay and at 6 hours in the presence of either PHA or the supernatant fraction from human peripheral blood leukocytes stimulated with LPS (See Figure I). Activity returned to base line values or less by 24 hours. It is interesting to note that the addition to the assay wells of cell-free peritoneal exudate from mice receiving 0.2 cc of normal saline intraperitoneally resulted in a decrease in the incorporation of tritiated thymidine by the thymocytes. This was also true for all samples from LPS treated mice, except for the 3 and 6 hour samples.

There was a definite relationship between the intraperitoneal dose of LPS and the mitogenic activity of the peritoneal washings (See Figure II). Two out of three mice that received 500 ug of LPS intraperitoneally died within 48 hours, while no animals given normal saline or 100 ug of LPS died.

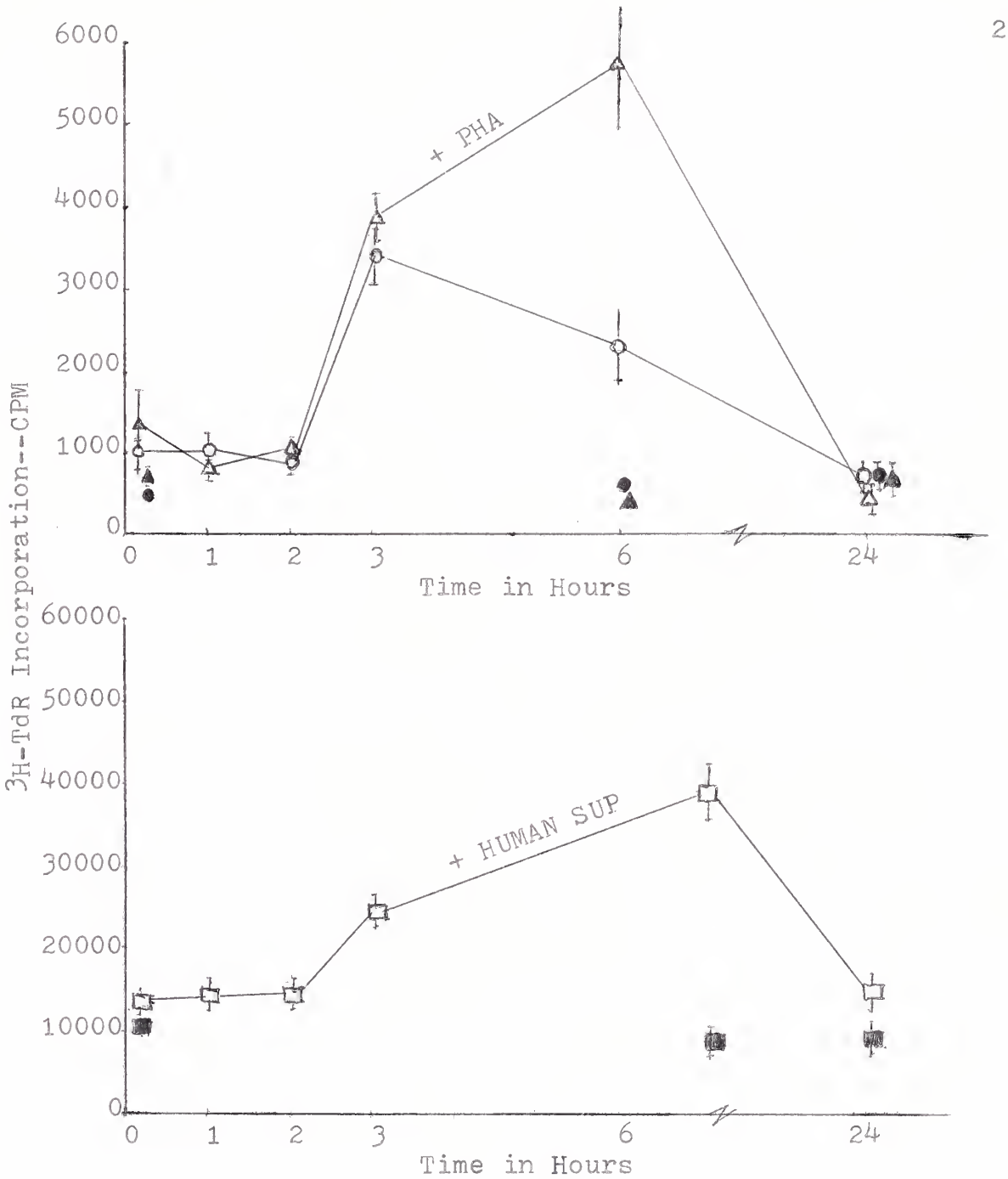


FIGURE I--TIME COURSE OF LAF PRODUCTION IN THE SUPERNATANT FRACTION OF PERITONEAL EXUDATE--B^6D^2F1 mice received 50 µg of LPS intraperitoneally at Time = 0 and were sacrificed after various intervals. The cell-free peritoneal exudate was tested for its ability to enhance thymocyte mitogenesis alone (O), in the presence of PHA (△), and in the presence of the supernatant fraction of LPS-stimulated human peripheral blood leukocytes (HUMAN SUP, □). Control mice received 0.2 cc of normal saline intraperitoneally. Cell free peritoneal exudate from these mice was assayed alone (●), with PHA (▲), and with HUMAN SUP (■). Tritiated thymidine (^{3}H-TdR) incorporation was 617±88 for thymocytes without a mitogen, 888±20 for thymocytes and PHA and 20,745±1,423 for thymocytes and HUMAN SUP. All values are ± standard error of the mean of 3 determinations.

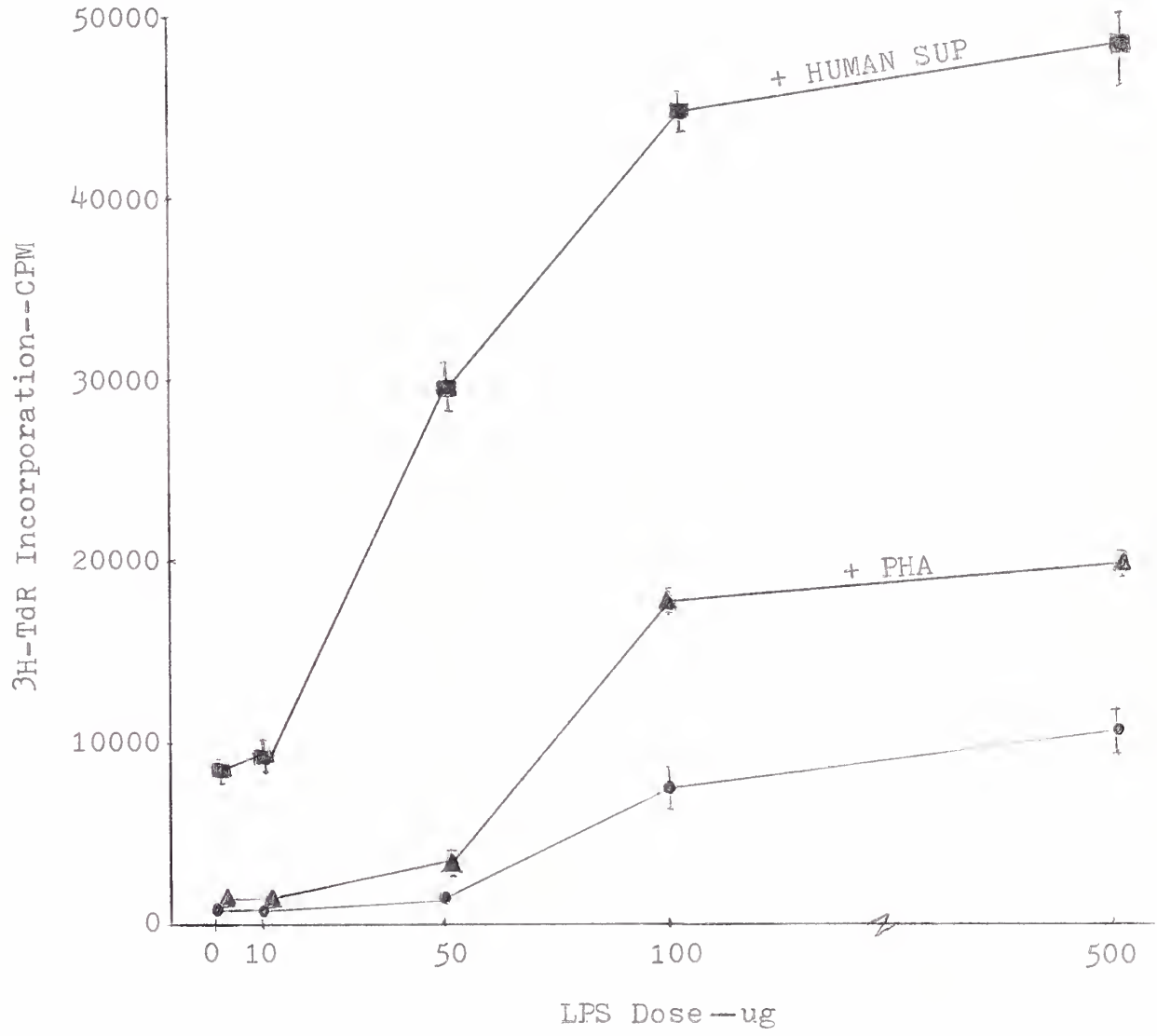


FIGURE II--RELATIONSHIP BETWEEN INTRAPERITONEAL LPS DOSE AND LAF PRODUCTION IN THE CELL-FREE PERITONEAL EXUDATE--Mice received various intraperitoneal doses of LPS suspended in 0.2 cc of normal saline. The animals were sacrificed after 4 hours. The supernatant fraction of the peritoneal exudate was assayed alone (●), in the presence of PHA (▲), and in the presence of the supernatant fraction of LPS-stimulated human peripheral blood leukocytes (HUMAN SUP, ■). Tritiated thymidine (^{3}H-TdR) incorporation was 1578±280 for thymocytes without added mitogen, 1871±101 for thymocytes and PHA, and 19,180±1,873 for thymocytes and HUMAN SUP. All are mean values are ± standard error of 3 determinations.

Mice given 100 ug of LPS were severely ill with diarrhea and diminished activity.

Injection with the methanol extraction residue of BCG (MER) also resulted in enhanced activity in the supernatant fraction of the peritoneal washings. Intraperitoneal MER injection did not by itself result in increased supernatant fraction activity after five days. However, in mice given a sub-optimal intraperitoneal dose of LPS (50 ug) five days after receiving 1 mg of MER there was a marked rise in activity in the peritoneal washings four hours after the LPS injection. There was no detectable LAF activity in the mice that had received an injection of saline 5 days before the LPS challange (See Table I.).

Studies using radioactively labelled LPS revealed that this substance is cleared rapidly from the peritoneum. Four hours after intraperitoneal injection only 0.15% of the initial dose was recovered. This can be compared to the 10.1% recovery immediately following injection. The addition of 0.1, 1, 10, and 25 ug of LPS to the assay wells in various experiments did not result in mitogenic activity significantly different from that of the control.

Splenic LAF

Spleen cells from mice stimulated with LPS were capable of releasing LAF when the cells were removed and cultured in medium without serum. To determine the time response curve for LAF production, spleens were excised at various intervals after intraperitoneal injection of LPS and cultured in a 37°C

TABLE I

ENHANCEMENT OF LPS INDUCED LAF PRODUCTION

BY TREATMENT WITH METHANOL EXTRACTION RESIDUE OF BCG (MER)

Treatment		^{3}H-TdR Incorporation (CPM)		
Day 0	Day 5	Perit. Sup. only	Perit. Sup +PHA	Perit. Sup. +Human Sup.
MER-1mg	LPS-50ug	3511±244	7200±604	43634±3887
MER-1mg	Saline	278± 31	483± 21	31043±1863
Saline	LPS-50ug	490± 76	636± 63	22155±1202
Saline	Saline	187± 15	483± 26	26392± 572
No Perit. Sup. Added		708±280	1073± 63 (PHA)	37372±2028 (Human Sup.)

Mice received either 1 mg of MER or 1 cc of normal saline intraperitoneally on day 0 and 50 ug of LPS or 0.2 cc of normal saline intraperitoneally on day 5. Four hours after the second injection the mice were sacrificed and a supernatant fraction of the peritoneal exudate prepared. All values reported are the mean of three determinations ± standard error.

incubator for a fixed interval (See Figure III). When all samples were dialyzed against HEPES buffer prior to assay, a biphasic activity curve resulted. There was a peak of production at 3 hours and a secondary rise in activity at 24 hours. (These times are the interval between LPS injection and the removal of the spleen from the animal. They do not include the 6 hour incubation period.)

The optimal incubation conditions were also determined. (See Figure IV.) The maximal rate of LAF release appeared to occur during the 1½ hours between the injection of LPS and the removal of the spleen from the mouse. (Compare the values at time 0 for the supernatant fractions from mice that had received LPS and saline with values obtained from supernatant fractions of spleen cells cultured for various lengths of time. To obtain time 0 values the spleen cell homogenate was centrifuged immediately and the supernatant promptly frozen.) A peak of activity occurred at 22 hours among the dialyzed samples. The mitogenic potential of the undialyzed samples was relatively constant. At 1½ and 4 hours the ratio of mitogenic activity of dialyzed to undialyzed samples assayed in the presence of PHA or CON A was 1.3-1.6. However, at 22 hours this ratio was 2.3. LAF release was also a function of cell concentration *in vitro*. At higher cell concentrations (2×10^7 cells/cc) less LAF activity per spleen cell was produced (See Table II).

Several unsuccessful attempts were made to stimulate LAF release in splenocytes not exposed to LPS *in vivo*. The

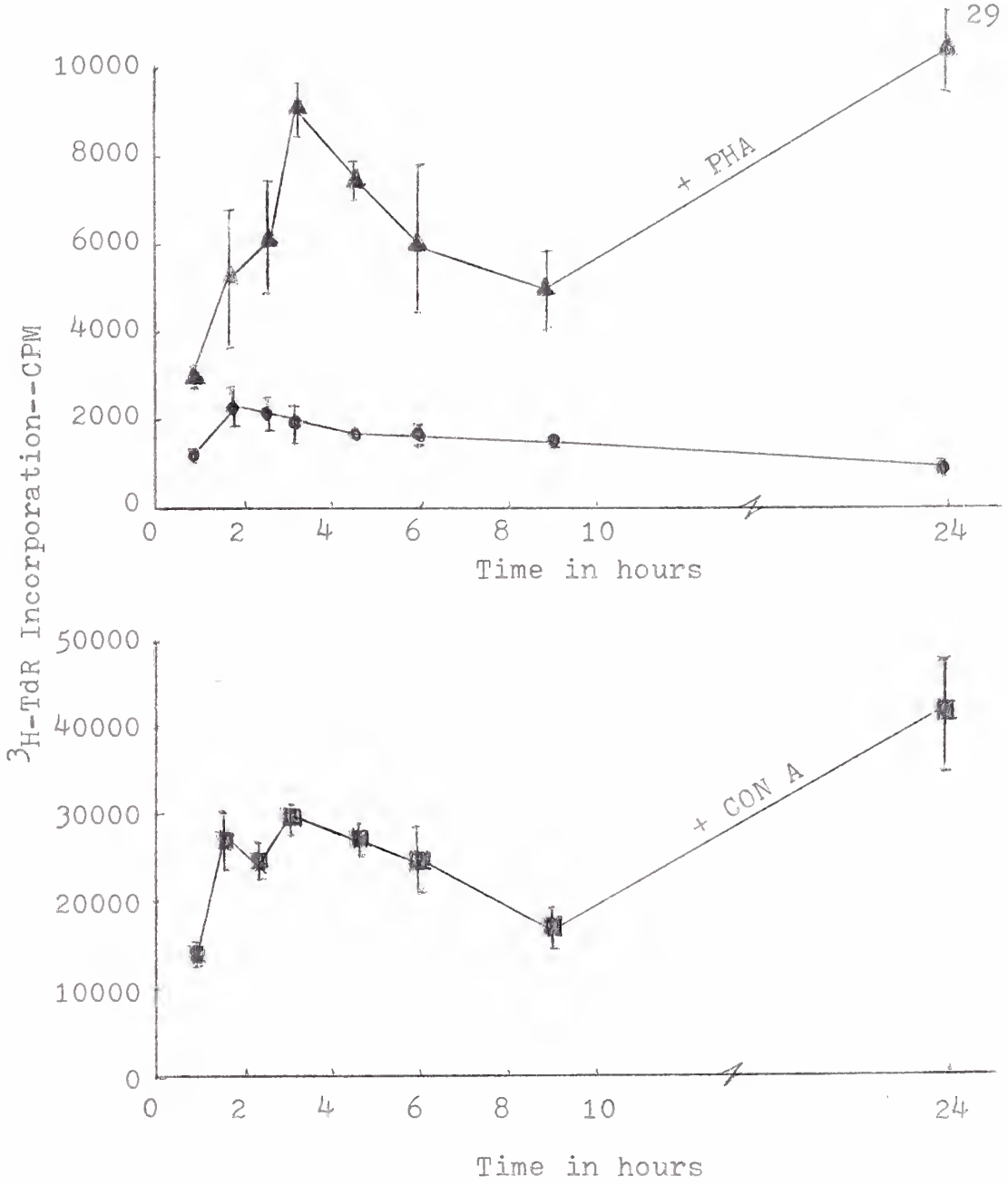


FIGURE III--LAF PRODUCTION BY DIALYZED SUPERNATANT FRACTIONS OF SPLEEN CELLS HARVESTED AT VARIOUS INTERVALS AFTER LPS INJECTION--Mice received 100 ug of LPS intraperitoneally and were sacrificed at various intervals after the injection. Spleen cells were incubated in MEM (without serum) for 6 hours at a concentration of 3.5×10^7 cells/cc. The dialyzed supernatant fractions of the cultures were assayed in the absence of additional mitogens (●), with PHA (▲) or with CON A (■). Spleen cells from mice given 0.2 cc of normal saline intraperitoneally were harvested after 1½ hours and cultured for 6 hours. Tritiated thymidine (^{3}H-TdR) incorporation was 455±26 for the supernatant fraction from these cells, 2743±198 for this fraction and PHA and 14,556±374 for this fraction and CON A. Values for the control assay were 1225±224 for the thymocytes without any mitogens, 1192±352 for the thymocytes and PHA, and 9850±1078 for the thymocytes and CON A. All values are the mean of three determinations ± standard error.

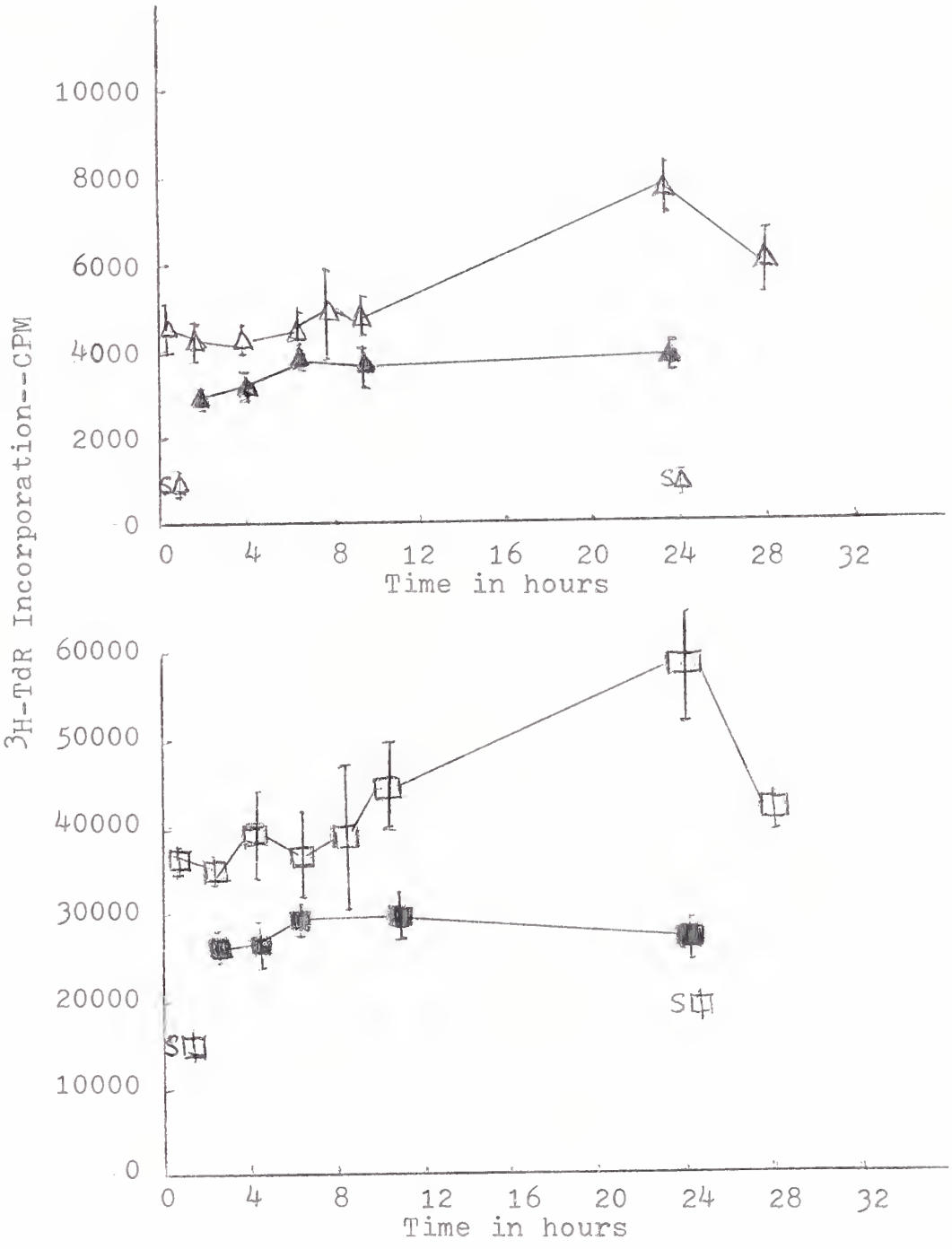


FIGURE IV--LAF PRODUCTION BY CULTURED SPLEEN CELLS EXPOSED TO LPS IN VIVO--DIALYZED VS. UNDIALYZED SUPERNATANT FRACTIONS--Mice received 100 ug LPS intraperitoneally and were sacrificed 1½ hours later. The spleens were homogenized and cellconcentration adjusted to 2 x 10^7 cells/cc. The cells were incubated in MEM (without seum) for various lengths of time. Samples were dialyzed against HEPES and assayed in the presence of PHA (Δ) or CON A (□). Undialyzed samples were also assayed with PHA (▲) or CON A (■). A group of mice received 0.2 cc of normal saline instead of the LPS. Supernatant fractions were prepared as described above and assayed with PHA (SΔ) or CON A (S□). Tritiated thymidine (^{3}H-TdR) incorporation was 1468±366 for the thymocytes without any mitogen, 1198±255 for thymocytes and PHA, and 10,800±1121 for thymocytes and CON A. All values are the mean of three detrminations ± standard error.

TABLE II

EFFECT OF SPLEEN CELL CONCENTRATION

IN VITRO ON LAF PRODUCTION

Treatment of mice	Spleen cell conc.	Tritiated Thymidine Incorporation (CPM) Spl. Sup. only	Spl. Sup. +PHA (50ug)	Spl. Sup. +CON A (2.5ug)
LPS-100ug	5×10^5	806±107	649±120	1923±189
LPS-100ug	5×10^6	577±231	1812±288	4225±310
LPS-100ug	2×10^7	579± 37	5541±1817	7555±341
Saline	5×10^5	629± 30	752± 93	1572±327
Saline	5×10^6	893±590	926± 16	1423±677
Saline	2×10^7	398± 73	660± 47	1005±327
No Spleen Sup. Added		689±262	936±164 (PHA only)	1649±154 (CON A only)

Mice received 100 ug of LPS or 0.2 cc of saline intraperitoneally and were sacrificed 1½ hours later. The spleens were homogenized and the cell concentration was adjusted with MEM (without serum). Spleen cells were incubated for 6 hours, and the supernatant fraction was obtained and assayed. All values reported are the mean of three determinations ± standard error.

addition to unstimulated spleen cells of 2 ug/cc or 20 ug/cc of LPS, 1 x 10^7 thymocytes, 0.05 cc/cc of human plasma or various combinations of these agents failed to cause LAF production.

Although the majority of the studies employed CD1 thymocytes in the assay of LAF produced by B6D2F1 mice, the allogenic nature of the source of LAF and its assay target was not essential for the demonstration of LAF activity. LPS stimulated spleen cells from CD1 and C3H mice produced LAF activity similar in magnitude (±25%) to that produced by B6D2F1 splenocytes (See Table III). CD1 thymocytes were used in the assay culture, the dose of LPS was adjusted to the weight of the mouse (3 ug LPS/gm mouse), and *in vitro* cell concentration and incubation time were held constant.

The direct addition of CD1, B6D2F1 or C3H spleen cells exposed *in vivo* to LPS to the CD1 thymocytes in the assay wells resulted in total thymidine incorporation greater than the algebraic sum of the thymidine incorporation by either cell type alone (See Table IV). In the presence of CON A the synergistic effect was also seen with unstimulated splenocytes and thymocytes. In other experiments (See Tables II and III) the conditioned medium from unstimulated splenocytes did not promote mitogenesis of thymocytes, and in some cases it was slightly inhibitory.

Characterization of Murine LAF

Preliminary data obtained by G-150 Sephadex column

TABLE III

PRODUCTION OF SPLEEN CELL LAF

IN DIFFERENT STRAINS OF MICE

Treatment of mice	Strain of mice	Tritiated Thymidine Incorporation (CPM) Spl. Sup. only	Spl. Sup. +PHA (50 ug)	Spl. Sup. +CON A (2.5 ug)
LPS (100ug)	B6D2F1	386± 78	1102±159	9767±663
LPS (100ug)	CD1	611± 13	1323±245	12058±471
LPS (100ug)	C3H	1096±322	917±114	7464±647
Saline	B6D2F1	987±472	493± 60	3478±213
Saline	CD1	486±189	890±208	3603±178
Saline	C3H	876±156	1092±230	3500±418
No Spleen Sup. Added		1130±277	1166±333 (PHA only)	4779±686 (CON A only)

Mice received 100 ug of LPS or 0.2 cc of saline IP and were sacrificed 1½ hrs after the injection. The spllen cells were cultured for 5 hrs at a concentration of 1.2×10^6 cells/cc. The undialyzed supernatant fractions were assayed. All values reported are the mean of three determinations ± standard error.

TABLE IV

EFFECT OF DIRECT SPLENOCYTE ADDITION ON THYMOCYTE DNA SYNTHESIS

Number of CD1 Thymocytes in Assay	Number of CD1 Splenocytes in Assay	Treatment of CD1 Splenocyte Donors	^{3}H-Thymidine Incoporation (CPM) No Added Stimulants	+PHA (50 ug)	+CON A (2.5 ug)
5×10^6	1×10^6	LPS-(100 ug)	3233± 55	5380±331	42737±2087
5×10^6	1×10^6	Saline	1764±129	1988±236	35297±1720
0	1×10^6	LPS-(100 ug)	298± 19	469±165	540± 82
0	1×10^6	Saline	417± 70	1130±116	360± 76
0	0	------	1130±277	1166±418	4779± 686

CD1 splenocyte donor mice (18 wks old) received either 100 ug of LPS or 0.2 cc of saline and were sacrificed after three hours. The spleens were homogenized and 1×10^6 cells in 0.1 cc of MEM was added to the appropriate 1.0 cc assay cultures. Thymocytes were obtained from 11 wk old untreated CD1 mice. The LAF assay was performed in the usual manner except for the addition of the splenocytes. All values reported are the mean of three determinations ± standard error.

chromatography suggests that peritoneal LAF has a molecular weight of 55,000 daltons (single determination of the ability of various fractions from the column to enhance thymocyte mitogenesis in the presence of CON A). There was a secondary peak of activity at 13,000 daltons (See Figure V .).

Splenic LAF appeared to have a molecular weight of 55,000 ± 15,000 daltons (two determinations-- See Figure VI..). In one experiment there was a secondary peak of activity corresponding to a molecular weight of 180,000.

The isoelectric point of splenic LAF was 5.0 ± 0.1 (two determinations).

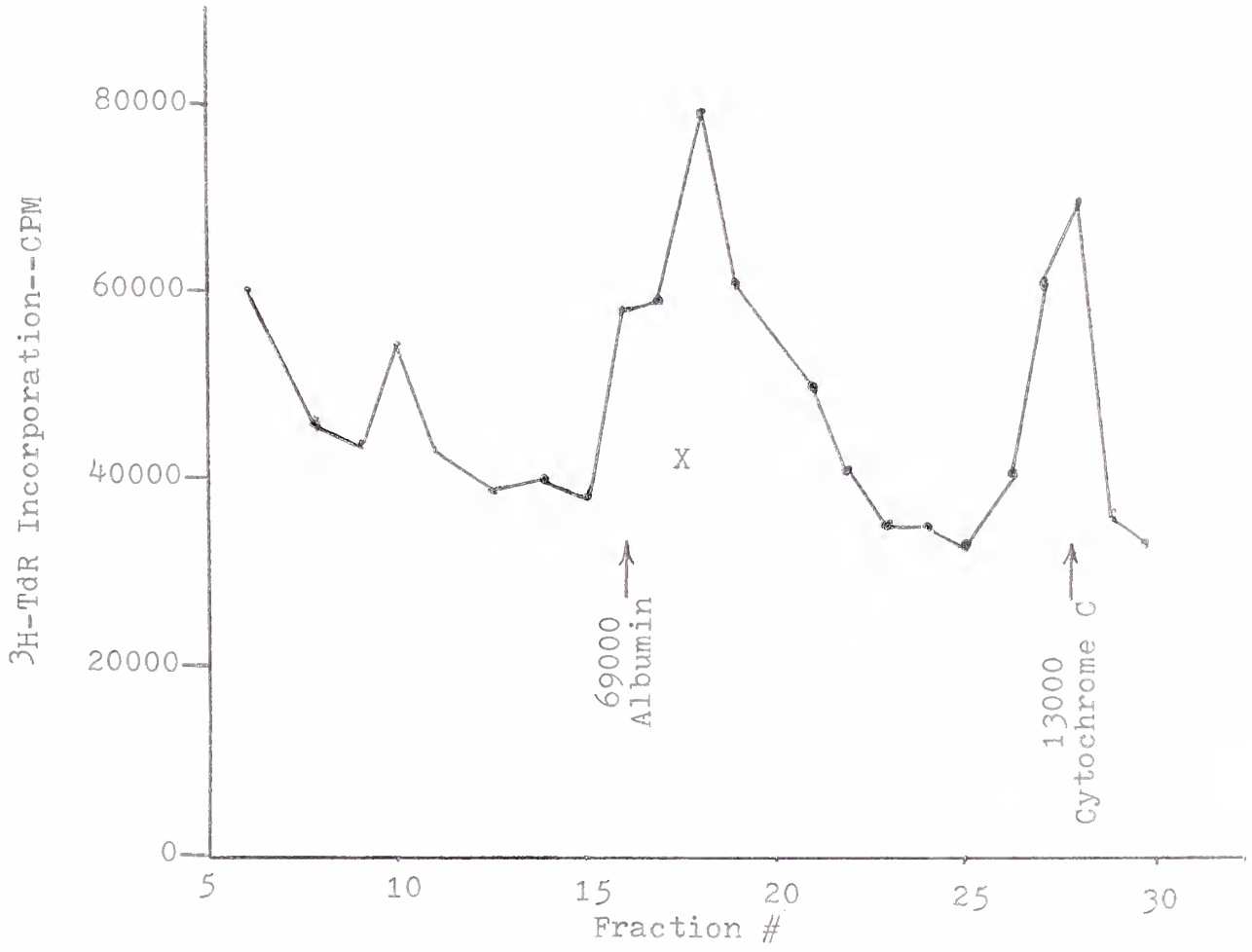
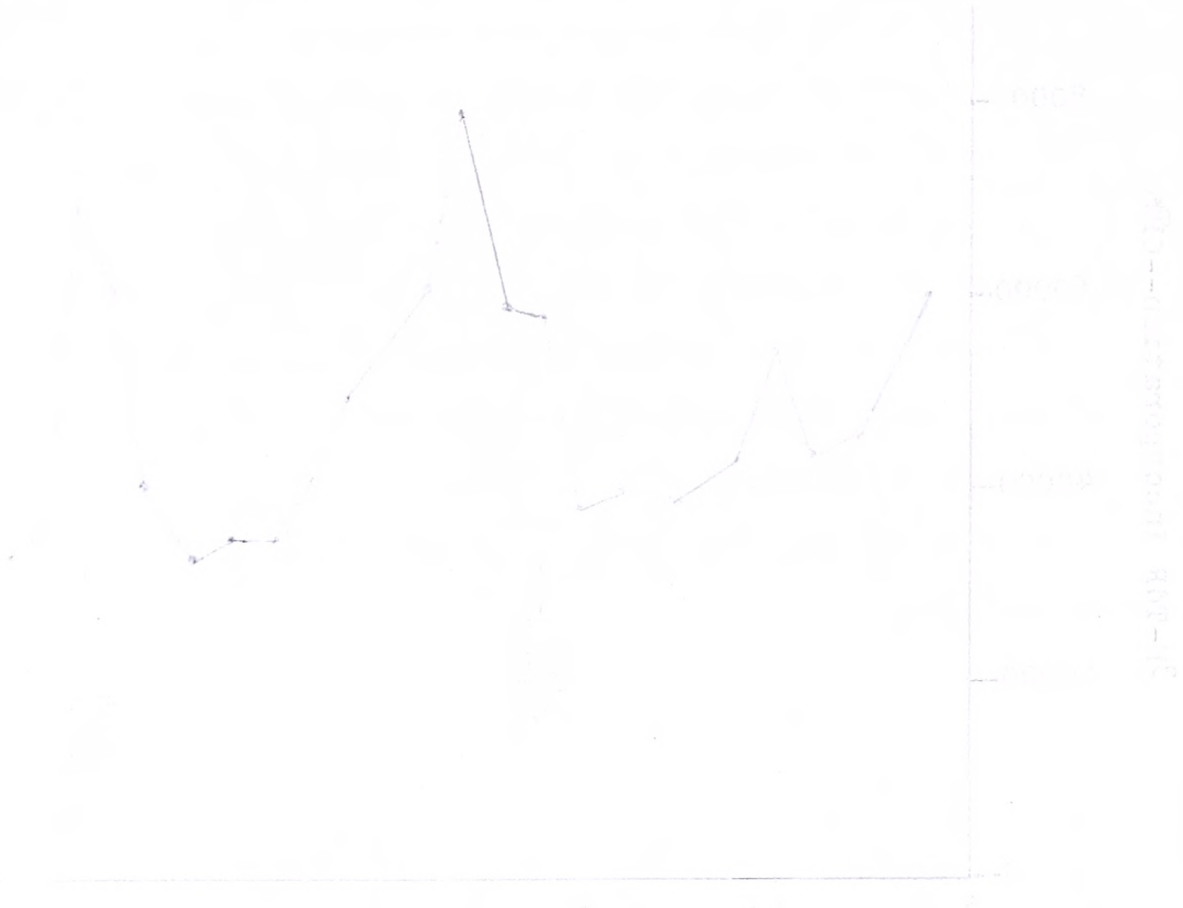


FIGURE V--G-150 SEPHADEX COLUMN CHROMATOGRAPHY OF PERITONEAL LAF
Peritoneal LAF was prepared by injecting 100 ug of LPS intraperitoneally into B6D2F1 mice. The mice were sacrificed 3 hrs after the injection and the peritoneums washed with 3.5 cc of MEM. The supernatant fraction was obtained and concentrated by a factor of 4. An aliquot (1.25 cc) was placed on top of a 25 x 0.5 cm Sephadex G-150 column and eluted with HEPES buffer. Each 1.0 cc fraction was assayed with 2.5 ug of CON A. Tritiated thymidine (^{3}H-TdR) incorporation for the thymocytes and CON A alone (X) was 42,308$\pm$9,667. All values are the mean of three determinations.



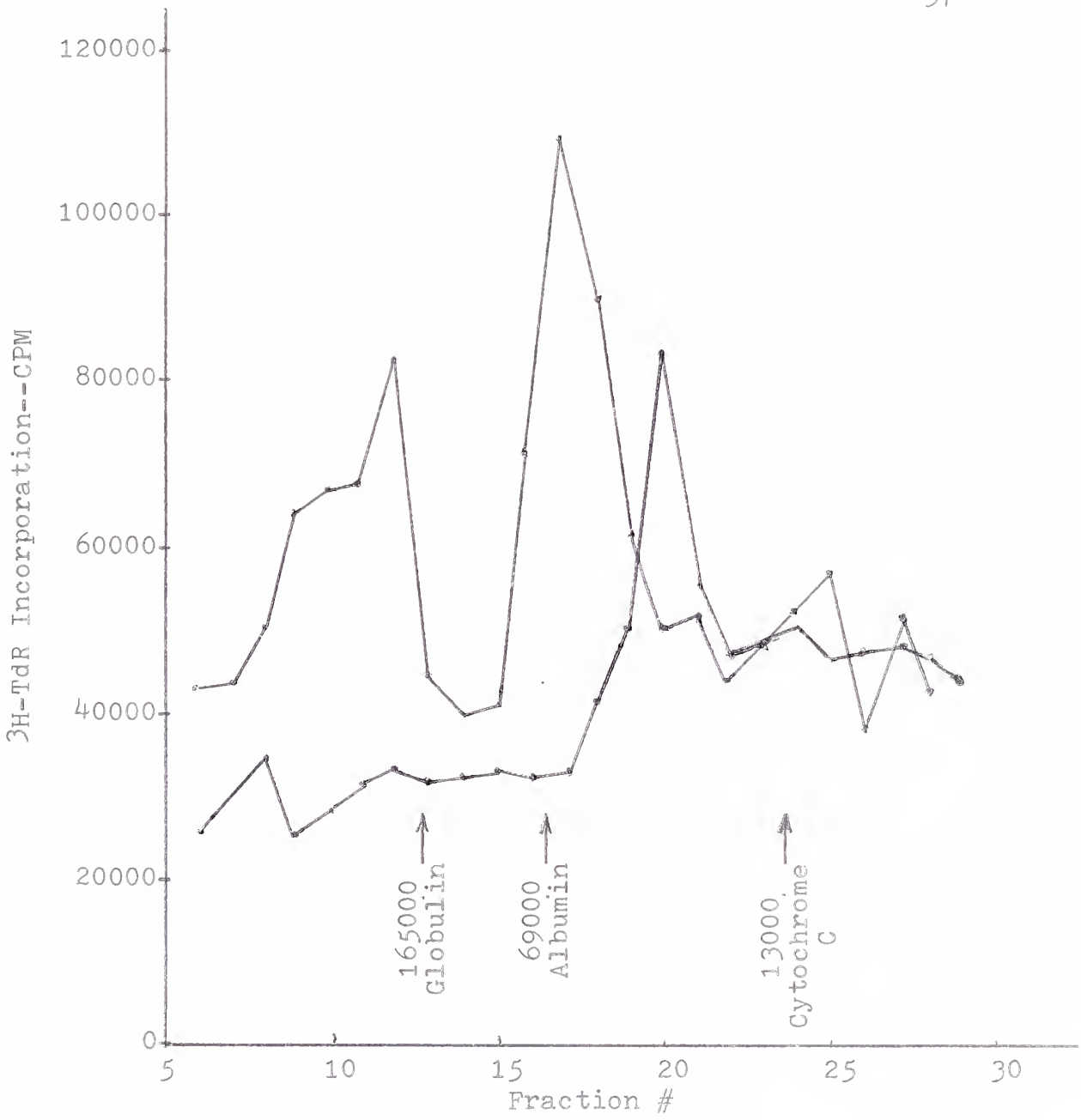


FIGURE VI--G-150 SEPHADEX COLUMN CHROMATOGRAPHY OF SPLENIC LAF
Splenic LAF was prepared by injecting 100 ug of LPS intraperitoneally into B6D2F1 mice. The mice were sacrificed after 1½ hours and the spleens removed and homogenized. The spleen cells were incubated for 2 hours at 37°C, and the supernatant fraction was obtained and concentrated by a factor of 4. An aliquot (1.25 cc) was placed on top of a 25 x 0.5 cm Sephadex G-150 column and eluted with HEPES buffer. Each 1.0 cc fraction was assayed with 2.5 ug of CON A and tritiated thymidine (^{3}H-TdR) incorporation by CD1 thymocytes was measured. All values represent the mean of three determinations.

DISCUSSION

These data confirmed the work of previous investigators, who demonstrated the production by leukocytes of a soluble factor, which is capable of enhancing lymphocyte mitogenesis. These experiments furthered this work by delineating an *in vivo* system in which LPS rapidly produced LAF within the peritoneum and spleen.

Peritoneal LAF

The rapid accumulation of LAF within the peritoneum of mice following the intraperitoneal injection of LPS is consistent with the results obtained by Yoshinaga *et al.*[14] They demonstrated that cell free peritoneal exudate caused maximal enhancement of thymocyte DNA synthesis when harvested 3 or 9 hours after the intraperitoneal injection of casein. Unfortunately, these cell-free peritoneal exudates were not dialyzed. It is possible that the release of thymidine by inflammatory cells interfered with the assay, which measured the incorporation of radioactively labelled thymidine by thymocytes. If a large amount of unlabelled thymidine were present during the assay, the measured rate of DNA synthesis would be decreased because of the dilution of the radioactive precursor.

The rapid production of LAF following challenge with an inflammatory agent raises a question regarding the cellular origin of LAF. During the first 24 hours of inflammation the predominant cell is the polymorphonuclear leukocyte.[14] Only after 3 days does the macrophage predominate. Yoshinaga et al.[14] measured the direct enhancing effect of peritoneal exudate cells on DNA synthesis by thymocytes stimulated with PHA (both cell types plus PHA in the same well). They found that early exudates (93% polymorphonuclear leukocytes and 2% macrophages) had a greater enhancing effect on DNA synthesis than the 96 hour exudate which contained 73% macrophages. An earlier report by Gery and Waksman[13] indicated that LAF was produced almost entirely by the plastic-adherent cell population (presumably macrophages). These data suggest that other leukocytes can produce LAF, although it is possible that the small number of macrophages present initially could have produced a large quantity of LAF.

Very large doses of LPS (50 µg per mouse or 2 ug/gm) were required to cause the appearance of LAF activity in the cell-free peritoneal exudate. The 100 µg dose used in most experiments caused the animals to become very ill and 500 ug was a dose greater than the LD_{50}. Some researchers have demonstrated that very small doses of LPS ($\leq$1 ug per mouse) inhibit the growth of certain tumors.[53, 58-60] Although it is possible that such small doses of LPS had a slight effect on LAF production, this was undetectable by current assay methods.

The intraperitoneal injection of MER 5 days before the LPS challenge resulted in greatly increased LAF production within the peritoneum. However, MER alone did not produce an increase in LAF production. The peritoneum in the normal mouse contains few macrophages or polymorphonuclear leukocytes, but during inflammation the number of cells increases. The chronic inflammation may also enhance the ability of LPS to release LAF by stimulating the formation and storage of LAF. It can be hypothesized that the injection of LPS into the already inflamed peritoneum provokes the rapid release of LAF, which has been manufactured in response to MER. In contrast, Gery et al.[20] have demonstrated that peritoneal exudate cells will release more LAF in culture when they are removed from a mouse that has been treated with MER than when they are derived from an unstimulated animal, and that no further stimulation is required.

Splenic LAF

Rapid production of LAF also occurred in splenocytes. Within 3 hours (1½ hours between injection and the start of incubation and 1½ hours of incubation with medium) significant activity was already present. The earliest activity noted by Blyden and Handschumacher[18] in the conditioned medium from in vitro stimulated human peripheral blood leukocytes occurred after 6 hours. The processing of LPS and stimulation of LAF production appear to be faster when the LPS is presented to the cells in an in vivo system.

Previous reports from this laboratory[18] have suggested that LAF is produced by leukocytes and not merely released. When unstimulated leukocytes were lysed osmotically and the supernatant fraction cultured with LPS for 24 hours, the activity was less than 10% that of intact leukocytes. This also suggests that the activity is not simply a degradation product of LPS, but it does not rule out the possiblity that LAF is the product of a larger precursor molecule, whose enzymatic breakdown is stimulated by LPS.

The biphasic production of LAF could be interpreted in several ways. There could be a precursor molecule available for rapid conversion or there could be rapid peptide syntesis. The secondary peak seen at 24 hours may reflect adaptation by the cell via enhanced protein synthesis. It is also possible that different cell types (polymorphonuclear leukocytes and macrophages) produce LAF at different times. In interpreting these data caution must be employed, since the amount of LAF activity represents the algebraic sum of the production and breakdown of LAF in the system. The lower value at 9 hours could simply be the result of the breakdown of the large amount of LAF produced within the first 2 hours.

The ability of unstimulated splenocytes to enhance mitogenesis in thymocytes when the two cell populations are cultured together with CON A suggests that some type of cellular cooperation (enhancement of LAF production by a thymocyte product or direct cellular contact) may be

involved. Conditioned medium from unstimulated splenocytes did not promote mitogenesis of thymocytes, and in some cases it was slightly inhibitory. Gery and Waksman[13] and Mokyr and Mitchell[62] postulated that CON A stimulates the thymocyte, which causes the splenocyte to produce LAF, which in turn further enhances thymocyte DNA synthesis. It would be worthwhile to perform a diffusion chamber experiment to determine if soluble factors were responsible for this synergistic phenomenon or whether direct cellular contact was required.

The LAF assay as developed by Gery, Gershon and Waksman[11, 12] and modified by Blyden and Handschumacher[18] was employed throughout this study. During the two years in which these studies were performed, there was considerable variation in the absolute number of counts of radioactive thymidine incorporated by the thymocytes. This occurred despite the fact that the reagents were not changed (all serum came from the same lot) and the mice were obtained from the same source. Gross bacterial contamination was quite obvious when it occurred and was a problem in only a few experiments. Inspection of the actual values obtained for cell-free peritoneal exudate and for splenocyte LAF reveals a substantial difference in the absolute amount of thymidine incorporation. Values for a standard mitogen (CON A) and supernatant fractions prepared from human peripheral blood leukocytes were substantially lower than the values seen at other times in this and other laboratories. The human leukocyte supernatant

fraction yielded ~20,000 CPM in the peritoneal exudate studies and only~2,000 in the splenocyte experiments (compared to values of ~1,000 for thymocytes cultured without any stimulants). It should be noted that these two sets of experiments were performed 12 months apart. Each component of the assay was reevaluated, but no reason was found for the discrepancy. It is possible that a subclinical viral infection could have resulted in a loss of immunocompetence. For example, it has been shown that human measles vaccination[63] and Epstein-Barr viral infection[64] (infectious mononucleosis) can result in decreased immunocompetence, as measured by anergy to the Mantoux tuberculin and Candida intradermal tests and the response of lymphocytes to PHA, pokeweed mitogen and allogeneic lymphocytes.

Since the supernatant fraction of cultured CD1 splenocytes caused mitogenesis of CD1 thymocytes, the role of strain differences as the sole cause of this phenomenon can be excluded. B6D2F1 thymocytes did not respond as well as those from the CD1 mice, and it is for this reason that CD1 thymocytes were used in the assay.

The effect of dialysis on the supernatant fractions assayed can be demonstrated by comparing the LAF activity of the dialyzed and undialyzed samples from splenocytes incubated with medium for various time intervals. The increasing difference in LAF activity between dialyzed and undialyzed samples could be explained by the production of increasing

quantities of a dialyzable inhibitor that obscures the elevation of LAF. Indeed Opitz and his group[30] studied a similar suppressor. They found that it merely reduced the incorporation of tritiated thymidine, but did not inhibit cell proliferation. The factor was heat stable and dialyzable and apparently was a cellular degradation product, since it was produced when T-lymphocytes and macrophages were cultivated together, but not when they were cultured in opposite sides of a diffusion chamber. This was consistent with the inhibitor being thymidine, which could interfere with tritiated thymidine uptake by increasing the ratio of cold thymidine to radioactive thymidine in the assay wells.

Characterization and Purification of LAF

Preliminary data indicates that the molecular weight of murine LAF (55,000 daltons) is much greater than that of the LAF obtained from human peripheral blood leukocytes (13,000 daltons)[18, 32] and values previously reported for murine LAF (5,000-25,000 daltons).[32, 34] The value obtained in these experiments appears to be similar to the molecular weight of the LAF from the murine cell line, P388-D1 (80,000 daltons).[35] The isoelectric point (pI) of the murine splenic LAF (5.0) is similar to that of the murine cell line LAF (pI = 4.8-5.0).[35] The pI of the human leukocyte LAF is 6.8-7.0.[18]

The molecular weight values were obtained with a relatively small column (25 x 0.5 cm) and each 1 cc fraction differed in molecular weight by 10,000 daltons in the range

of the peak values. Molecular weight determination with larger columns would be useful. The apparent molecular weight of the splenic LAF was only approximately twice the magnitude of the baseline activity, and the significance of the secondary peak at 180,000 daltons in one run can not be determined. These molecular weights represent peaks of activity in the presence of the mitogen, CON A. The fractions in the absence of mitogens failed to enhance DNA synthesis. Since CON A stimulates immature thymocytes, the molecule which enhances the CON A response may be different from that which affects another portion of the thymocyte population. It is conceivable that the fractions assayed in the absence of a mitogen would have produced a peak of activity corresponding to a different molecular weight. However, the fractions of human LAF which maximally enhanced CON A induced mitogenesis had the same molecular weight as those which enhanced thymocyte mitogenesis in the presence of PHA and in the absence of any mitogen. The murine LAF was obtained 4 to 6 hours after the animals were injected with LPS, and it is possible that LAF with a different molecular weight could appear later.

Implications for Immunotherapy

This research demonstrates that an intraperitoneal endotoxin challenge to a living animal can lead to the rapid production of LAF by cells in the peritoneum and spleen. The LPS resulted in considerable toxicity, and it would be

worthwhile to determine if a derivative of this complex material could produce the same LAF response with less toxicity. Different routes of endotoxin administration (intravenous or subcutaneous) might also be considered.

The experiments performed measured the amount of LAF in the immediate proximity of the cells producing this substance. It would be valuable to measure the concentration of this substance in the blood stream of mice receiving intraperitoneal LPS.

One of the distant objectives of these studies is to purify LAF and determine if non-specific stimulation of the immune system of cancer patients could effect the course of their disease. The apparent differences between human and murine LAF indicate the need for caution in applying the results from one species to another. In preliminary experiments (data not included), very crude preparations of human and murine LAF were unable to inhibit the growth of L1210 leukemia in B6D2F1 mice. Better preparations of LAF and different dosage schedules may prove more successful, and other tumors may be more responsive than L1210.

Another possible use of LAF is in the treatment of congenital immunodeficiency disease. Although LAF by itself will not replace the many components of the immune system, it might prove valuable as an adjuvant in treating certain diseases. Two diseases in which cell-mediated immunity is deficient, mucocutaneous candidiasis and the Wiskott-Aldrich

syndrome (eczema, thrombocytopenia and immunodeficiency), have responded in some cases to another substance, transfer factor.[65, 66] This substance is a dialyzable extract of sensitized leukocytes which transfers reactivity from skin test positive donors to skin test negative recipients.[67] It is possible that the use of LAF might cause these patients to respond immunologically to a wider variety of antigens.

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