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GENERATION, ISOLATION AND ASSAY METHODS FOR HUMAN LYMPHOCYTE MITOGENIC FACTOR

A Dissertation
Presented to
the Faculty of the Department of Biochemistry
Quillen-Dishner College of Medicine
East Tennessee State University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy in Biomedical Sciences

---

by
Thomas Edwin Seay
December, 1982

APPROVAL

This is to certify that the Graduate Committee of

THOMAS EDWIN SEAY

met on the

Thirtieth day of September, 1982.

The committee read and examined his dissertation, supervised his defense of it in an oral examination, and decided to recommend that his study be submitted to the Graduate Council and the Dean of the School of Graduate Studies in partial fulfillment of the requirements for the degree Doctor of Philosophy in Biomedical Sciences.

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Abstract

GENERATION, ISOLATION AND ASSAY METHODS FOR HUMAN LYMPHOCYTE MITOGENIC FACTOR

by

Thomas Edwin Seay

Activated lymphocytes secrete many products including the lymphokine human lymphocyte mitogenic factor (HLMF). In preliminary experiments lymphocytes from peripheral blood and palatine tonsils were evaluated as possible sources of HLMF by evaluating their level of activation through screening their spontaneous and concanavalin A (con A)-induced blastogenic responses. Tonsil lymphocytes (TL) were found to have high spontaneous proliferation as compared to peripheral blood lymphocytes (PBL). Cells from both sources responded to con A by undergoing a typical blastogenic response.

Because TL must be obtained septically, they are frequently cultured in the presence of the antimycotic agent, Amphotericin B (Am B). Since the primary and induced blastogenesis of TL were greatly inhibited by even low concentrations of Am B, those lymphocytes were considered unacceptable sources of HLMF. In contrast to TL the induced blastogenic responses of PBL were found to be augmented by concentrations of Am B less than 5 μg/ml, but the drug appeared to provide no beneficial effect on the quantity of HLMF produced by the cells.

HLMF appeared to be produced optimally in the first 48 hr of culture by 10^7 PBL/ml, cultured in Neuman-Tytell serumless medium which had been adjusted to 5×10^{-5} M 2-mercaptoethanol, and 5-35 μg con A/ml.

Stability of the HLMF activity could best be maintained by immediate dialysis against 0.05 M NH_4HCO_3 solution, followed by lyophilization and storage of the dried material at -80° C until use. Activity was retained at -80° C for greater than 3 months. The activity was diminished after exposure to 56° C for 30 min, and completely lost after treatment at 80° C for 10 min or 100° C for 5 min. HLMF was insensitive to trypsin and exposure to pH ranges 2-7.

Separation of HLMF and con A blastogenic activities was accomplished by addition of ovalbumin followed by Bio-Gel P-100 column chromatography. HLMF activity eluted in the 12,000-20,000 d and 30,000-50,000 d ranges. The lower molecular weight material was active in the pH range 3.4-4.6 as demonstrated by isoelectric focusing. The larger molecular weight fractions had a pI of 4.14 ± 0.97.

HLMF activated T cells, B cells and unfractionated PBL in assay, with the T cell response being generally, but not always greater. The factor behaved in a dose dependent fashion when assayed against unfractionated PBL.

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ABBREVIATIONS

α-mm	α-methylmannoside
20-α-DHP	20-α-hydroxypregn-4-en-3-one
20-α-SDH	20-α-hydroxysteroid dehydrogenase
Am B	Amphotericin B
AMoL	Acute monocytic leukemia
con A	concanavalin A
C	control
cpm	counts per minute
DIFP	diisopropylfluorophosphate
DNA	deoxyribonucleic acid
DNCB	dinitrochlorobenzene
E	experimental
HBSS	Hanks balanced salt solution
HIABS	heat inactivated AB^+ serum
HIFCS	heat inactivated fetal calf serum
HLMF	human lymphocyte mitogenic factor
IL-1	Interleukin -1
IL-2	Interleukin -2
IL-3	Interleukin -3
LAF	lymphocyte activating factor
LPS	lipopolysaccharide
MASH	multiple automated cell harvester
MCGF	mast cell growth factor
MI	mitogenic factor
MLC	mixed lymphocyte culture
MNL	mononuclear leukocytes

N-T	Neuman-Tytell serumless medium
P	preincubated
PBL	peripheral blood lymphocyte
PBS	phosphate buffered saline
P cell	persisting cell
PHA	phytohemagglutinin
pI	isoelectric point
PMA	phorbal myristic acetate
PPD	purified protein derivative of Tuberculin
PWM	pokeweed mitogen
R	reconstituted
RNAase	ribonuclease
RPMI	Roswell-Park Memorial Institute
SDS	sodium dodecyl sulfate
SI	stimulation index
SRBC	sheep red blood cell
TCGF	T-cell growth factor
TL	tonsillar lymphocyte
TT	tetanus toxoid

CHAPTER 1

INTRODUCTION

Although the earliest observations of immunity were recorded by Thucydides during an epidemic of the plague in Athens, the ability to induce immunity deliberately as a protective measure was not accomplished until the 18th century by the English physician Edward Jenner. Since that time there has been active research into the mechanism and regulation of the immune phenomena.

The existence of soluble, antigen-specific products now known as immunoglobulins was recognized early on, but the mechanisms by which the cellular and humoral immune systems communicated were poorly understood. The observation by Lawrence and Rapaport (1) that a permanent, specific immunity could be transferred from one individual to another in a cell- and immunogen-free supernatant from cultured human leukocytes led to the concept of immunoregulation by soluble mediators. The soluble mediators which act in a hormonal fashion to regulate the immune response are referred to as lymphokines (2). The study of lymphokines is now a well established parameter of immunological investigation.

The advent of *in vitro* cell culture and activation techniques allowed investigators to examine specific components of the immune response in isolated biological systems. Biological assays usually consisted of testing crude or partially purified supernatant fluids from mitogen- or antigen-stimulated immune system cells for the ability to induce the biological activity of interest in a target cell population. Target cell populations have included peripheral blood lymphocytes (PBL), T-cell and B-cell enriched subpopulations from peripheral blood, precursors of T and B lymphocytes, basophils, eosinophils, transformed cell lines, macrophages and mast cells.

Observation of a particular phenomenon in a biological assay was taken as presumptive evidence for the existence of a soluble mediator of the activity; consequently over 90 lymphokines are now named for their purported biological activity. Unfortunately, lymphokine bioassays may cumulatively measure the similar effects of several factors simultaneously. Also a single molecular entity may show varied effects that are dependent on the assay system employed. It is, therefore, unlikely that 90 different molecular entities of lymphokines exist.

Interpretation of the available information has been impeded by apparent contradictions in the literature, which may have been due, in part, to the similarity of data from many different *in vitro* systems and in the generation, isolation and assay of the various activities. Although isolation to homogeneity of some of the lymphokines have unequivocally established their existence and allowed some physiochemical characterization, that has not been possible for the majority of factors due to insufficient quantities of biologically active material.

Several authors have attempted to clarify the present mass of lymphokine literature by categorizing the various factors according to cellular origin, production conditions, biological activity, isolation methods, assay criteria and limited physiochemical data (3-7). Certainly, a more precise concept of the functional responsibilities of a given factor within the confines of the immune response can be gained by more carefully defining the generation and assay conditions. Investigations into optimum sources, production methods and isolation procedures offer prospects for procuring larger quantities of individual lymphokines. This is necessary to provide sufficient homogeneous material to allow specific antibody production and physiochemical characterization. It is to these ends that my work has examined the generation, isolation, and assay methods of one of the lymphoproliferative lymphokines, human lymphocyte mitogenic factor (HLMF).

A comprehensive review of all parameters of the presently cited lymphokines is beyond the scope of this dissertation and the reader is referred to the several previously mentioned reviews (3-7). This literature review will focus on HLMF and the related human lymphoproliferative factors. Finally, a limited review of the mitogen concanavalin A is included to familiarize the reader with the physiochemical characteristics as they are presently cited in the literature. Because of apparent widespread misconceptions, the mechanism of lymphocyte activation by con A is specifically addressed.

HLMF is so named because of its ability to stimulate directly cellular incorporation of radiolabeled thymidine in cultures of autologous or allogeneic lymphocytes in the absence of all other stimulants (7). Several investigators have described mitogenic factor assays (8-11) which quantitate cellular proliferation by measuring DNA synthesis (12). Generally, replicating cells are exposed to exogeneous radiolabeled thymidine for specific periods of time, and the levels of incorporated radiolabeled materials are determined by scintillation counting. Modifications of culture periods, target cell source and assay conditions give substantial versatility in design and application to accomodate definition of HLMF's functional responsibilities.

Several lines of evidence suggest T cells as the source of HLMF. Geha and Merler (13) demonstrated HLMF production in agammaglobulinemic children who lack B lymphocytes. Rocklin et al. (14) discovered that only immunoglobulin-negative subpopulations of lymphocytes could produce HLMF. Geha et al. (15) showed that of the sucrose density separated T-and B-cell populations, only the T-cell enriched population produced HLMF in response to the purified protein derivative of Tuberculin (PPD). Blomgren (16) demonstrated that fractionated T-cells produced HLMF after stimulation with mitogens; however, he also reported HLMF production by B-enriched subpopulation with Sepharose-bound concanavalin A. Reinherz et al. (17) using monoclonal antibodies directed against cell surface antigens, separated the T-lymphocyte population into OKT4$^+$ and OKT4$^-$ subsets. They found HLMF to be produced by antigen-stimulated OKT4$^+$, but not OKT4$^-$ subpopulations.

Other investigators have provided indirect evidence supporting this concept, including loss of HLMF production after depletion of certain T-lymphocyte subpopulations (18), and failure of purified B-cells (19, 20) or adherent cells (4) to produce HLMF activity. Larsson (21) and Littman et al. (11), however, demonstrated a macrophage requirement for HLMF production *in vitro*. This is in contrast to evidence by Insel et al. (22) that suggests HLMF can be generated in the absence of macrophages. To date, there have been no reports of endogeneous or inducible production of HLMF by an established cell line.

Maini et al. (23) reported as early as 1969 that lymphocyte activation was necessary for HLMF production. Various methods of cellular activation have been used to induce HLMF production including mixed lymphocytes culture (MLC) (24-30) in which PBL from histoincompatible donors are co-cultured for up to 120 hours. Some investigators have also used a one way MLC with the intent of reducing the sources of the lymphokines to a single donor's cells (25-27, 29, 30). De Weck et al. (30) reported the detection of HLMF activity more often and at greater levels using the one-way MLC. Either method results in the occasional failure to produce HLMF, which is probably due to insufficient foreign antigen presentation to either set of lymphocytes.

Many investigators prefer the use of subpopulation specific, polyclonal mitogens, such as pokeweed mitogen (PWM) (20, 28, 31), phytohemagglutinin (PHA) (20, 31-33) or concanavalin A (con A) (10, 19, 32-38). These mitogens induce blastogenesis as well as HLMF production in the generating cell cultures, and, therefore, it is essential that they be removed or completely inhibited in any subsequent assay for HLMF. Methods have been defined for the removal of PHA (39) and con A (40, 41), but some investigators have reported difficulty with complete removal of con A (10). Unlike PWM and PHA, con A can be specifically blocked in bioassay without concomitant loss of HLMF activity (42).

Additionally, HLMF has been generated from lymphocytes activated by specific antigens, other than the previously mentioned histocompatibility antigens. PPD (14, 16, 23, 30, 31, 43, 44) and tetanus toxoid (TT) (13, 28, 45-48) have been widely used. The use of specific antigens, however, necessitates the production of HLMF from antigen-sensitive donors, while assay of unpurified HLMF-containing supernatants requires lymphocytes from antigen-insensitive donors (25). This requires extensive donor pre-testing and additional internal assay controls.

HLMF production has also reportedly been induced by dinitrochlorobenzene (DNCB) (13, 46, 48), tumor promoting agents (49), bacterial products (27) and extracts (50) with moderate degrees of success.

Some conditions affecting the *in vitro* production of HLMF have been examined. The lymphokine is produced by cells from most lymphoid organs, including peripheral blood, lymph nodes and spleens (51). Jacobsson and Blomgren (52) reported, however, that cortical thymocytes do not produce any HLMF activity. Several investigators reported that HLMF could not be produced in serum-free media (53, 54). This is in contrast to reports by Duncan (55), Hodge and Inman (unpublished data), and others (10, 25) who have demonstrated HLMF activity in supernatants generated in various systems in the absence of serum. Cell concentrations varying from 3-10 x 10^6 cell/ml have been reported as optimum for HLMF production (3). Hart et al. (53) reported increases in yield of HLMF after inclusion of the reducing agent L-cysteine.

Production of HLMF is observed within 6 hours of culture with maximum activity being detectable 1-3 days after lymphocyte activation (11, 13, 56). Duncan et al. (55) examined the HLMF production timetable and reported optimal generation on days 2 and 3 of incubation in serumless medium. This is analogous to the generation kinetics reported for HLMF produced by antigen-stimulated mononuclear leukocytes (MNL) in the presence of serum (13, 21). The report by Hiramine and Hojo (57) of the inhibition of HLMF appearance by puromycin and cyclohexamide suggests that active protein synthesis is required for production rather than release of a preformed HLMF by activated cells. This does not, however, rule out the possibility that HLMF may be produced as an inactive, preformed precursor, which requires cleavage by an induced enzyme for activity (3). The failure of cytosine arabinoside, vinblastin and mitomycin C to block HLMF production suggests that HLMF may be generated independently of lymphocyte proliferation (57).

Reports regarding partial characterization of HLMF are difficult to compare because of the divergent conditions utilized in the assays (58). Initially, Rocklin et al. (14) observed that the 15,000-31,000 molecular weight fraction of an antigen-produced HLMF supernatant chromatographed on Sephadex G-100 induced antigen-independent proliferation of unfractionated human PBL. Littman and David (11) detected mitogenic activity in the 29,000-56,000 dalton (d) and the 15,000-31,000 d fractions; however, they qualified the report by stating that B-cell mitogenic activity was detected in significant quantity in all fraction pools above 15,000 d. De Weck et al. (30) reported HLMF activity in the 40,000-45,000 d range. Rutenberg et al. (47) observed HLMF activity from Sephadex G-200 column chromatography of HLMF-active supernatants only in the post-albumin fraction as was previously reported by Geha et al. (13). Amicon ultrafiltration of HLMF-active supernatants reported by the same group (47) suggested a molecular weight of 50,000-100,000. HLMF appeared to have a molecular weight of 21,500 on sodium dodecyl sulfate polyacrylamide gel electrophoresis (47). Very recently Duncan et al. (55) confirmed the existence of HLMF in the 27,000-35,000 molecular weight range after Bio-Gel P-100 column chromatography of 30-fold concentrated HLMF-active supernatants.

Limited thermostability and protease sensitivity data suggested that HLMF is relatively stable at 56° C, and is destroyed by proteolytic enzymes but is not affected by RNase, neuraminidase of α-amylase (13, 14, 59). Rutenberg et al. (47) later reported loss of HLMF activity after heating at 70° C for 5 min. Sundar et al. noted the loss of activity after heat treatment of 100° C for 10 min (49).

The majority of these data were obtained by assaying HLMF active, unfractionated supernatants after various treatments. The results from these experiments could have been altered by certain constituents of the serum which often were added to the medium prior to supernatant generation. Duncan et al. (55) demonstrated, however, that assay of unfractionated supernatants did in fact accurately represent the partially purified activity in serum-free systems.

Seravalli et al. (50) reported the isoelectric point (pI) of HLMF to be heterogenous between pH 8.0-8.5. Recently, this has been contradicted by Wood et al. (60) who reported pIs of 7.6-8.4 for the B cell HLMF with molecular weight greater than 26,000. The B cell HLMF smaller than 26,000 d was found to have a very broad range of pIs from 4.5-7.5 (60). These investigators also showed T-cell growth factor (TCGF) activity on isoelectric focusing to be restricted to the 6.5-7.5 pH range.

HLMF appears to exert its effect for at least 48 hours after direct contact with the responder cells (51). Since HLMF can be absorbed out by an excess of target cells it is likely that it mediates its effect via a receptor, however, the mechanism by which HLMF accelerates DNA synthesis in unstimulated lymphocytes is completely unknown (51).

Originally, investigators attributed two types of proliferative activities to HLMF; that of (a) direct induction of cellular proliferation and (b) the maintenance of ongoing proliferative action induced by prior exposure of target cells to specific antigens or mitogens (51). Lately, some investigators have suggested that only the first activity is a valid criterion for HLMF identification, since it has been possible to remove the maintenance activity without concomitant loss of the direct mitogenic activity (55).

Literature regarding target cell identification for HLMF has been extremely contradictory but progressive. Earlier reports suggested that HLMF induced proliferation in both T and B lymphocytes (13, 14, 19), however, the B lymphocyte response is often (11), but not always (32), quantitatively greater than the response of T lymphocytes. HLMF has also been reported to stimulate the proliferation of thymocytes as well as immunocompetent T lymphocytes (61). Several investigators have suggested that thymocytes are stimulated to a greater degree than lymph node, peripheral blood or peritoneal lymphocytes (62, 63).

These apparent contradictions have been, to some extent, resolved with the demonstration of several soluble mediators which potentiate ongoing proliferative actions, but fail to induce direct mitogenesis. The separation of these activities, which appear to be directed toward the T-lymphocyte population (55), has led several investigators to suggest that B lymphocytes are the primary target of HLMF (28, 47, 55, 60, 64).

The consensus is not complete, however, and there are several notable exceptions which suggest T-lymphocytes may, in fact, be the target (16, 59). Reinherz et al. (17) reported direct proliferative induction by HLMF of all major lymphocyte subclasses (T, B, and Null cells), with the proliferative response of the null subclass being maximal and significantly greater than either the T- or B-cell fractions.

Certainly, the methods of cell separation used in any of the above cited investigations allow cross-contamination among subpopulations.

The distinct possibility remains that several HLMF entities may exist, and that small variations in production or assay methods may selectively enhance for one over the other (58, 65).

Morgan et al. (66) observed that proliferation was induced in a homogeneous population of non-leukemic lymphoblasts from human leukemic blood or bone marrow by the addition of supernatant from mitogen stimulated PBL. This led to an active search for the responsible factor. Several parameters of lymphoproliferation were attributed to this factor including (a) induction of thymocyte proliferation in the presence (60, 67) or absence (68) of suboptimal concentrations of a T-cell mitogen, (b) augmentation of proliferation and generation of cytotoxic cells by allo-antigen-stimulated T-cell populations (68, 69), and (c) contribution of a second signal to activated antigen-specific helper T cells and maintenance of the cells in long-term culture (70). In 1978, Gillis et al. (71) further investigated the production and assay parameters and suggested the term T-cell growth factor (TCGF) for the responsible lymphokine. In 1979, investigators at the Second International Lymphokine Workshop adopted a classification system for the more thoroughly characterized lymphokines (72). Under the system the activities previously attributed to TCGF (71), as well as the activities of thymocyte mitogenic factor (68), co-stimulator (73), killer cell helper factor (68), and secondary cytotoxic T cell-inducing factor (69) were collectively ascribed to a single molecular entity now referred to as Interleukin-2 (IL-2) (72). Presently, the terms IL-2 and TCGF are used synonomously and interchangeably in lymphokine literature and discussion (74).

The functional criterion for distinguishing HLMF and TCGF is the ability of the former to induce direct blastogenesis in PBL (7, 55), while TCGF cannot (75, 76). TCGF can, however, act as a selective mitogen for T cells that have undergone blast transformation as a result of exposure to a lectin or antigen (74-76).

Several assays have been described for TCGF which involve lectin or specific antigen-induced peripheral T-cell blasts as targets (9, 77-81). The presently accepted definitive biological assay for IL-2 measures the lymphokine's ability to maintain the growth of factor-dependent cytotoxic T-cell lines (71, 79, 82-84).

The production of TCGF appeared to be T-cell specific in that only T-cell lectins (PHA, con A, and PWM) or antigens (alloantigens, tumor antigens) elicited TCGF release from mononuclear cell populations (71). Smith et al. (85) reported an adherent cell requirement for production. The adherent cell requirement can be substituted by lymphocyte activating factor (LAF) (see next section) in mitogen-activated systems (86). This macrophage product released after mitogen or antigen stimulation in turn stimulates T cells to produce TCGF in the presence of mitogen or antigen (87). The cellular source of TCGF has now been unequivocably confirmed with the discovery of several T-cell lines which produce TCGF constitutively (88, 89), or after mitogen stimulation (90, 91).

As is the case of HLMF, lymphocyte activation is required for TCGF production (71), but DNA synthesis is not (69). Many of the methods used for HLMF induction also stimulate TCGF production. There are reports of the use of PHA (9, 71, 75-79, 81, 84, 90, 91), con A (55, 71, 80, 90-92) and PWM (71), as activators of TCGF production. Bonnard et al. (93) reported con A to be a relatively poor stimulant of IL-2 production in cultures containing PBL. However, addition of phorbal myristic acetate (PMA) resulted in approximately a 10-fold increase in the amount of IL-2 produced above that produced by con A alone (94). Alvarez et al. (95) reported optimal production parameters for TCGF to include high cellular concentrations (5 x 10^6 cells/ml), use of serum-free media, and 1% PHA. These investigators also reported significantly less TCGF production when con A was used as the inducing lectin.

Small amounts of TCGF activity can be detected in con A-stimulated cultures within four hours, with maximal activity occurring in the 18-24 hours interval and dropping substantially thereafter (96). The peak production time of 18-24 hours was confirmed by Alvarez et al. (95) and Bonnard et al. (93). The sharp drop in production has been investigated by Gullberg et al. (96, 97) who demonstrated that abrogation of TCGF-production in primary cultures is due to cellular suppression and not to lectin-dependent killing of the TCGF-producing T cells.

Physiochemical characterization of TCGF has been significantly more thorough than for HLMF. The following characteristics have been reported by Gillis et al. (98). Molecular weights on AcA 54 and Sephadex G-100 were 15,000 d, isoelectric point of the major species was 6.5, and the activity was maintained after exposure to 6 M urea, 50 mM dithiothreitol, 0.1% sodium dodecyl sulfate (SDS), DNase I, RNase A, and neuraminidase. Activity persisted in pH ranges from 2.2-10.0 and after exposure to temperatures of 70° C for 15 minutes. Activity was lost after treatment with trypsin, chymotrypsin, subtilisin, and leucine amino peptidase (98). Duncan et al. (55) reported coelution of TCGF and HLMF in the 27,000-35,000 molecular weight fractions from Bio-Gel P-100 column chromatography. Most investigators concur with the protease sensitivity results except for Yung et al. (99) who reported TCGF to be relatively insensitive to trypsin treatment at 37° C for 15 minutes. George et al. (100) reported trypsin and chymotrypsin sensitivity for TCGF, and noted major peaks on isoelectric focusing at pH 6.8 and 8.0 and a minor peak at pH 7.4. They concluded that human IL-2 is a glycoprotein(s) with heterogeneous biochemical properties. Robb and Smith (101) showed that treatment with neuraminidase caused loss of isoelectric point heterogeneity, which also suggests protein glycosylation.

IL-2 apparently mediates its effects via a specific cell surface receptor (102). Time dependent IL-2 absorption studies have been performed which show loss of IL-2 activity from supernatants after they were incubated at either 37° C or 4° C with antigen- or lectin-activated murine spleen and thymus cells (85, 102, 103), lectin-activated human PBL (104, 105), and a murine IL-2 dependent, cytotoxic T-cell line (106). However, non-activated spleens cells, lipopolysaccharide (LPS)-activated spleen cells (85), and a non-T-cell line do not absorb IL-2 activity in a time-dependent manner (82). Absorption of IL-2 by appropriate target cells is linear with cell number (106), and the absorption of IL-2 by CT-6 cells (a murine IL-2-dependent cytotoxic T-cell line) is saturable since repetitive incubation with three aliquots of a partially purified IL-2 preparation leads to diminished IL-2 absorption (106). The absorbed IL-2 activity can be eluted from the target cell either by washing laden target cells with pH 7.0 glycine buffer (106) or heating laden glutaraldehyde-fixed cells to 56° C (102).

Several TCGF dependent cell lines have been reported (71, 79, 82-84), and are of great importance for definitive assay purposes. The target cell of biological importance, however, appears to be cytotoxic and helper T cells which have developed TCGF specific membrane receptor sites secondary to exposure to antigen or lectin (85, 103, 107, 108). Freshly isolated T cells will neither bind nor proliferate in response to TCGF, whereas T cells that have had prior exposure to antigen or lectin do absorb TCGF and proliferate apparently indefinitely, as long as TCGF is present (109). TCGF-responder precursors have been found in both immature, prothymocyte populations, and mature, peripheral, thymic-dependent T-cell populations (110, 111).

Although TCGF is well established in unique biological and biochemical parameters, it is not the only immunoproliferative factor described that potentiates via an antigen- or lectin-primed mechanism. The previously mentioned monokine, lymphocyte activating factor (LAF), was first described in 1971 by Gery et al. (112) as an activity produced by cultured human peripheral adherent leukocytes that was mitogenic for murine thymocytes but not for PBL. It was also demonstrated to augment synergistically the mitogenic effects of the lectins con A and PHA on murine thymocytes as well as splenic, thymic-derived (T) lymphocytes (74) and human T cells (113).

Investigators of the Second International Lymphokine workshop classified the LAF under the new title of Interleukin 1 (IL-1) (72). Under this system, the activities previously attributed to LAF, as well as the activities of mitogenic protein (114), helper peak-1 (115), T cell-replacing factor III (116), T cell-replacing factor-mø (61), B cell-activating factor (117), B cell-differentiation factor (118), were collectively ascribed to a single molecular entity now referred to as IL-1. Presently, LAF and IL-1 are used synonomously and interchangeably in the literature and discussion (72, 74).

HLMF and LAF can be distinguished by the ability of HLMF to induce direct mitogenesis in PBL (7, 55), while LAF can only augment, but not sustain an antigen- or mitogen-induced proliferation (72). Both LAF and TCGF appear to augment synergistically antigen- or mitogen-stimulation of lymphocytes. They can be distinguished, however, on the basis of TCGF's ability to promote and maintain *in vitro* long term cultures of T cells, while LAF lacks the capacity (72).

There is, in fact, a functional relationship between LAF and TCGF, in that the ability of LAF to augment antigen- or mitogen-stimulation is mediated by its preeminent capacity to augment production of IL-2 (TCGF) (119, 120, 121, 122). In this respect, LAF functions by inducing the synthesis and secretion of the TCGF (94, 120, 121). This link between IL-1 and IL-2 is an essential element in the cell activation sequence because it involves the conversion of a primary macrophage-derived maturational signal into a secondary T cell-derived proliferative signal that results in the amplification of specific immune responses (123).

Assays for LAF (IL-1) utilize mitogen-activated mouse thymocytes (124, 125, 126). Briefly, mouse thymocytes are incubated in medium supplemented with 5-10% pooled heat-inactivated serum, 2-mercaptoethanol (10^{-5}M), submitogenic concentrations of mitogens (con A or PHA) and LAF-active samples for 72 hrs at 37° C in a 5% CO_2 incubator. Tritiated thymidine is added after 48 hours and after 24 hours more of incubation the assays are harvested and radioactivity measured. This quantitates the ability of LAF samples to augment the suboptimal mitogen effect on the thymocytes.

Early investigations contributed considerable evidence that LAF was produced by adherent macrophages and monocytes from peripheral blood, spleen, peritoneal and pulmonary alveolar regions (126-128). Calderon et al. (129) demonstrated production by peritoneal cells even after treatment with anti-theta antiserum and complement to eliminate T cells. Similarly, Hoessli and Waksman (130) showed LAF production by T-depleted spleen cells. Confirmation of the cell responsible for LAF synthesis was achieved in 1977 by Lachman et al. (131) who showed production by the murine macrophage cell line P 388D1. To date, four cell lines have been found that produce LAF after stimulation with lipopolysaccharide (LPS), also suggesting the need for activation before synthesis (131).

Although LPS is the primary stimulant used for LAF induction (112, 114, 126, 127), other B-cell stimulants such as PPD (132), and mycobacterial peptidoglycolipids (133) may be used also. Similar to the induction of HLMF and TCGF, con A (55, 112), and PHA (112) induce LAF production in PBL cultures. Mizel et al. (134) found there was LAF production after macrophage stimulation with PMA.

The cell line P 388D1 has been a significant source of LAF for study. Initially, PMA was used alone to induce P 388D1 to make LAF. The levels of IL-1 produced, however, were not high enough to preclude an eventual requirement for hundreds of liters of culture fluid to obtain a few hundred micrograms of purified IL-1 (123). Mizel et al. (135) modeled a superinduction protocol after the method used by Belliau et a. (136) for interferon production. Generally, P 388D1 cells are incubated for 4 hours with PMA and cyclohexamide prior to a one-hour incubation with actinomycin D. Using this protocol the production of

IL-1 during the next 24 hour was dramatically increased relative to the IL-1 levels obtained with cells incubated with only PMA for five hours (123).

In PBL systems, more LAF was produced in serum-containing, rather than in serum-free, medium (51). Although it was reported that human mononuclear cell cultures failed to produce LAF in the absence of serum (137), others (138) demonstrated LAF production with rat and mouse adherent cells in the absence of serum if 2.5×10^{-5} M mercaptoethanol was added to the culture medium. Oppenheim et al. (51) reported that LAF production occurred at cell concentrations significantly less than that required for HLMF production, and they noted a decrease in LAF production with increasing cell densities.

LAF activities can be demonstrated in culture supernatants of human leukocytes within six hours of activation, but the maximum level of bioactivity is not reached until 24-48 hours of culture (126). Greater production is noted in the human system if the stimulant is continuously exposed to the producing cells, but the cells must be left exposed at least 6-8 hours to produce a detectable amount of LAF (51).

Human IL-1 is a 12,000 to 15,000 d polypeptide when obtained from human peripheral blood buffy coat mononuclear cells or acute monocytic leukemia (AMoL) cells (139). Lachman (140), reported purification to homogeneity using hollow fiber filtrations, ultrafiltration, and isoelectric focusing which resulted in a single peak with a pI of 7.0. This is in contrast to the three peaks of activity with pI's of 5.2, 6.0 and 6.9 reported by Oppenheim et al. (74). The charge heterogeneity of IL-1 may be due to the same type of posttranslational glycosylation that has been described for human IL-2 (101). Purification of IL-1 has been hampered by its predisposition to adsorb to proteins, glass surfaces, and anion exchangers, all of which significantly reduce yield (125).

IL-1 and IL-2 were not resolved by gel filtration, but were distinguished by isoelectric focusing (121). Togawa et al. (125) reported LAF activity in two distinct fractions after Bio-Gel P-100 column chromatography. These fractions differed also in biological activity. The 12,000-22,000 d fraction contained only the predescribed activities of LAF; however, the 50,000-70,000 d fraction was reported to have the unique *in vitro* functions of induction of human lymphocyte blastogenesis and production of a lymphokine that was chemotactic for monocytes (141). The supernatant from which the two peaks of activity were generated contained 5% heat-inactivated human serum. Togawa et al. (125) reported the ability to generate the 50,000-70,000 molecular weight form by incubation of the 12,000-22,000 d fraction with 2% human serum, suggesting that the higher molecular weight form of LAF may actually have been a complex of the lower molecular weight LAF and a human serum component(s) (125). Lachman (113) recently reported purification to homogeneity of IL-1 from cells obtained by therapeutic leukophoresis from a patient with acute monocytic leukemia (AMoL). The IL-1 produced by culturing AMoL cells in serum-containing medium yielded only a single peak of biological activity at approximately 11,000 d on sodium dodecyl sulfate polyacrylamide gel electrophoresis (113).

The role of IL-1 in T-cell proliferation (112, 141, 142), and the generation of helper (58) and cytotoxic T cells (143) is well established. Although IL-1 is an antigen-nonspecific mediator, it functions as an essential activating signal in all T-cell dependent, antigen-specific immune responses; the specificity of a given response being defined by the eliciting antigen (142, 143). Farrar et al. (119, 144) demonstrated that IL-1 can be substituted for the macrophage requirement in mitogen-activation of T-cells, however, the stimulatory effects of IL-1 are totally dependent upon the presence of macrophages in the antigen specific responses (142).

Investigators have suggested that IL-1 may convert its target cells into a primed state during which they develop the capacity to bind specific antigen (123). The IL-1-mediated activation step may actually involve the induction of the synthesis of cell surface receptors for antigen on T-cells (123). Evidence for this theory was presented by Puri et al. (145) with the demonstration that IL-1 increased the frequency of T cells binding the synthetic antigen, poly [L-Tyr, L-glu-poly (DL-ala)-poly (L-Lys)] (145).

The mechanism by which IL-1 exerts its effects on target T cells is currently under intensive investigation by Mizel et al. (123). It is clear, however, that expression of IL-1 activity in immune responses is dependent on the presence of a second signal, either antigen or mitogen, but that IL-1 possesses the ability to modulate the surface phenotype of T cells in a manner that is independent of an antigen or mitogen requirement (123). Stadler et al. (146) presented evidence that PMA (an *in vitro* analog of IL 1) promotes the progression of T cells to a specific part of the G_1 phase of the cell cycle and holds the cell at this stage. In this respect, IL-1 may stimulate quiescent T cells to enter a specific early phase of the cell cycle within which the cells are not committed to DNA synthesize and division, but rather to initiate the synthesis of specific cell surface components as well as lymphokines that are essential for the participation of these cells in immune responses (123).

In the regard that IL-1 can function as a thymocyte mitogen (112), or augmentor of antigen- or mitogen-proliferation (74) via its IL-2-production enhancement capability (119-122), the behavior is one of a clonal expander after specific challenge (123). The IL-1 activity of specific cell surface receptor induction may be considered one of differentiation, in that it causes quiescent T cells to progress to the final stage of readiness prior to specific antigen challenge (123). Recently, other lymphokines have been reported, whose apparent primary function is essential in the T-cell lineage differentiation.

Ihle et al. (147) reported isolation and purification to homogeneity of a factor which induces 20-α-hydroxysteroid dehydrogenase (20-α-SDH) enzyme activity on the cell surface of thymocytes. This enzyme reversibly reduces progesterone to 20-α-hydroxypregn-4-en-3-one (20-α-OHP), and is strongly associated with maturational sequences along the T-cell lineage (148). The existence of this factor is well established in the mouse, but to date, the human equivalent has not been observed (147). Since this factor is decidedly different from IL-1 and IL-2 in physiochemical and biological parameters, but certainly of lymphocyte origin, it is referred to by Ihle et al. (148) as Interleukin-3 (IL-3).

Recent experiments indicated that IL-3 may also be involved in the regulation of mast cell/basophil growth (147). The appearance in IL-3-dependent cell lines after 2-3 months exposure to IL-3 of heavily granulated, toluidine blue-positive cell types was suggestive of mast cell/basophil presence.

Recently, several groups reported isolation of continuous cell lines with basophil/mast cell characteristics whose growth was readily supported by partially purified IL-3 preparations (147, 149). Schrader et al. (150) demonstrated that persisting (P) cells similarly had the characteristics of mast cells. In this case, P cells were generated from normal splenic lymphocytes grown in the presence of T-cell growth factor preparations after removal of T cells with T lymphocyte characteristics.

In addition to the above studies, Yung et al. (99) demonstrated that conditioned medium containing IL-3 was capable of supporting the long term growth of mast cells, and thus it was named mast cell growth factor (MCGF). Proof that IL-3 unequivocably was the responsible factor for the mast cell growth in this system remains to be shown, but evidence suggests that was the case (147). It is widely recognized that although the interleukins may be present in lymphocyte culture supernatants, there appear to be several other factors involved in lymphocyte activation (151).

Sumner et al. (154) investigated the ability of some plant lectins to specifically agglutinate certain bacteria. These investigators were the first to crystalize a lectin from the jack bean (*canavalis ensiformis*), which was originally termed concanavalin fraction A. This fraction of the jack bean became known as concanavalin A or con A and could be used to agglutinate certain bacteria such as Mycobacterium and Actinomyces (155).

During the past ten years this globular lectin has been suggested to possess numerous interesting properties (155). Con A forms insoluble complexes with many polysaccharides (156), and glycoproteins (157). This precipitation reaction of con A has been utilized to assay many dextrans, lipopolysaccharides and other carbohydrate containing substances (156, 158, 159). Con A is also a potent mitogen (160, 161, 162) and it stimulates the blast formation of lymphocytes, as well as inhibiting the migration of tumor cells in *in vitro* cultures (163).

Current literature suggests the molecular structure of con A to be one of a subunit protein composed of identical subunits associated to form predominantly 55,000 d dimers below pH 6 and tetramers of 110,000 d above pH 7.0 (165). Each subunit contains one manganese ion, one calcium ion, and one saccharide molecule, and the metals are necessary for saccharide-binding activity (166, 167).

Lis et al. (168) reported that con A bound glucose, mannose, glucosamine and other structurally-related residues. Other investigators reviewed binding of con A and glycoproteins, as well as methods of inhibition and reversal of the binding (169). These have included extensive studies on the interaction of normal and pathological serum proteins and con A (170) and has been extended to include investigations of con A-serum protein interactions of non-human species (171). Lis et al. (168) reported the most effective method of con A-glycoprotein dissociation to be by addition of the hapten sugar, α-methylmannoside (α-mm).

The usefulness of plant lectins such as con A in the study of the control of cell proliferation in lymphocytes was recognized early on by immunologists (172). Studies described the effect of con A on target lymphocytes in terms of increases in membrane fluidity (172), glucose consumption (173), membrane protein synthesis (174), biological cellular cytotoxicity (175, 176, 177), biological suppression (33, 178, 179), and induction of DNA synthesis (180). A major part of recent immunological investigation focused on the molecular and biological aspects of the lymphokines produced by lymphocytes after activation by con A and other mitogens (10, 19, 32-37, 55, 71, 80, 90-92, 112). In order to define the functional and biological responsibilities of many of the induced lymphokines (181-184) it was necessary to investigate the mechanism by which con A promoted lymphocyte activation and blastogenesis (181). Power et al. (162) described the necessity of the mitogen's binding via carbohydrate binding sites to the lymphocyte surface as a prerequisite to activation. This was later confirmed by Novogrodsky et al. (185) and Dillner et al. (186). Internalization of con A does not appear to be required for activation (187, 188). 10-20 hours of cell-mitogen contact has been reported necessary for irreversible committment to DNA synthesis (162, 189). Bloom et al. (190) demonstrated that lymphokine production could occur independent of mitosis; others demonstrated differential production of lymphokines after con A challenges (181, 191).

Recently, however, it was demonstrated that the production of certain lymphokines and blastogenesis was, in fact, inseparable. The previously held simplistic version of proliferation of T cells in response to con A as a model for the proliferative aspect of the immune responses gave way to a significantly more complex model (192). Among the complexities were the interactions between distinguishable types of cells and the presence of cell-produced soluble factors (Interleukins) (193).

It is now widely accepted that con A binds to and activates T lymphocytes (71, 186) and macrophages (85, 86, 180). Macrophages respond by producing IL-1 (55, 112), which augments the con A-induced production of IL-2 (119-122) by a T-cell subpopulation. Con A simultaneously activates an IL-2-responding population of T lymphocytes to develop IL-2 receptors and the cell cycle then stops in this primed state (85, 103, 107, 108). Subsequent binding of the IL-2 (generated in the same supernatant but by a different subpopulation of T cells) results in a characteristic blastogenic response of the IL-2-reactive primed cells (109, 192-194). This requirement for IL-1 and IL-2 involvement in the con A blastogenic response may serve as a point of distinction between con A and the mechanism of HLMF blastogenesis, since it appears that the same accessory lymphokines are not required for HLMF action (55).

The presence of con A in lymphokine supernatants led to problems in distinguishing the direct effects of con A from those mediated by other lymphokines. Attempts were made to separate con A from the various lymphokine activities by a series of steps involving ammonium sulfate precipitation. Most of the lymphokine activity was located in the 50-70% salt fractions, while con A remained in solution (195). Others who used this procedure noted significant con A contamination of the resulting lymphokine-rich ammonium sulfate fractions (36). Subsequent gel filtration chromatography using Sephadex G-75 and G-100 reduced the amount of contaminating con A, but did not completely remove it (36).

Some investigators used a procedure for removal based on the affinity interaction of con A with crossed-linked dextran gels (37).

In this procedure aliquots of con A generated supernatants were passed through columns of Sephadex G-50 or G-100 at appropriate flow rates (10, 35, 35). Early reports suggested complete removal of con A was possible by this method (34, 35); however, Taylor et al. (10) showed there was significant con A contamination even after doubling column lengths and reducing con A concentrations applied to the gels (10). They further demonstrated that protein added prior to cell culture was responsible for the failure to remove con A by gel filtration presumably because the added glycoprotein effectively completed with the polyglucose gel for glycoprotein binding sites on the con A.

Recently, Duncan et al. (55) showed that con A removal from HLMF can be accomplished by Amicon ultrafiltration, followed by Bio-Gel P-100 column chromatography. They also confirmed the finding by Taylor et al. (10) that con A-activity in lymphokine supernatants can be abrogated in assay by addition of α-methylmannoside at 0.05 to 0.10 molar final concentrations (55). This allowed distinction between HLMF and contaminating con A activity in untreated or partially purified lymphokine supernatants in that the majority of HLMF activity is not abrogated while the con A blastogenic response is apparently completely blocked by the sugar (55).

Certainly, the established interleukins, as well as HLMF, and others are unique lymphokines which regulate both differentiation and proliferation of lymphocyte populations. The exact mechanisms by which these factors regulate the well orchestrated immune response are now matters of urgent and active investigation (151, 152). Understanding the products of activated lymphocytes and their mode of action in molecular terms is crucial for permitting immunologists and clinicians the use of these regulatory molecules to intervene selectively in altering, amplifying, or suppressing specific components of immune responses (153).

OBJECTIVES

The major problem with obtaining definitive biological and physiochemical information on all lymphokines is the inherent difficulty created by the small initial amount of lymphokine material. This difficulty is compounded by poorly defined handling and assay procedures which result in substantial losses before isolation and purification. Initially, it was hoped that alternate sources of cells, such as human palatine tonsils, could replace peripheral blood and provide larger numbers of starting cultures for supernatant generation. This, however, was not possible, since tonsillar lymphocytes (TL) are activated *in vivo*, and are continuously producing many different lymphokines. Secondly, TL are greatly affected by antimycotic drugs which are necessary to avoid fungal contaminations.

With the resolution of PBL as the source of HLMF and the realization that limited quantities of the lymphokine would be available as a starting material, experiments were designed to define the optimum generation, handling and assay procedures that would result in minimum loss. Ultimately, the desire was to procure enough HLMF-active material to purify the lymphokine to homogeneity. This report describes our observations and presents biochemical data defining some molecular parameters of HLMF.

CHAPTER 2

MATERIALS AND METHODS

Preparation of Peripheral Blood Lymphocytes (PBL). Whole blood was obtained in heparinized (10 U/ml) syringes by phlebotomy from normal adult human donors. The lymphocytes were collected following flotation on Ficoll-Paque (Pharmacia Fine Chemicals). They were washed twice with Hanks balanced salt solution (HBSS; Microbiological Associates), and recovered each time by centrifugation at 400 x g for 10 min at 20° C or 100 x g for 15 min at 20° C, as indicated. The washed cells were suspended in 10 mls of RPMI-1640 (without glutamine; Flow Laboratories) or Neuman-Tytell Serumless Medium (N-T; Grand Island Biologicals Company), supplemented to single strength (IX) with penicillin (100 U/ml), streptomycin (100 μg/ml), and HEPES (25 mM; Grand Island Biological Company) (supplemented medium). Glutamine was added to RPMI-1640 to 2 mM but not to N-T medium. Viability was determined by trypan blue exclusion (12). The cell concentration was adjusted to 1.25 x 10^6, 2.5 x 10^6, 5 x 10^6 or 10 x 10^6 viable cells/ml by addition of the appropriate supplemented medium. Cell preparations with less than 85% original viability were not utilized.

Preparation of Tonsillar Lymphocytes (TL). Human palatine tonsils were obtained from patients younger than 25 years after routine elective surgery for recurrent tonsillitis. The surgery generally was performed at least two weeks post-infection, during which time the patients received antibiotic chemotherapy. The excised organs were placed immediately in ice cold RPMI-1640 medium, supplemented to triple strength (3X) with penicillin (300 U/ml), streptomycin (300 μg/ml), gentomycin (100 μg/ml), amphotericin B (10 μg/ml) (Am B; Grand Island Biological

Company), and HEPES (25 mM) for transport from the hospital to the laboratory. Within 2 hours each tonsil was minced in a petri dish containing 30 ml of (1X) supplemented RPMI-1640, and then the fragments were pressed gently through an 80-mesh screen (Cellector; E.C. Apparatus) into a petri dish to obtain a single cell suspension. The cell suspension was brought to a final volume of 50 ml with supplemented RPMI and centrifuged at 20 x g for 2 min to sediment larger debris. The remaining suspension was layered on Ficoll-Paque and centrifuged at 400 x g for 40 min at 20° C. The mononuclear cells were recovered, washed twice with HBSS, and recovered each time by centrifugation at 400 x g for 10 min. The final washed cell pellet was suspended in 40 ml of supplemented RPMI. Viability was determined by trypan blue exclusion, and the concentration was adjusted to 1.25 x 10^6, 2.5 x 10^6, or 5 x 10^6 viable cell/ml with supplemented (1X) RPMI. Tonsil cell preparations with less than 85% original viability were not used.

Primary culture Blastogenesis. PBL or TL isolated as previously described were suspended in supplemented (1X) RPMI-1640 in concentrations of 1.25 x 10^6, 2.5 x 10^6, and 5.0 x 10^6 viable cells/ml. PBL and TL were each divided into four equal volumes. Am B was added to the appropriate stock cell suspension of PBL or TL so that during culture in the tissue culture plate, final concentrations respectively were 2.5, 5.0, and 10.0 μg/ml. Additional supplemented RPMI was added to control (0 μg Am B/ml) and other cultures to bring all cultures to the same volume.

Cultures were done in 96-well Falcon micro Test II sterile tissue culture plates (Becton-Dickenson and Co.). Each test well received 0.2 ml of the cell suspension containing Am B delivered by a 100 μl

Hamilton repeating syringe. All wells received 0.025 ml of heat-inactivated pooled human AB$^+$ serum (HIABS: Flow Labs.). Con A (Miles Laboratories) was suspended in sterile 1 M NaCl at a concentration of 750-850 μg/ml. This was diluted with supplemented RPMI to 50, 200, or 250 μg/ml working stock solutions. The concentration of the lectin was measured with a Beckman Model 35 spectrophotometer (Beckman Instruments) using $E^{1\%}_{280\text{ nm},\ 1\text{ cm}} = 13.0$ (Miles Laboratories). Appropriate wells were given 0.025 ml of the con A solution which resulted in a final concentration in the well of 5, 10, or 15 μg con A/ml. Wells containing control cells received only supplemented (1X) RPMI. The final density of cells was 1×10^6, 2×10^6, and 4×10^6 per ml.

After 72 hr incubation at 37° C in 95% air-5% CO_2, 0.025 ml of a ^{3}H-thymidine solution in supplemented RPMI (40 μCi ^{3}H-thymidine/ml; 6 Ci/mM; New England Nuclear) was delivered to each well. The cultures were incubated an additional 18 hr before harvesting. All cultures were done in quadruplicate.

Determination of Radioactivity. Cells were harvested on a MASH II (Microbiological Associates) sample harvester using 934 AH or GF/C glass fiber media strips (Whatman, Inc.). The strips were wet with 0.16 M NaCl before the cells were harvested.

The cell-laden discs punched from the media strips were placed in plastic mini-scintillation vials (Kimble) without complete drying. Aliquots of 5 ml of Scintisol (Isolab) were added to each vial and radioactivity was determined with a Beckman LS-3155 T liquid scintillation counter (Beckman Instruments). The radioactivity in lectin-stimulated cells was determined to 1% counting error, and that in unstimulated (control) cultures to 5% error. The data are presented as means of quadruplicate counts on each set of samples with standard deviations. Background counts per minute (cpm) determined with vials containing Scintisol were 25 ± 5. Counting efficiency using ^{3}H-thymidine standards (Beckman Instruments) consistently was 62% ± 2%.

Data are presented in the manner most easily interpreted for the specific investigation. Generally, the mean and standard deviation in quadruplicate samples were determined. From these, the data may be presented in the following way. The mitogenic index (MI) or stimulation index (SI) may be calculated using the formula:

$$\text{M.I. or (S.I.)} = \frac{\text{mean cpm of experimental}}{\text{mean cpm of control}}$$

In some cases, the values are presented as cpm above control. In these cases, the value is determined by the following formula:

[Cpm above control = cpm experimental - cpm control]

In either case the experimental value (E) represents the cpm of supernatants or treated material, or partially purified lymphokine preparation. The control value (C) may in some cases be the paired reconstituted control (R), but generally is either the AB$^+$ serum control, or the α-methylmannoside (α-mm) paired AB$^+$ serum control. In each case, however, the source of every variable is specified.

Occasionally, it is most appropriate to use the difference between the matched, rank-ordered experimental and control values to determine the mean and standard deviation. The following general protocol is used to do this:

Let X, Y, and Z equal representative cpm of either experimental (E) or control (C) samples. Then rank $X_E > Y_E > Z_E$ and

$X_C > Y_C > Z_C$ and match

$$\begin{aligned} X_e - X_c - &= X' \\ Y_e - Y_c - &= Y' \\ Z_e - Z_c - &= Z' \end{aligned}$$

and the differences X', Y', and Z' are used as single entry units to give a mean and standard deviation of differences between experimental and control values.

Determination of the Effects of Cell Density, Mercaptan, Mitogen, Am B and Culture Time on HLMF Production. The effects on HLMF production of several variables were tested using a microsupernatant generation method. Generally, PBL were obtained as previously described, except that cells were collected from HBSS washes by centrifugation at 100 x g for 15 min at 20° C, and were resuspended in supplemented N-T medium. For the cell density and mercaptan experiments, PBL cell suspensions of 12.5×10^6, 6.25×10^6, and 3.13×10^6 in the presence and absence of 5×10^{-5}M 2-mercaptoethanol were made. 0.2 ml of the suspension was delivered into designated wells of a 96-well microtiter tray. Test wells received 0.025 ml of a 200 μg/ml con A stock solution (final culture concentration was 20 μg/ml) and 0.025 ml additional supplemented medium to give a total culture volume of 0.25 ml. Control wells received 0.050 ml of supplemented medium. The cultures were incubated for 24 hr at 37° C in 95% air-5% CO_2. At the end of this period, the microtiter trays were spun at 100 x g for 15 min at 20° C in a Beckman TJ-6 Table Top centrifuge equipped with a TH-4 rotor and microtiter plate swinging bucket attachment (Beckman Instruments). 100 μl of supernatant were removed in two aspirations of 50 μl each with a Finnpipette (Titertek). The 100 μl aliquots of each test supernatant were placed aseptically in corresponding positions in a new sterile 96-well microtiter plate and frozen at -10° C until assay. 0.1 ml of fresh supplemented medium without additional con A was added to the cells in each original microsupernatant well with a 100 μl Hamilton repeating syringe. The process of removing aliquots was repeated after a total of 48 and 72 hr, with each being frozen at -10° C for at least 24 hr prior to assay.

Optimum con A dose experiments were conducted by adding 0.2 ml of 12.5×10^6 cell/ml solution to designated wells of a 96-well microtiter well. Test wells received 0.025 ml of con A stock solutions of 50, 100, 150, 200, 250, or 350 μg/ml so that final culture concentrations of 5, 10, 15, 20, 25, and 35 μg/ml were achieved. Test wells received an additional 0.025 ml of supplemented medium to reach a total culture volume of 0.25 ml. Control wells received 0.050 ml of supplemented medium to obtain the 0.25 ml cell culture volume. Samples of each test and control well were obtained and stored (as described above) at designated time intervals of 24, 48, and 72 hr. In addition to the test and control wells used for microsupernatant generation, identical corresponding tests and controls were set up in matching 96-well microtiter plates, to evaluate the blastogenic response over time of cells exposed to the same varying concentrations of con A. In these experiments, the blastogenic test and control plates were handled exactly as the microsupernatant plates, except that 0.025 ml of ^{3}H-thymidine was added for the last 18 hrs of each test period. At the end of the designated 24, 48, and 72 hr test periods, the samples were harvested and radioactivity was determined as previously described.

In the Am B experiments, 0.2 ml of the 12.5×10^6 cell/ml suspension was added to designated wells of a 96-well microtiter plate. Test wells received 0.025 ml of Am B stock solutions of 12.5, 25, 50, 100, 150, 200, or 250 μg/ml so that final culture concentrations of 1.25, 2.5, 5.0, 10.0, 15.0, 20.0, and 25.0 μg/ml were achieved. Additionally, each test well received 0.025 ml of a 200 μg/ml con A stock solution to give a final concentration of 20.0 μg con A/ml. Control wells were brought to a total volume of 0.25 ml by addition of the appropriate amount of supplemented medium. 100 μl aliquots of each test and control well were harvested as described previously, and stored at -10° C for at least 24 hr prior to assay.

In evaluating the effect after 24 hr culture of adding fresh mitogen, a different protocol was necessary because of the difficulty in removing all the culture fluid from microtiter wells while maintaining sterility and viability. This was accomplished in the following manner: PBL were obtained from several donors as described, and were washed twice in HBSS at 100 x g for 15 min at 20° C. Individual donor's cells were suspended in supplemented N-T at 10×10^6 viable cells/ml in tissue culture flasks. Cultures were designed to be preincubated (P) in which con A was added to a concentration of 20 μg/ml immediately prior to incubation, or reconstituted (R) in which con A was not added until after the 48 hr incubation. After 24 hr of incubation the P culture supernatants were harvested by centrifugation at 400 x g for 10 min, and the cells were resuspended to 10×10^6 viable cells/ml and the supernatants frozen at -10° C. One half of the individual P cultures received fresh mitogen to a concentration of 20 μg con A/ml added to the cells, and the other half received only supplemented medium and no additional con A. After an additional 24 hr incubation under the described conditions, cell culture supernatants were harvested by centrifugation, as described above and stored at -10° C for at least 24 hr before they were assayed.

HLMF Assay. Generally, samples that were to be assayed for HLMF

Figure 1. Schematic representation of assay of HLMF.

Heparanized (10 μ/ml) blood; diluted 1:1 HBSS

↓

Layer on Ficoll-Paque; 400 x g x 40 min; 20° C

↓

Collect PBL (Mononuclear fraction)

↓

Wash 2 x HBSS

↓

Suspend PBS at 4 x 10^6 viable cells/ml in Supplemented (1X) Neuman-Tytell

↓

0.1 ml cell suspension in microtest plate well

↓

Add test or control reagents to wells to give concentrations as indicated below. Final volume = 0.25 ml/well

Assay Cultures	α-D-Methylmannoside (α-mm) paired control cultures
A. AB Control: 2 x 10^6 cells/ml 10% HIABS	A. AB Control: Assay culture + 0.1 M α-mm
B. Con A Control: 2 x 10^6 cells/ml 10% HIABS 10 μg con A/ml	B. Con A Control: Assay culture plus 0.1 M α-mm Added before con A
C. "Test" Wells: 2 x 10^6 cells/ml 10% HIABS 0.1 ml of test supernatant	C. Test Wells: Assay culture plus 0.1 M α-mm Added before test supernatant

↓

Incubate 37° C, 95% Air, 5% CO_2 x 72 hrs

↓

add 1 μCi ^{3}H-thymidine (6 Ci/mm)/well

↓

Incubate 37° C, 95% Air, 5% CO_2 x 18 hrs

↓

Harvest Cells (MASH II)

↓

Determine Radioactivity

activity were delivered to the designated wells of a 96-well microculture tray in 0.1 ml volumes. For the previously described microsupernatant experiments, however, the -10° C frozen samples already in trays were thawed and allowed to equilibrate to 37° C for 30 min. Controls were created by placing 0.1 ml of supplemented medium into the extra unused wells.

Each test supernatant was assayed in the presence and absence of 0.1M α-mm (Fig. 1). In the assay wells, 0.1 ml of the supernatant fluid was placed in a microculture well, followed by 0.025 ml of HIABS. An additional 0.025 ml of supplemented medium was used to bring the total volume of 0.150 ml. Con A control wells received 0.025 ml of HIABS plus 0.025 ml of a 100 μg con A/ml stock solution. AB^+ serum controls received 0.025 ml of HIABS plus 0.025 ml supplemented medium. The α-mm paired controls received the same reagents, except that 0.025 ml of 1 M α-mm was added instead of the supplemented medium.

PBL obtained as previously described were washed twice in HBSS, and collected by centrifugation at 100 x g for 15 min at 20° C. They were then suspended in supplemented N-T medium at a concentration of 5×10^6 viable cells/ml. 0.1 ml of the PBL suspension was added to every well.

Cultures were incubated for 72 hr at 37° C in 95% air-5% CO_2 then pulsed with 1 μCi/well of ^3H-thymidine. After 18 hr more incubation, cells were harvested and radioactivity was determined as described.

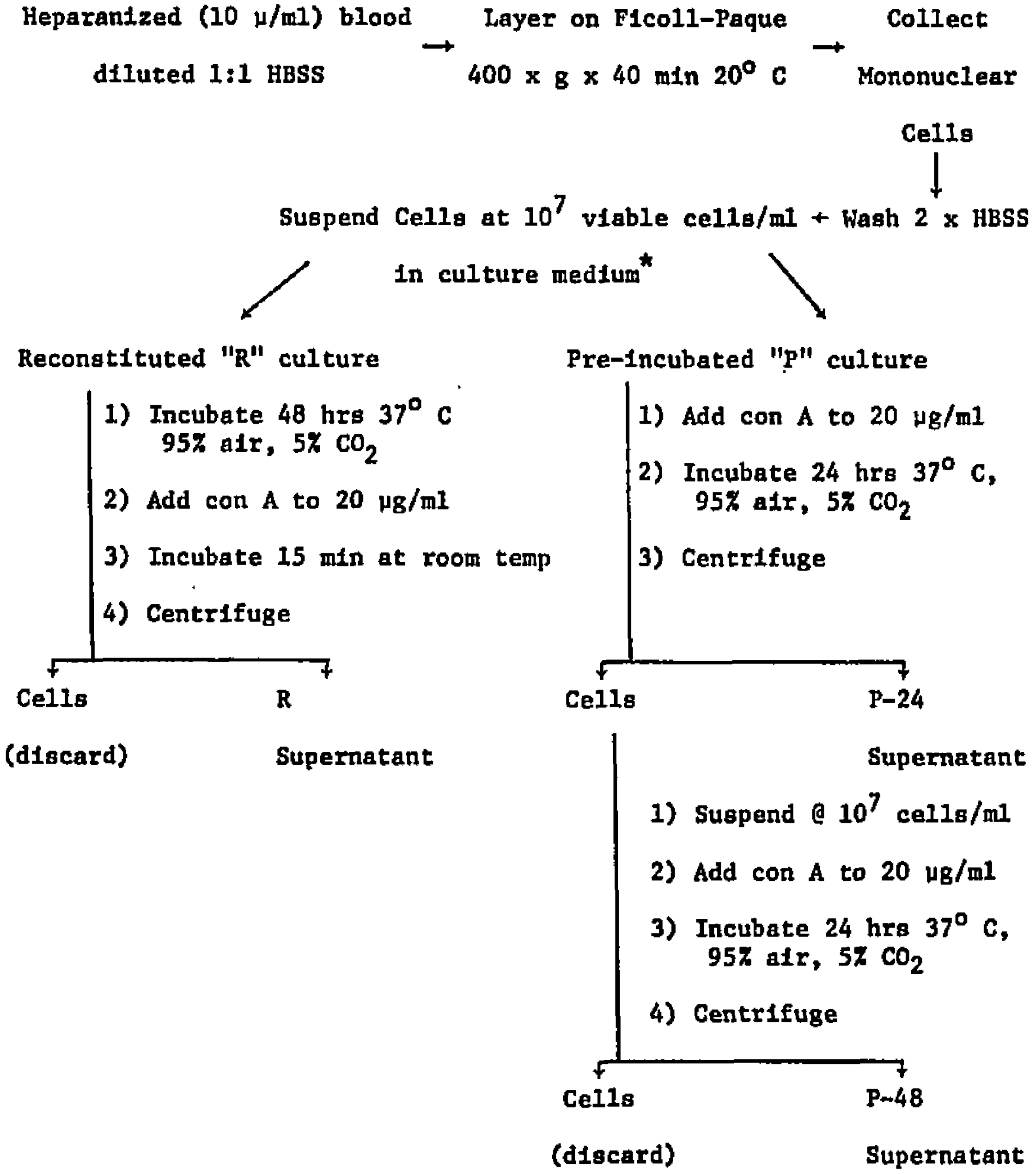
HLMF Supernatant Generation. PBL obtained as previously described were suspended at a concentration of 10^7 viable cells/ml in supplemented N-T medium containing 5×10^{-5} M 2-mercaptoethanol (Fig. 2). Viability, as judged by trypan blue exclusion, had to be at least 85% or the PBL were discarded.

Usually 5 ml of the suspension were removed and cultured separately at 37° C, 95% air-5% CO_2 for 48 hr in Corning 25 cm^2 tissue culture flasks (Corning Glass Works). After the 48 hr incubation period con A was added to a concentration of 20 μg/ml and incubated for 15 min at room temperature. The supernatant was harvested by centrifugation at 400 x g for 10 min at 20° C, and processed or frozen for assay. This is known as the reconstituted (R) supernatant and served as a control. The cells collected during centrifugation were discarded.

The remaining cell suspension was cultured at 37° C, 95% air-5% CO_2 in 75 cm^2 tissue culture flanks (Lux Scientific Corporation) for 24 hr in the presence of 20 μg con A/ml of culture fluid. At 24 hr the cells were collected by centrifugation at 400 x g for 10 min at 20° C. The supernatant fluid (P-24 supernatant) was processed or frozen for assay. The cells were resuspended at a concentration of 10^7 viable cells/ml with supplemented medium. Con A was added to a final concentration of 20 μg/ml and the cultures were incubated for 24 hr more. The supernatants were then harvested by centrifugation and were processed or frozen as P-48 supernatants. The pelleted cells were discarded (Fig 2).

Processing of Culture Supernatants. Several Methods of volume reduction were evaluated, and so was the stability of frozen lymphokine-active supernatants. 24 and 48 hr microsupernatants were obtained by culturing cells as previously described and they were frozen at -10° C without further processing. Cells from the same donors were cultured in larger flasks as described in the previous section yielding larger

Figure 2. Schematic representation of production of HLMF.



Heparanized (10 μ/ml) blood diluted 1:1 HBSS → Layer on Ficoll-Paque 400 x g x 40 min 20° C → Collect Mononuclear Cells

↓

Wash 2 x HBSS → Suspend Cells at 10^7 viable cells/ml in culture medium*

Reconstituted "R" culture

1) Incubate 48 hrs 37° C 95% air, 5% CO_2
2) Add con A to 20 μg/ml
3) Incubate 15 min at room temp
4) Centrifuge

Cells (discard)

R Supernatant

Pre-incubated "P" culture

1) Add con A to 20 μg/ml
2) Incubate 24 hrs 37° C, 95% air, 5% CO_2
3) Centrifuge

P-24 Supernatant

Cells

1) Suspend @ 10^7 cells/ml
2) Add con A to 20 μg/ml
3) Incubate 24 hrs 37° C, 95% air, 5% CO_2
4) Centrifuge

Cells (discard)

P-48 Supernatant

*RPMI-1640 requires 10% HIABS for optimum HLMF production; there appears to be no requirement for serum in Neuman-Tytell serumless medium.

volumes of 24 and 48 hr supernatants. All supernatants except those stored for stability studies were assayed the same number of days after harvest. All supernatants were assayed at approximately one-half the original concentration and in the presence of 0.1 M α-mm.

Supernatants raised in culture flasks were divided into several portions, each of which was processed in a different way and assayed for HLMF activity. One portion was stored at -80° C for 1 week, with aliquots set aside for assay at 6 weeks, 3 months, 6 months. Another portion was concentrated 30-fold by diafiltration using a DiaFlo YM-10 Ultrafiltration membrane (Amicon Corporation) under 40 psi N_2 pressure and stored at -80° C. A third portion was lyophilized without prior dialysis and stored in powder form at -80° C. A fourth portion was dialyzed against 0.05 M NH_4HCO_3 solution, pH 7.3, for 24 hr, lyophilized and stored at -80° C.

On the day prior to assay, the lyophilized samples from each protocol were rehydrated to their original volume, and dialyzed against supplemented N-T medium for 24 hr in preparation for assay. On the following day, the dialyzed samples were filter-sterilized with a 0.22 μm filter (Gelman Science), and assayed according to the standard mitogenic factor assay. Microsupernatants were thawed, equilibrated and assayed according to the described assays for microsupernatants against target cells from the same donor. Assays were begun on the seventh day after the original cultures were harvested.

Sephadex G-100 Affinity Chromatography. In the optical density experiments, a known amount of con A contained in 8-10 mls of RPMI was loaded by gravity flow onto a (1.5 x 30 cm) 30 ml Sephadex G-100 column equilibrated with PBS, pH 7.2. After 30 min incubation at room temperature without flow, elution commenced and one V_t (30 ml) was collected. The OD_{280} was measured. Initial concentration and eluate concentrations were determined by OD_{280}, $E^{1\%}_{280,\ 1\ cm} = 13.0$.

For the ^{3}H-con A trace experiments, con A concentrations of 10-20 μg/ml were trace labeled with ^{3}H-con A (34.5 Ci/mm; New England Nuclear). After the trace labeled con A stock solution cpm were determined, a known amount was loaded on the column, incubated and eluted as above. Radioactivity was determined in a Beckman LS 3155 T Liquid Scintillation counter.

In the bioassay for con A experiments, an 8 ml sample of a stock solution of con A at 4 or 10 μg/ml was loaded on G-100 columns. The eluate was concentrated to 1/2 the volume of the original stock solution and assayed according to the standard mitogenic factor assay, except that the processed eluates were used as test supernatants.

To determine the effect of serum on removal of con A from RPMI-1640, a bioassay evaluation was also used. Nine ml of RPMI-1640 containing 20 μg con A/ml and 10% serum were loaded by gravity flow onto a (1.5 x 30 cm) 30 ml Sephadex G-100 column. Incubation, elution, and bioassay were as previously described, except that a 20 μg con A/ml control was included and the assay was accomplished in the presence and absence of α-mm.

Molecular Sieve - Affinity Chromatography with Sephadex G-100. Concentrated HLMF-active supernatants were fractionated using a (1.5 x 92 cm) 145 ml column of Sephadex G-100 (Pharmacia Fine Chemical). The gel matrix was equilibrated with PBL, pH 7.2. Ascending chromatography was accomplished at room temperature using gravity flow at a rate of 12-15 ml/hr with a pressure head of 50 cm H_2O. The column was equipped with an interchangeable 0.22 µm in-line filter (Gelman Sciences), and 3-ml fractions were collected using an ISCO (Instrumentation Specialties Co.) model 328 fraction collector. The column was calibrated with the following molecular weight standards: bovine serum albumin (mol wt 67,000), ovalbumin (mol wt 43,000), chymotrypsinogen A (mol wt 23,000), and ribonuclease A (mol wt 13,000). Ferritin (1 µg/ml: mol wt 440,000) was used to determine the exclusion volume. A single two ml sample of 30-fold concentrated supernatant or control supernatant, which consisted of 150 µg con A/ml in PBS, pH 7.2 in the presence and absence of 0.1 M glucose, as well as 150 µg con A/ml in 30X N-T medium were fractionated and bioassays performed according to the HLMF assay.

Since the Sephadex G-100 also acted as an affinity column for con A, it was necessary to strip the bound con A by eluting it with one column volume of 0.1 M α-mm in PBS, pH 7.2. This was followed by re-equilibration of the column with two volumes of PBS, pH 7.2 prior to each supernatant fractionation.

Affigel-Ovalbumin Affinity Chromatography. Two ml of 20 µg/ml con A stock solution, or MF-active supernatant were loaded onto columns (0.7 x 20 cm) comprising 8 ml Affigel-ovalbumin equilibrated with PBS, pH 7.2, (Bio-Rad Laboratories). The exclusion volume of these column was three ml. Loading was accomplished by gravity flow at a rate of 1 ml/15 min. After loading, the columns were incubated at room temperature for 30 min without flow. The subsequent elution with one column volume of PBS was done at a rate of 1 ml/15 min. Samples were concentrated and assayed biologically as described in the previous section.

Bio-Gel P-100 Column Chromatography. A (2.6 x 100 cm) 375 ml column of Bio-Gel P-100, equilibrated with PBS, pH 7.2 was used for molecular sizing and fractionation. Ascending chromatography was accomplished at 4° C using gravity flow at a rate of 12-15 ml/hr and a pressure head of 75 cm H_2O. The column was equipped with an interchangeable 0.22 μm in-line filter (Gelman Sciences), and three ml fractions were collected with an ISCO Model 328 fraction collector. The column was calibrated with the following molecular weight standards: bovine serum albumin (mol wt 67,000), ovalbumin (mol wt 43,000), chymotrypsinogen A (mol wt 23,000) and Ribonuclease A (mol wt 13,700). Ferritin (1 μg/ml, mol wt 440,000) was used to determine the exclusion volume. Three-five ml samples of con A at known concentrations, or 30-fold concentrated P supernatants were fractionated, and the OD_{280} read on a Beckman Model 35 spectrophotometer.

Isoelectric Focusing. Preparative isoelectric focusing in flat beds was accomplished using Pharmalytes 3-10 (LKB) with Ultradex (LKB) serving as the support medium in the plate.

Supernatant culture fluids demonstrating HLMF activity in bioassay were concentrated by lyophilization after dialysis for 24 hr against NH_4HCO_3 solution (0.05 M; pH 7.3) at 4° C. They were rehydrated to 1/30th the neat volume with PBS, pH 7.2. Con A was removed from some samples by Affigel affinity chromatography. Other samples were fractionated by Bio-Gel P-100 column chromatography and the HLMF-active fractions pooled. Samples from each column were again dialyzed against NH_4HCO_3 for 24 hr at 4° C, lyophilized and rehydrated with PBS, pH 7.2 to 1/30th the original supernatant volume.

Plates three mm in depth with Ultradex were prepared and allowed to dry to the evaporation limit at room temperature. A 2.0 cm x 9.5

cm section of the gel was removed and mixed with the rehydrated sample. The gel-sample slurry was repoured into the section and the plate was allowed to equilibrate for 30 min at room temperature. The gels were then focused at 10° C using an LKB multiphor apparatus (LKB, Biochem). Electrophoresis was at 8 watts constant power until no further drop in milliamperage could be detected.

The gel was sliced into 15 equal sections and each was placed in a plastic funnel. The gel slices were eluted with 3-5 ml of degassed, deionized water. The pH of the eluates was determined with the use of a standard reference electrode and Brinkman Model 103 pH meter (Brinkman Instruments). The eluates were then dialyzed against 10 volumes of PBS, pH 7.2 to remove Pharmalytes, and then against 10 volumes of supplemented N-T medium in preparation for assay. Samples were then assayed using the pre-described standard HLMF assay.

pH Stability. Biologically active, NH_4HCO_3-dialyzed supernatants were dialyzed at room temperature against sodium barbitol and sodium acetate-HCl buffers (196) which were adjusted to pH values of 2.0, 3.0, 5.0, and 7.0. When the pH inside the control dialysis bag equilibrated to the designated buffer pH, timing was begun. After 30 min at the designated pH, samples were removed and placed into supplemented N-T medium which had been adjusted to pH 7.0, at 4° C. Control samples were dialyzed against the supplemented N-T only but at appropriate temperatures. All samples were dialyzed overnight in the cold against fresh supplemented N-T medium in preparation for bioassay. All samples were assayed for HLMF activity according to the described procedure.

Thermostability: HLMF-active supernatants were tested for thermal stability at 56° C for 30 min, 80° C for 10 min, and 100° C for 5 min. Aliquots of two ml of unconcentrated supernatants were placed in 1-ml pyrex test tubes with stoppers. Each sample was placed in a water bath set at the appropriate temperature. Timing was begun after the sample had reached the test temperature. The samples were immediately cooled to 10° C in an ice bath, removed, dialyzed against supplemented N-T medium and assayed for HLMF activity.

Trypsin Sensitivity. Immobilized trypsin (1238 U/gm; Millipore Corporation) was washed repeatedly in several volumes of 0.1 M Tris-HCl solution, pH 8.0. HLMF-active supernatants that had previously been dialyzed against large volumes of NH_4HCO_2, pH 7.2 were dialyzed against the 0.1 M Tris-HCl buffer. A small aliquot of each dialyzed supernatant was reserved to serve as a control. Two-ml fractions were treated with 10 µg of the washed enzyme for 1 hr at room temperature. The reaction was terminated by the addition of 10 µl of diisopropylfluorophosphate (DIFP) (0.54 M). Each sample was then centrifuged at 100 x g for 30 min to remove the immobilized trypsin and the treated supernatants were dialyzed against PBS, pH 7.2 to remove unreacted DIFP. Dialysis against supplemented N-T medium was performed in preparation for bioassay.

Optimum Bioassay Parameters. PBL obtained as previously described were suspended in supplemented RPMI-1640 medium at densities of 4×10^6, 2×10^6, and 1×10^6 viable cells/ml. Cells were stimulated with concentrations of 5, 10, and 15 µg con A/ml, in the presence of 10% HIABS.

In the experiments designed to test the media and serum support capabilities cells at 2×10^6 viable cells/ml were suspended in supplemented RPMI-1640 or supplemented N-T. Cells were stimulated with 10 μg con A/ml in the presence of 2.5, 5.0, or 10% HIABS.

Finally, cells at 2 x 10^6 viable cells/ml were suspended in supplemented N-T and stimulated with 10 μg con A/ml in the presence of 10% HIABS or heat-inactivated serum autologous to donor cells. After 72 hr incubation at 37° C in 95% air-5% CO_2, 0.025 ml of a 40 μCi ^{3}H-thymidine/ml solution in the appropriate medium was delivered to each well. The cultures were incubated 18 hr more.

Cells at a density of 2 x 10^6 viable cells/ml in supplemented RPMI-1640 fortified to 10% with HIABS and stimulated with con A as described above were harvested on a MASH II sample harvester equipped with 934-AH, GF/A, GF/C, or GF/F glass fiber media strips (Whatman, Inc.) (Table 1). Radioactivity was determined as previously described.

Log transformation of the filter media raw data was performed according to Dei et al. (197). A two way analysis of variance (ANOVA) was performed on the log transformed data to evaluate sources of variation. Multiple comparisons were made by construction of Bonferroni 95% confidence intervals about means and difference between means to evaluate performance of the filter media. The confidence limits were constructed using Dunnett's Q' (198) statistic and the standard error of the difference used to establish the limits was computed from the error mean square of a one-way ANOVA. When individual comparisons were made between experimental control values, Dunnett's test was used (PL. 05).

HLMF Dose Response. Biologically active fractions from Bio-Gel P-100 column chromatography were pooled according to peaks of activity.

TABLE 1

CHARACTERISTICS OF WHATMAN GLASS MICROFIBER FILTER MEDIA[a]

Grade	Weight (g/m^2)	Thickness (mm)	Particle size retention (μ)	Flow rate (ml/sec)
934-AH		0.30		
GF/A	52	0.25	1.5	13.0
GF/C	55	0.26	1.2	10.5
GF/F	75	0.44	0.7	6.0

[a] Taken from Whatman Inc., 1975 and 1979.

Samples were dialyzed against NH_4HCO_3 (0.05 M; pH 7.2), lyophilized, and rehydrated with distilled water to 1/30th the original supernatant volume. In preparation for bioassay, samples were dialyzed against supplemented N-T. Serial dilutions were made in supplemented N-T medium and were assayed for HLMF biological activity as described.

Biological assays for human TCGF were performed concomitantly according to the protocol of Warren et al. (9). Briefly human PBL were obtained as described and were incubated for 5 days at 37° C, 95% air-5% CO_2 at a density of 1×10^6 viable cells/ml in supplemented N-T medium made 10% in heat-inactivated fetal calf serum (HIFCS: Grand Island Biological Company) and containing con A at a concentration of 20 μg/ml. At the end of five days, the cells were washed X 3 with supplemented N-T medium, and resuspended at 2×10^5 viable cells/ml.

Serial dilutions of the 30-fold concentrated supernatants were made and assayed for their ability to sustain the proliferative activity of the con A-activated PBL. Control supernatants of TCGF-rich material were obtained commercially from Bethesda Research Laboratories.

Assays were incubated for 48 hr at 37° C, 95% air-5% CO_2 and pulse labeled with ^3H-thymidine. Radioactivity was determined as described for other bioassays after 18 hr more incubation.

Subpopulation Target Identification. PBL were separated into T-cell enriched or B-cell enriched populations. The T-cell enriched population was obtained using the nylon wool separation method of Julius et al. (199) except that HBSS was used instead of Eagles minimum essential medium. For each experiment, 3.5 μg pieces of nylon wool (Leukopak Laboratories), were washed in 0.2 N HCl, rinsed, and dried at 100° C in a drying oven. Then each 3.5 g sample was packed into a 60 cc sterile syringe and autoclaved for 20 min at 15 pounds pressure. Before use, the syringe was filled with N-T containing 10% HIFCS. After incubation of the packed column for 25 min at 37° C, 2×10^6 cells in 3 ml N-T containing 10% HIFCS were added. After a 30 min incubation at 37° C, the non-adherent cells were eluted by addition to the wool of 150 ml of HBSS containing 10% HIFCS. The cells which did not adhere were termed the T-lymphocyte-enriched population. E-rosette testing, with 10% sheep red blood cells (SRBC) (96) for T-lymphocyte markers showed 83% rosetting cells and viability of the eluted cell population was 90% as determined by trypan blue exclusion.

B-enriched cell populations were obtained by a combination of SRBC E-rosetting, and Ficoll-Paque density gradient centrifugation as described by Jencks et al. (200). PBL at a density of 4×10^6 cell/ml were mixed 1:1 with freshly washed SRBC at a concentration of 0.5% (0.05 cc packed cells in 10 ml medium) and incubated at 37° C for 15 min in a heated water bath. The mixture was then layered on Ficoll-Paque and centrifuged in a TJ-6 table top centrifuge for 10 min at 200 x g at 20° C. The remaining mononuclear layer was collected and the procedure repeated. After the second E-rosette, Ficoll-Paque centrifugation, floating MNL cells were collected, washed twice, and examined for T-lymphocyte markers and viability. The floating MNL layer of cells were termed B-cell enriched population. The cells had <1% E-rosetting cells and were >95% viable.

The standard HLMF assay was performed on whole HLMF-active supernatants, pooled active Sephadex G-100 chromatography fractions and the pooled active Bio-Gel P-100 chromatography fractions using unfractioated PBL, T-enriched, or B-enriched cells from the same donor as target cells. The cell density in each case was adjusted to 2×10^6 viable cell/ml in the wells for assay.

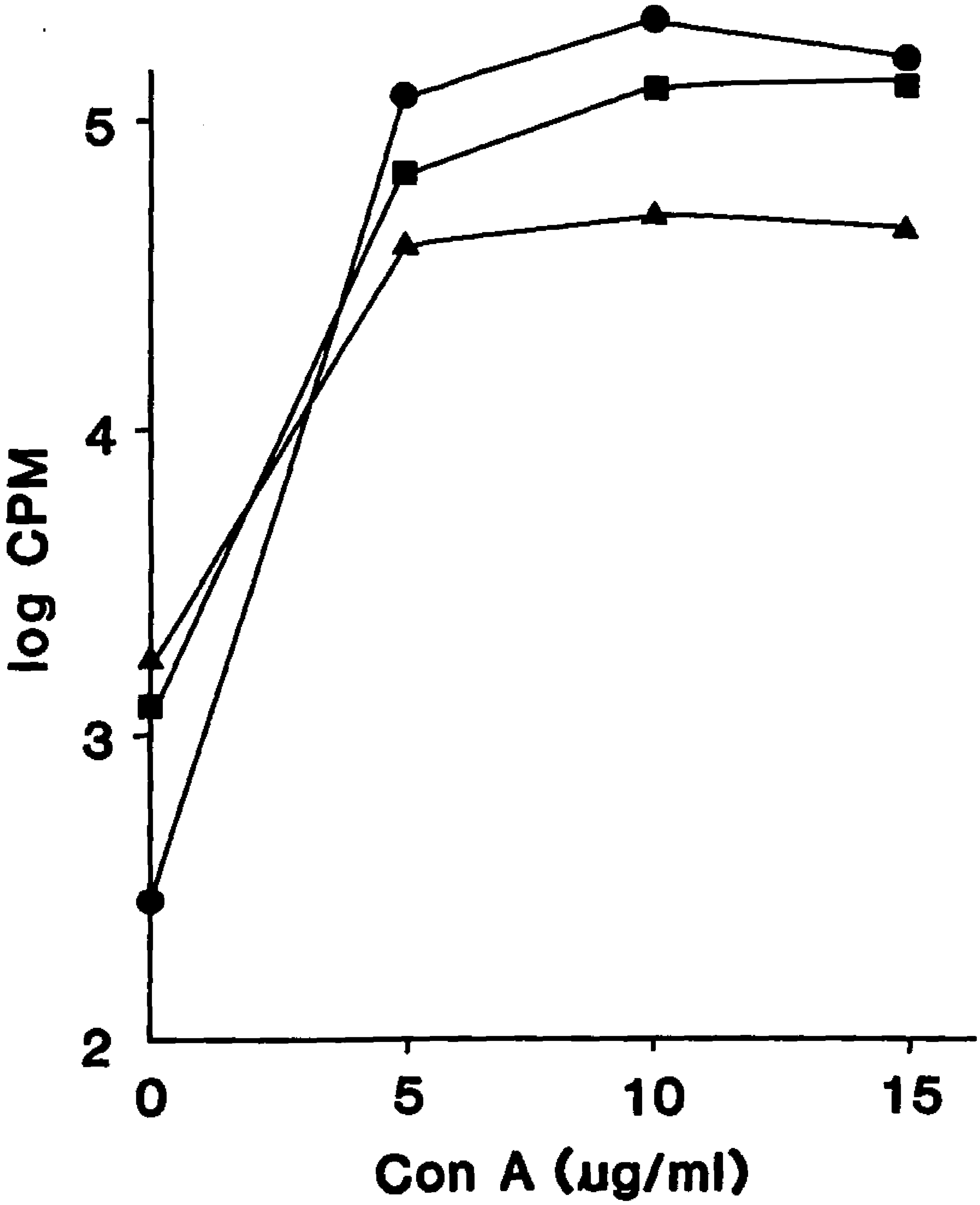
CHAPTER 3

RESULTS

Primary Blastogenic Responses of Peripheral Blood Lymphocytes. The responses of PBL to con A are shown in Figure 3. Three different concentrations of cells were utilized in the experiments. The concentration of con A ranged from 0-15 μg/ml. In the absence of con A the PBL were not very active. The magnitude of the incorporation of ^{3}H-thymidine was, of course, greater as the concentration of the cells increased. When cultured with con A, the cells assimilated much more of the ^{3}H-thymidine. The maximum response of stimulated cells became smaller as cell concentration increased. Peak responses were achieved at 10 μg con A/ml and 1 x 10^6 PBL/ml.

The range of modifications of the PBL response to 10 μg con A/ml induced by Am B is illustrated in Figure 4. The incorporation of ^{3}H-thymidine by unstimulated cells was not affected by Am B (Table 2). The drug had a profound effect on the responses of the cells to the optimal dose of con A, however. Although the proliferative response to con A usually was enhanced when the concentration of Am B was 2.5 or 5.0 μg/ml (Table 3), there were a few cases of negligible effect or even suppression noted at these intermediate doses (Fig 4). At these concentrations of the drug, up to 66% greater incorporation of ^{3}H-thymidine was noted when compared to the incorporation induced by con A in the absence of Am B (Table 3). When Am B was present at 10 μg/ml, however, there was a significant decrease (up to 65% in the data reported; Table 3) in the proliferative response when compared to the responses at 0, 2.5, or 5 μg Am B/ml.

Figure 3. The incorporation of ^{3}H-thymidine by PBL stimulated with 0, 5.0, 10.0, or 15.0 μg con A/ml culture fluid. Single representative experiment selected from a group of ten; SD $\gtrsim$ ± 10% mean. ●, 10^6 PBL/ml; ■, 2 x 10^6 PBL/ml; ▲, 4 x 10^6 PBL/ml.



log CPM
2
3
4
5
0
5
10
15
Con A (µg/ml)

Figure 4. The effect of various concentrations of Am B (0, 2.5, 5.0, or 10.0 μg/ml) on the incorporation of ^{3}H-thymidine by PBL (2 x 10^6/ml) activated with 10 μg con A/ml culture fluid. Three representative experiments selected from a group of ten. S.D. ≤ ± 10% mean. ●, Exp. 1; ■, Exp. 2, ▲, Exp. 3.

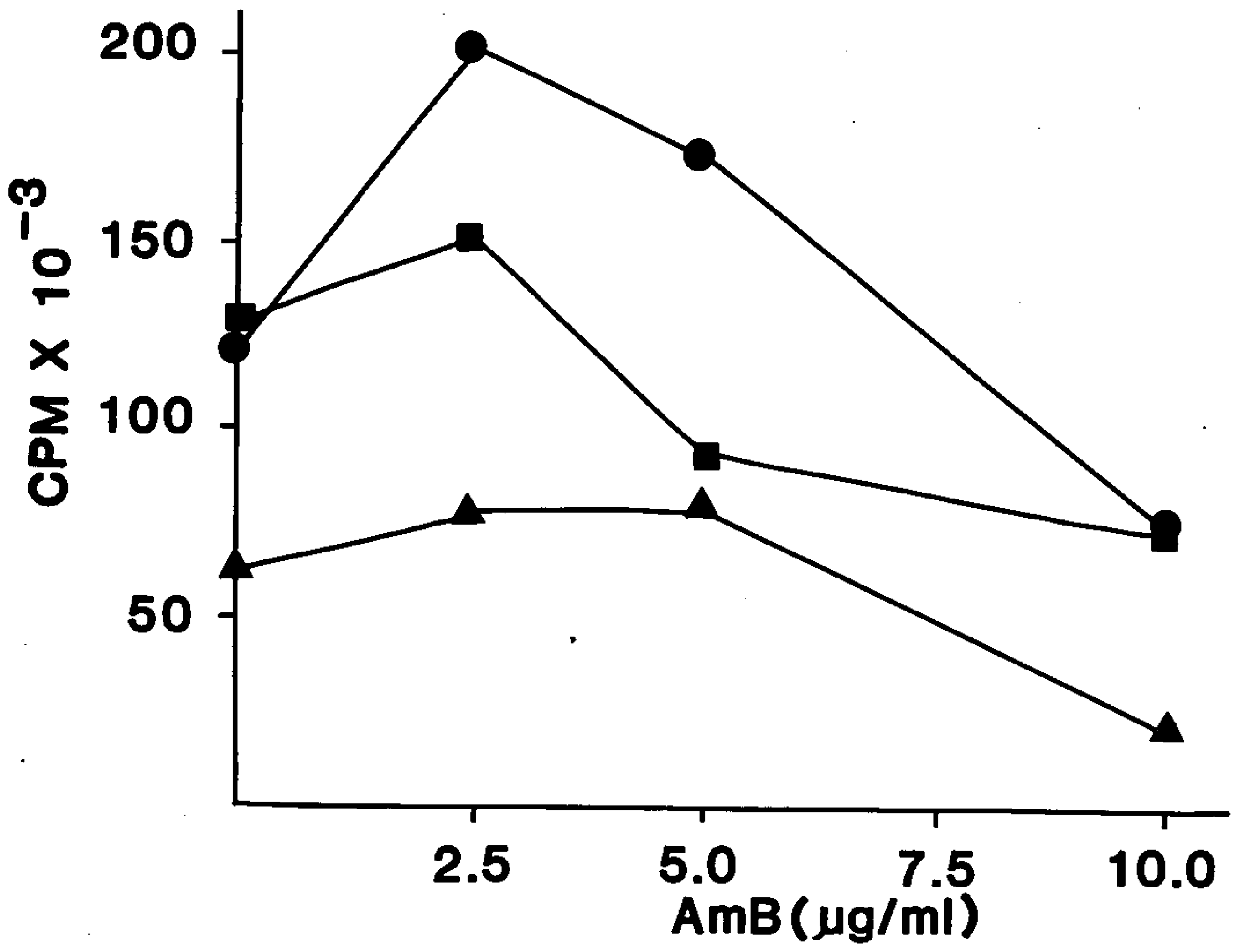

200
150
100
50
CPM X 10⁻³
2.5
5.0
7.5
10.0
AmB (µg/ml)

TABLE 2

Am B Modification of Spontaneous Proliferation of Peripheral Blood Lymphocytes

Experiment	*Spontaneous proliferation (cpm) means ± S.D.; η = 4	Proliferation in presence of Am B at indicated μg/ml (cpm) 2.5	5.0	10.0
1	543 ± 85	489 ± 35	400 ± 28	363 ± 74
2	255 ± 44	285 ± 49	263 ± 31	189 ± 19
3	307 ± 12	293 ± 44	300 ± 49	317 ± 67
4	325 ± 36	383 ± 72	299 ± 51	245 ± 31

*PBL at 2 x 10^6 viable cells/ml.

TABLE 3

Am B Modification of Con A-induced proliferation of Peripheral Blood Lymphocytes

Experiment	*Sponteneous proliferation (cpm) means ± S.D.; n=4	+Con A-induced proliferation (cpm)	% Change in con A-induced proliferation in presence of Am B at indicated µg/ml 2.5	5.0	10.0
1	543 ± 85	120,532 ± 8,933	50	33	-28‡
2	255 ± 44	62,409 ± 7,614	24	25	-65
3	307 ± 12	127,706 ± 18,505	16	26	-41
4	325 ± 36	119,990 ± 5,569	66	45	-35

*PBL at 2 x 10^6 viable cells/ml.

+Con A at 10 µg/ml.

‡Negative sign indicates that the cpm were less than con A control.

TABLE 4

Am B Modification of Spontaneous Proliferation
Response of Tonsillar Lymphocytes

Experiment	*Spontaneous proliferation (cpm) means ± S.D.; η=4	% Change spontaneous proliferation in presence of Am B at indicated μg/ml 2.5	5.0	10.0
1	27,563 ± 2,961	-61[+]	-80	-95
2	15,217 ± 2,482	-58	-89	-98
3	31,682 ± 1,711	-88	-98	-99
4	6,650 ± 961	-21	-64	-98

*TL at 2 x 10^6 viable cells/ml.

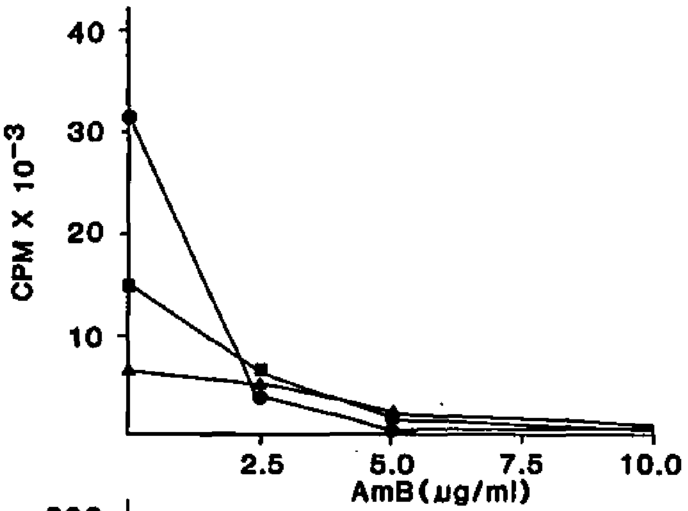
[+] Negative sign indicates that the cpm were less than control.

Primary Blastogenic Responses of Tonsil Lymphocytes. Tonsil cells cultured in the absence of con A and Am B invariably exhibited significant incorporation of ^{3}H-thymidine (Table 4) as compared to PBL (Table 3). The magnitude of incorporated radioactivity ranged from a few thousand cpm to tens of thousands. It always was greater than that expressed by unstimulated PBL (Fig 3) at a similar cell concentration (2×10^6/ml). Am B at all concentrations tested significantly reduced this incorporation (Table 4, Fig 5A). When the concentration of the drug was 10 μg/ml, the incorporation of radioactivity was reduced by 95%-99%, and the radioactivity was at the level of that seen in unstimulated PBL at the same cell concentration.

When tonsil cells were stimulated with con A in the absence of Am B, the stimulation indices (SI) ranged from 5 to 9. This was much less than the stimulation indices one often noted for PBL similarly treated. The latter indices ranged from about 50 to 500. The low indices of tonsil cells reflected the high background radioactivity, however, and were the result of the formula for calculating SI. If, for example, the indices were calculated using the radioactivity of control cells cultured with 10 μg Am B/ml, they would fall in the range of the indices noted for PBL. The magnitude of the radioactivity in the stimulated TL was as great or greater than that characteristic of stimulated PBL.

Am B markedly reduced the proliferative responses of tonsil cells stimulated with 10 μg con A/ml (Fig 5 B). All levels of the drug significantly and progressively reduced the incorporation of ^{3}H-thymidine. At the highest concentration of the drug, the incorporation of radioactivity was diminished about 72% to 97% (Table 5).

Figure 5. The effect of various concentrations of Am B (0, 2.5, 5.0, or 10 μg/ml) (a) on the spontaneous incorporation of ^{3}H-thymidine by TL, and (b) on the incorporation induced by the presence of 10 μg con A/ml culture fluid. The TL were at 2 x 10^{6}/ml. Three representative experiments selected from a group of six; S.D. ≤ ± 10% mean. ●, Exp. 1; ■, Exp. 2; ▲, Exp. 3.


CPM X 10^{-3}
40
30
20
10
2.5
5.0
7.5
10.0
AmB(μg/ml)

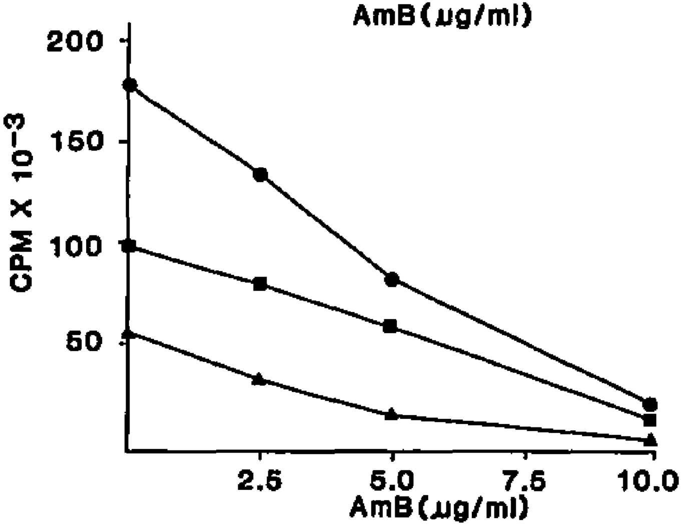

CPM X 10^{-3}
200
150
100
50
2.5
5.0
7.5
10.0
AmB(μg/ml)

TABLE 5

Am B Modification of Con A-induced Proliferation of Tonsillar Lymphocytes

Experiment	*Con A-induced proliferation (cpm) means ± S.D.; n=4	% Change in con A-induced proliferation in presence of Am B at indicated µg/ml		
		2.5	5.0	10.0
1	212,290 ± 12,729	-33+	-50	-72
2	98,505 ± 10,536	-19	-33	-87
3	176,737 ± 9,539	-21	-54	-88
4	60,952 ± 6,911	-34	-75	-97

*Con A at 10 µg/ml; TL at 2 x 10^6 viable cells/ml.

+Negative sign indicates that the cpm were less than con A control.

Effect of Con A on Amphotericin B-Inhibited Tonsil Cells. Although Am B reduced the incorporation of ^{3}H-thymidine, there was still some incorporation, the amount of which varied depending on the source of the TL. It was of interest to examine the response to con A of the drug-suppressed population of TL. In these experiments the density of the cells was 1, 2, or 4 x 10^6/ml. The cells were cultured in the presence of 5 or 10 μg Am B/ml, and their responses to various concentrations of con A were measured by cellular incorporation of ^{3}H-thymidine.

At a drug concentration of 5 μg/ml, the shape of the dose curves (Fig 6) resembled those of PBL stimulated in the absence of the drug (Fig 3). In the absence of con A, the incorporation of radioactivity at cell densities of 1-2 x 10^6/ml was very low, and at a density of 4 x 10^6 cells/ml ^{3}H-thymidine was taken up much less than one would have expected by uninhibited spontaneous proliferation (Table 4). Am B evidently suppressed proliferation as was shown earlier (Table 4). The optimal dose of con A in the presence of 5 μg Am B/ml was 5-10 μg/ml (Fig 6). Unlike the response of PBL (Fig 3), the maximum incorporation of radioactivity occurred with the higher densities of cells (Fig 6).

When TL were cultured in 10 μg Am B/ml, spontaneous proliferation was essentially ablated, even at the highest cell density (Fig 7). In spite of the presence of the drug, however, con A induced incorporation of ^{3}H-thymidine. The shape of the dose curve (Fig 7) resembled that for the response of TL (Fig 6) in the presence of 5 μg Am B/ml. At the lowest cell density (10^6/ml), uptake of radioactivity was about one log unit lower than it was at double (2 x 10^6 cells/ml) that cell

Figure 6. The effect of Am B (5 μg/ml) on the response of TL to con A at 0, 5.0, 10.0 or 15 μg mitogen/ml culture. The response was measured by the cellular incorporation of ^{3}H-thymidine. Single representative experiment selected from a group of three; S.D. ≤ ± 10% mean. ●, 10^6 TL/ml; ■, 2 x 10^6 TL/ml; ▲, 4 x 10^6 TL/ml.

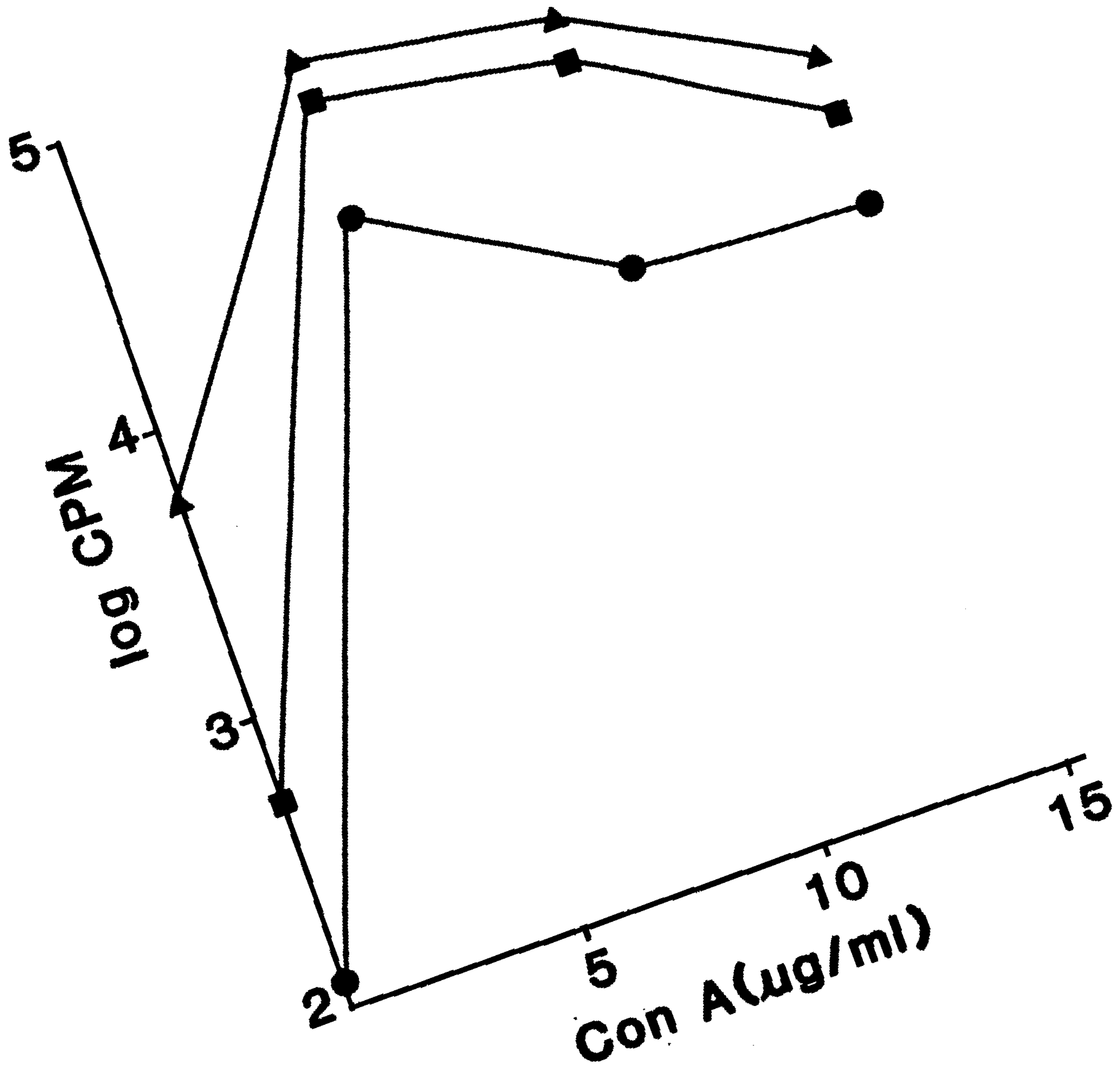
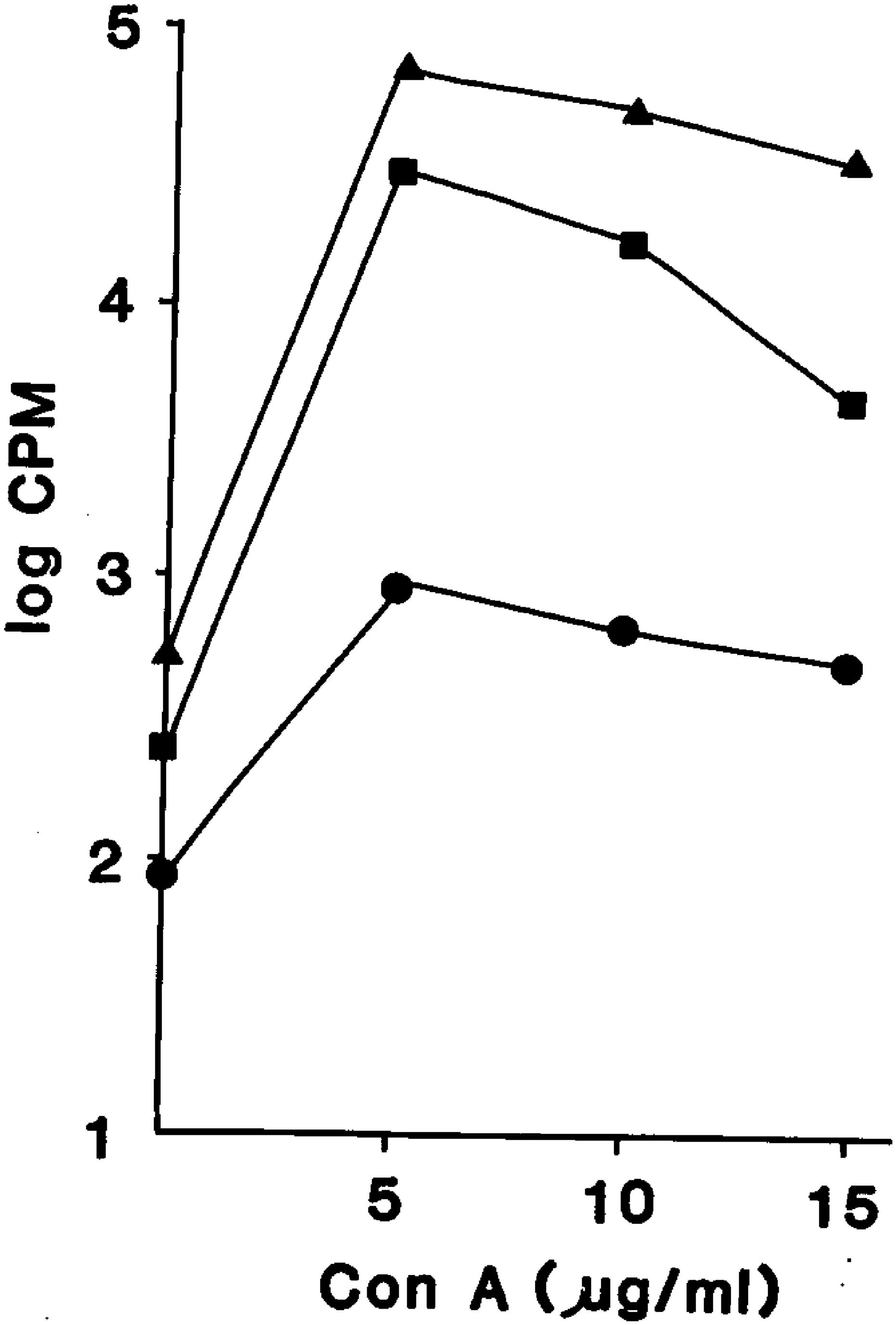

log CPM
5
4
3
2
5
10
15
Con A(µg/ml)

Figure 7. The effect of Am B (10 µg/ml) on the response of TL to con A at 0, 5.0, 10.0 or 15 µg con A/ml culture fluid. The response was measured by the cellular incorporation of ^{3}H-thymidine. Single representative experiment selected from a group of three; S.D. ≤ ± 10% mean. ●, 10^6 TL/ml; ■, 2 x 10^6 TL/ml; ▲, 4 x 10^6 TL/ml.



log CPM
5
4
3
2
1
5
10
15
Con A (µg/ml)

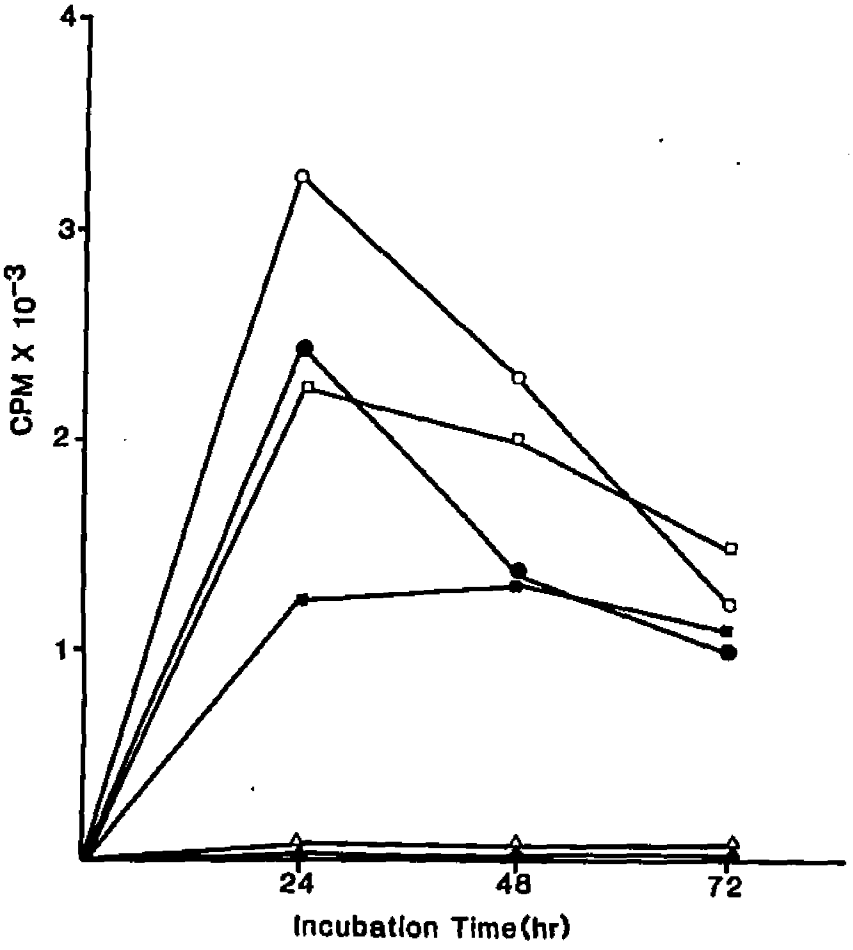
density. Incorporation of ^{3}H-thymidine at each dose of con A was greater with increasing cell density.

The Effect of Cell Density and Mercaptan on Production of HLMF. The effects of the density of the lymphocytes cultured in serumless medium on production of HLMF was studied. The density of the lymphocytes was 2.5 x 10^6, 5 x 10^6, or 10 x 10^6 per ml in the wells of tissue culture plates, and the concentration of con A was 20 μg/ml. There was no detectable production of the lymphokine at the lowest density of cells (Fig 8), and the greatest lymphokine activity occurred in microsupernatants raised at the highest cell density. Microsupernatant HLMF activity in several experiments tended to decrease after 24 hr culture. Samples for assay were removed after 24, 48, and 72 hr. The wells were replenished with an equal volume of fresh medium after the 24 and 48 hr samples of microsupernatant were taken, but additional con A was not presented to the cells.

Mercaptoethanol boosted the lymphokine activity about 50% after 24 hr of culture at cell densities of 5-10 x 10^6 per ml. It had no significant effect on HLMF activity in microsupernatants taken at 48 or 72 hr although it was present in the appropriate replenishment medium. After the first 24 hr, there was a decrease in lymphokine activity which occurred even in the absence of the mercaptan (Fig 8).

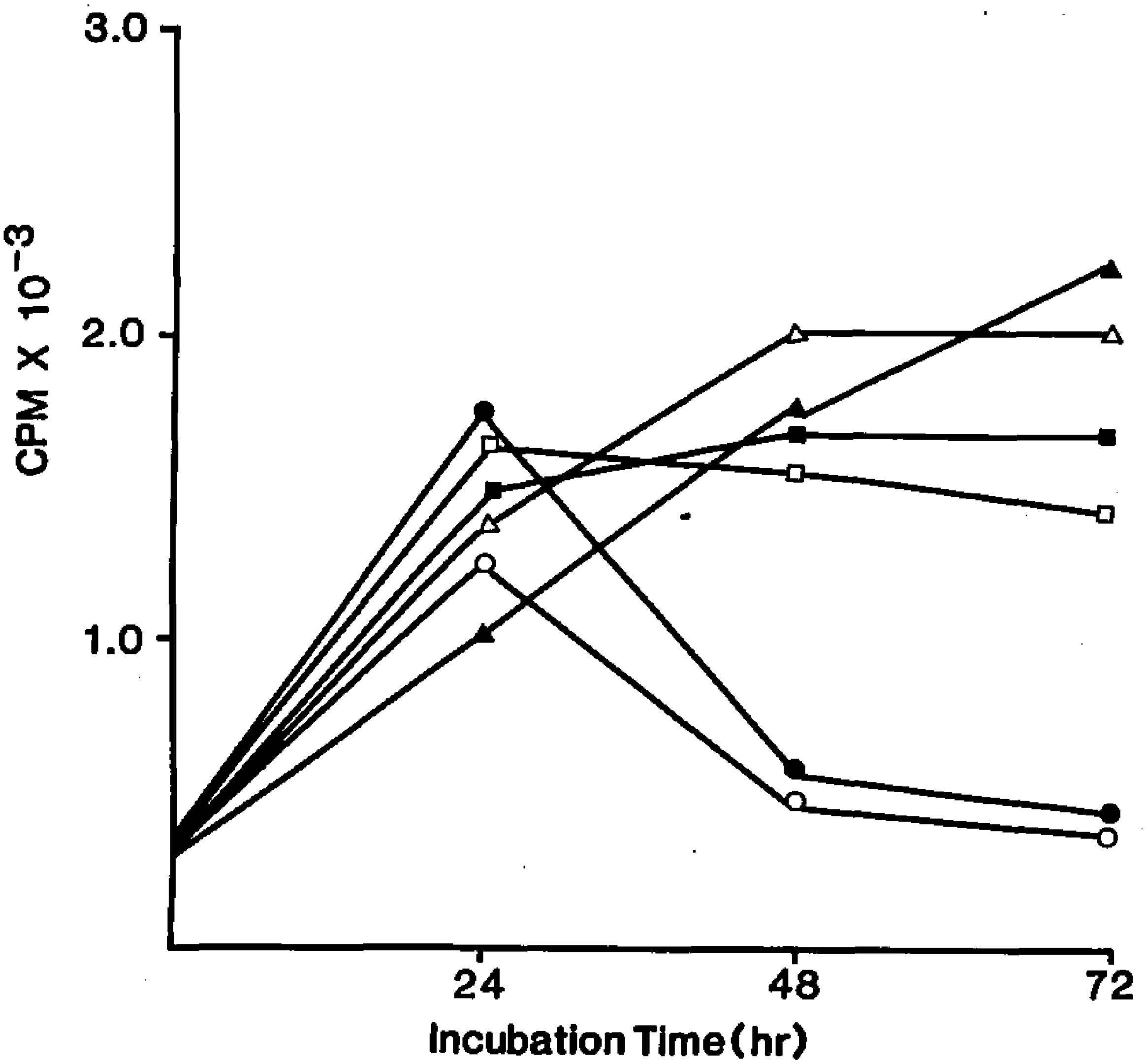
The Concentration of Con A Optimal for Production of Mitogenic Factor. PBL were cultured in Neuman-Tytell medium with mercaptoethanol for 24 hr in the presence of 5-35 μg con A/ml (Fig 9). The microsupernatants were recovered and assayed in the presence of α-mm for HLMF activity. The volume withdrawn was replaced with an equal volume of medium containing mercaptoethanol but no con A. After an additional

Figure 8. Effect of cell density and mercaptan on production of HLMF. Cell densities are as indicated in the absence (-) or presence (+) of 5×10^{-5} M, 2-mercaptoethanol. o, 10×10^6 cells/ml; (-). ●, 10×10^6 cells/ml; (+). □, 5×10^6 cells/ml; (-). ■, 5×10^6 cells/ml; (+). Δ, 2.5×10^6 cells/ml; (-). ▲, 2.5×10^6 cells/ml; (+).



4
3
2
1
CPM X 10^{-3}
24
48
72
Incubation Time(hr)

Figure 9. Effect on HLMF production by 10 x 10^6 cells/ml in response to varying concentrations of con A. o, 5 μg/ml; ●, 10 μg/ml; □, 15 μg/ml; ■, 20 μg/ml; Δ, 25 μg/ml; ▲, 35 μg/ml.


3.0
2.0
1.0
CPM X 10^{-3}
24
48
72
Incubation Time (hr)

24 hr another sample was taken, and the medium was replenished as described. Finally, a third sample was taken after a total of 72 hr culture. When the stimulating dose of con A was 5 or 10 μg/ml, HLMF activity was recovered in the 24 hr microsupernatants, but the factor apparently was no longer produced by the cultures stimulated with those doses of the lectin (Fig 9). HLMF was produced not only during the first 24 hr by cultures stimulated with 15, 20, 25, or 35 μg con A/ml, but production apparently continued during the next two 24-hr sampling periods. There was greater mitogenic activity in the 48 and 72 hr microsupernatants than there was in the 24 hr microsupernatants when the dose of con A was 20, 25, or 35 μg/ml.

A comparison of producer cell population proliferation with HLMF production as a function of dose of con A was made. During the first 24 hr only the cultures given 5 μg con A/ml signaled proliferation by incorporating a little ^{3}H-thymdiine. HLMF activity in the microsupernatants was maximal when the dose of con A was 10 μg/ml although there was no proliferation during that time at that dose (Fig 10). HLMF was produced at all doses of con A regardless of whether there was proliferation in the producing cell population. After 48 or 72 hr culture in the presence of con A, the dose responsible for peak proliferation remained at 5 μg con A/ml, but the extent of incorporation of ^{3}H-thymidine increased greatly with increasing time of culture (Fig 11 and 12). Further, proliferation at higher doses of con A began to be apparent and that phenomenon progressed with time. The HLMF activity was greatest in the microsupernatants corresponding to the cells showing the least proliferation after 48 or 72 hrs culture.

Figure 10. Temporal relationship of proliferation and HLMF production with varying doses of con A during the 1st 24 hr period of incubation. ●, HLMF production; o, proliferation.

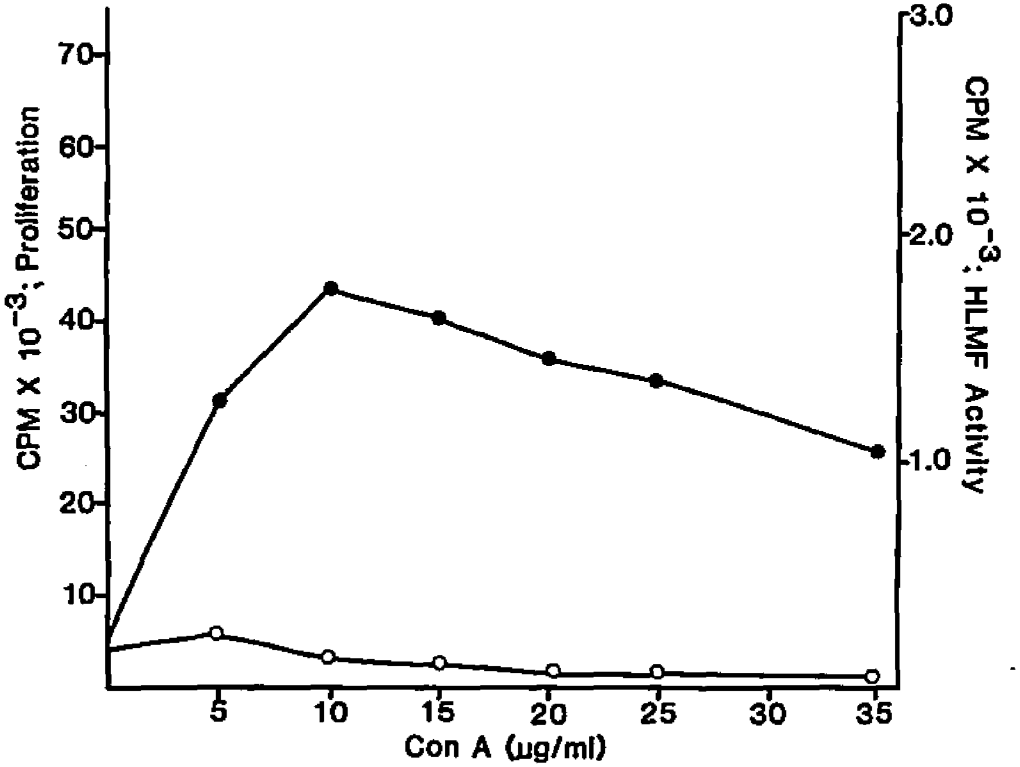

CPM X 10^{-3}; Proliferation
70
60
50
40
30
20
10
3.0
2.0
1.0
CPM X 10^{-3}; HLMF Activity
5
10
15
20
25
30
35
Con A (µg/ml)

Figure 11. Temporal relationship of proliferation to HLMF production with varying doses of con A during the 24-48 hr period of incubation. ■, HLMF production; □, proliferation.

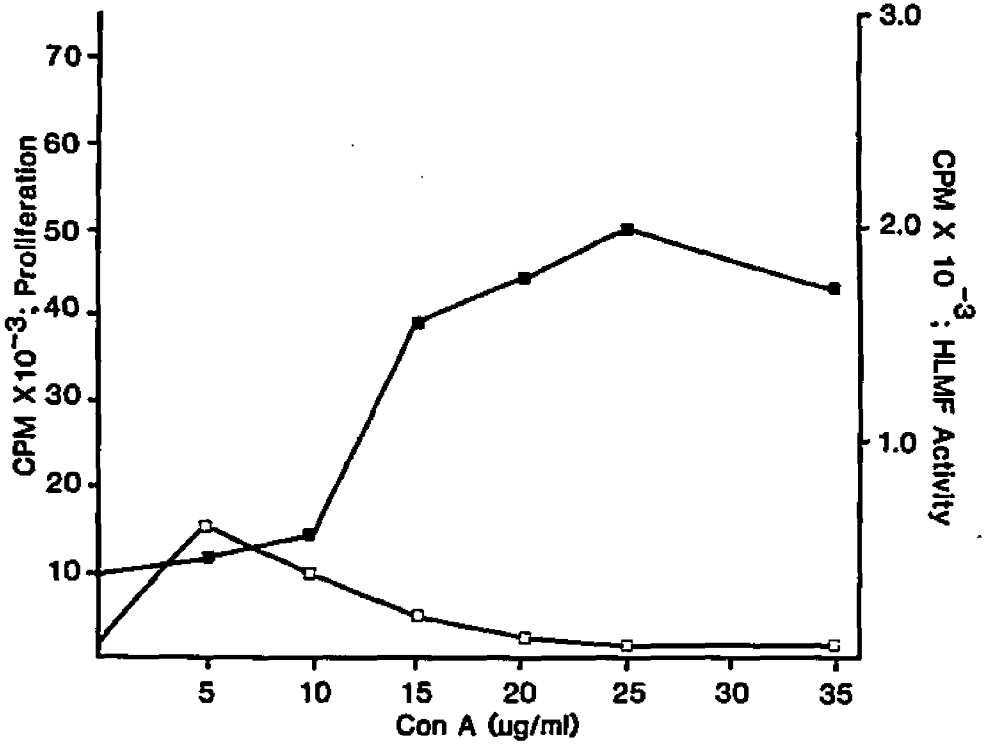
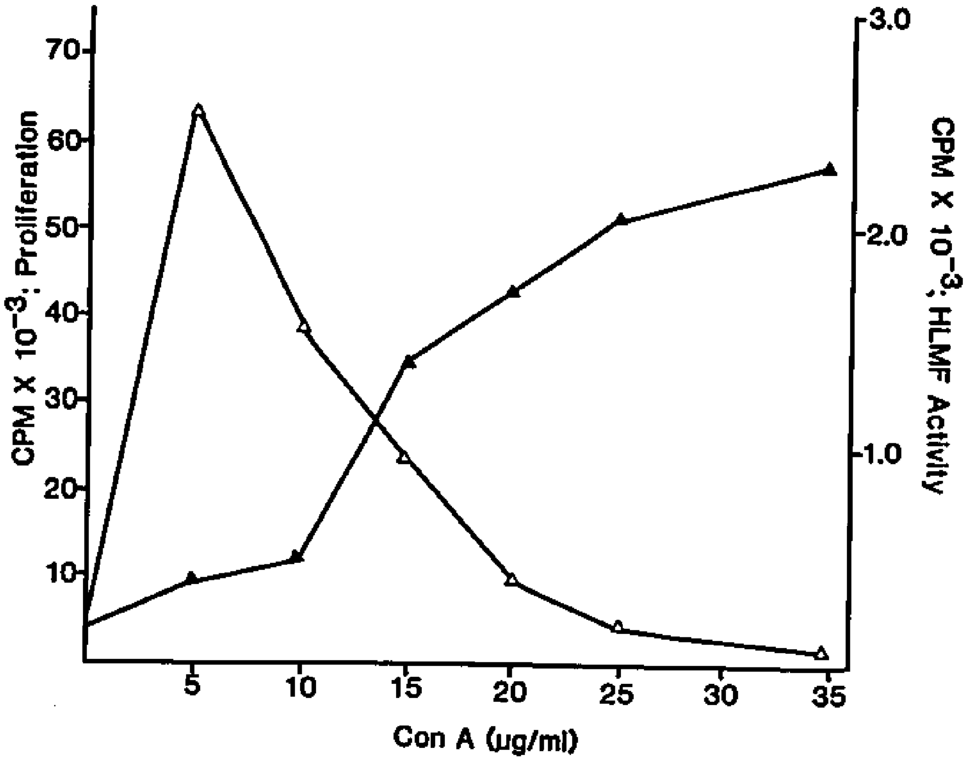

CPM X10^{-3}; Proliferation
70
60
50
40
30
20
10
5
10
15
20
25
30
35
Con A (ug/ml)
3.0
2.0
1.0
CPM X 10^{-3}; HLMF Activity

Figure 12. Temporal relationship of proliferation to HLMF production with varying doses of con A during the 48-72 hr period of incubation. ▲, HLMF production; △, proliferation.



70
60
50
40
30
20
10
CPM X 10^{-3}; Proliferation
3.0
2.0
1.0
CPM X 10^{-3}; HLMF Activity
5
10
15
20
25
30
35
Con A (µg/ml)

The Effect of Amphotericin B (Am B) on HLMF Production. Various doses of Am B were given to microcultures of PBL stimulated with con A. The supernatants were assayed for HLMF activity. In 4 out of 7 experiments, each examining the effect of several doses on Amphotericin B on different donors' PBL, HLMF activity in the supernatants was progressively depressed with increasing doses (Fig 13). In the other experiments, however, there was no effect by the drug on production of this lymphokine.

The Effect of Fresh Mitogen on HLMF Production. The cells from several donors were cultured. One half of the cultures received fresh mitogen after 24 hr culture, while the remainder received only supplemented medium. After harvesting the culture supernatants, they were assayed for HLMF activity. All the P-24 hr supernatants induced similar levels of blastogenesis in target cells and contained significant levels of HLMF as evidenced by the number of α-mm-resistant counts. Mitogenic indices in all cases were greater than 3.0 (Table 6).

In the cultures which did not receive additional con A after 24 hr, there was a significant drop in both the test and α-mm-paired controls. The MI of the 48 hr cultures were significantly less than for the corresponding 24 hr cultures.

For the cultures which received fresh mitogen there was only a slight decrease in the 48 hr test supernatants relative to the 24 hr cultures. The α-mm control demonstrated, however, there was an equivalent or greater amount of HLMF produced during the 48 hr culture if fresh mitogen was added. There appeared to be no endogenous HLMF produced in the R cultures in any case.

Figure 13. Representative experiments showing the two general effects of Am B on HLMF production. o, Exp. 1; ●, Exp. 2.

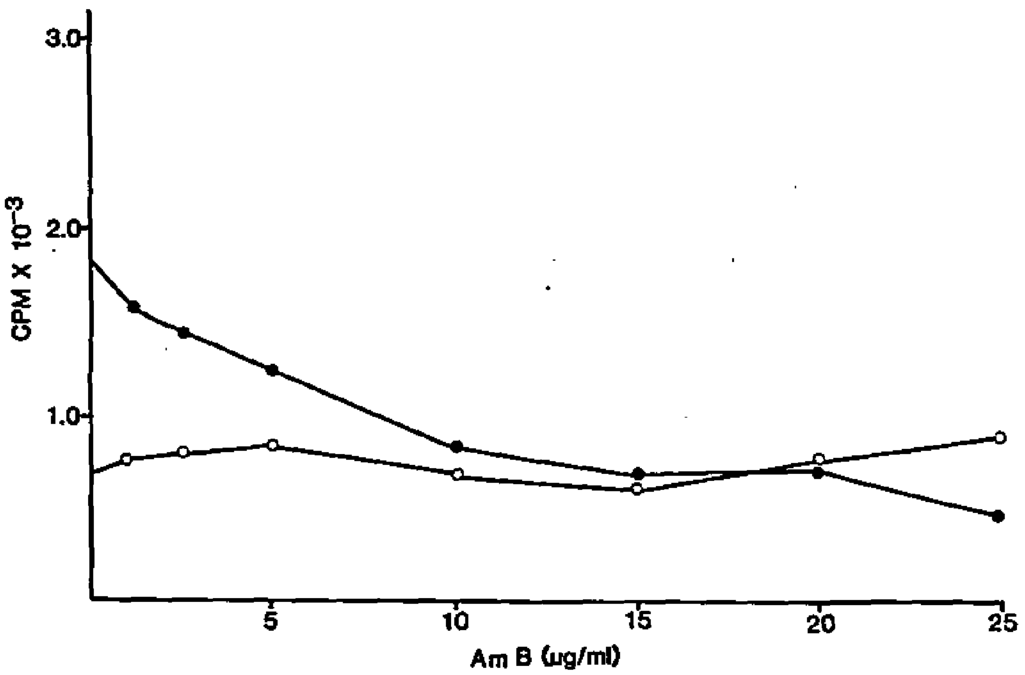

3.0
2.0
1.0
CPM X 10^{-3}
5
10
15
20
25
Am B (ug/ml)

TABLE 6

HLMF Generation in Presence and

Absence of Additional Mitogen

Source		α-mm Test	 Paired Control	MI^a
AB Control		1,162	656	
Con A Control		36,468	693	
Donor 1	P 24 hr[b]	76,709	3,699	3.4
(no Con A)	P 48 hr	12,005	1,309	1.2
	R[c]	74,692	1,096	-
Donor 2	P 24 hr	110,275	3,593	4.9
(no Con A)	P 48 hr	16,072	1,937	2.6
	R	127,104	738	-
Donor 3	P 24 hr	109,455	5,629	6.5
(+ Con A)	P 48 hr	89,759	5,013	5.7
	R	118,846	868	-
Donor 4	P 24 hr	112,112	2,559	3.2
(+ Con A)	P 48 hr	94,068	4,183	5.3
	R	93,294	793	-

$^a\text{MI} = \dfrac{\alpha\text{-mm paired P}}{\alpha\text{-mm paired R}}$

[b] P = Preincubated culture supernatant; con A was added at culture initiation.

[c] R = Reconstituted culture supernatant; con A was added 15 min before culture termination

Effects of Processing of Culture Supernatants on Expression of HLMF. Large volumes of culture supernatants are required to obtain even small quantities of lymphokines. Reduction of supernatant volumes to convenient levels usually is necessary. There also are long periods of time during which supernatants must be stored while samples are undergoing assay. Therefore, it is necessary that supernatants be processed (concentrated or stored) with minimal loss of activity. Several handling procedures were investigated to determine which were most advantageous toward preserving HLMF activity.

Microsupernatants were obtained from cells cultured in microtiter trays for 24 hr, and they were stored at -10° C without further processing. The residual cells were replenished with medium and con A, and the microsupernatants were recovered after an additional 24 hr and were stored frozen. Cells from the same donors were cultured also in flasks. This permitted larger volumes to be obtained compared to those taken from microcultures. Supernatants were recovered after 24 hr, and the cells were replenished with medium and con A. After another 24 hr the supernatants were harvested. When appropriate, these large volumes were reduced prior to storage. Ultimately all supernatants except those stored frozen for six weeks or three months were assayed the same number of days after harvest. All supernatants were assayed at one-half the neat concentration and in the presence of 0.1 M α-mm.

Supernatants raised in microcultures and stored frozen activated target PBL (Table 7). The supernatants were neither dialyzed nor concentrated prior to assay. The most active usually were those harvested after 48 hr culture.

TABLE 7

Effect of Various Treatments of Supernatants on the Activity of HLMF

Sample	Microculture Supernatant (cpm)	Freeze -80° (cpm)
Cell Control	745 ± 76[a]	626 ± 102[a]
Cell Control & α-mm	579 ± 99	360 ± 143
Con A Control	79,890 ± 8,104[b]	61,838 ± 4,001[b]
924	2,189 ± 910[c]	648 ± 157[c]
948	2,356 ± 246	603 ± 122
1024	2,309 ± 940	644 ± 511
1048	1,649 ± 833	147 ± 42
1124	1,579 ± 239	315 ± 197
1148	2,480 ± 652	281 ± 52
1224	1,496 ± 647	314 ± 10
1248	2,396 ± 696	730 ± 105
R-24	-	975 ± 226
R-28	-	777 ± 269

[a] mean ± standard deviation; (n = 4).

[b] Radioactivity (cpm) was corrected for that of the cell control.

[c] Radioactivity (cpm) was corrected for that of the corresponding R control and α-mm for this and all following cpm in this column.

TABLE 7 (cont)

Effect of Various Treatments of Supernatants on the Activity of HLMF

Sample	Freeze 6 wk -80° (cpm)	Freeze 3 mo -80° (cpm)
Cell Control	278 ± 17[a]	536 ± 318[a]
Cell Control & α-mm	302 ± 30	424 ± 28
Con A Control	99,728 ± 11,156[b]	49,001 ± 7,783[b]
924	2,448 ± 579[c]	1,158 ± 536[c]
948	2,666 ± 1,096	2,963 ± 272
1024	2,152 ± 810	1,587 ± 592
1048	1,531 ± 109	2,108 ± 1,125
1124	1,438 ± 363	1,715 ± 401
1148	876 ± 702	2,151 ± 300
1224	1,202 ± 252	1,553 ± 682
1248	1,140 ± 98	1,076 ± 517
R-24	802 ± 152	1,224 ± 105
R-28	1,615 ± 536	460 ± 69

[a] mean ± standard deviation; (n = 4).

[b] Radioactivity (cpm) was corrected for that of the cell control.

[c] Radioactivity (cpm) was corrected for that of the corresponding R control and α-mm for this and all following cpm in this column.

TABLE 7 (cont)

Effect of Various Treatments of Supernatants on the Activity of HLMF

Sample	Ultrafilter freeze -80° (cpm)	Lyophilize freeze -80° (cpm)	Dialyze, lyophilize, freeze -80° (cpm)
Cell Control	600 ± 53[a]	719 ± 118[a]	998 ± 232[a]
Cell Control & α-mm	501 ± 74	386 ± 24	486 ± 62
Con A Control	55,698 ± 3,525[b]	55,032 ± 4,655[b]	83,523 ± 10,176[b]
924	0[c]	582 ± 415[c]	2,442 ± 618[c]
948	980 ± 337	602 ± 117	2,969 ± 216
1024	532 ± 152	539 ± 406	3,318 ± 208
1048	830 ± 275	216 ± 75	3,319 ± 691
1124	0	201 ± 91	2,556 ± 192
1148	491 ± 78	592 ± 176	1,422 ± 486
1224	429 ± 201	805 ± 116	2,855 ± 1,230
1248	459 ± 59	273 ± 84	675 ± 231
R-24	922 ± 286	835 ± 257	910 ± 57
R-28	666 ± 132	727 ± 235	891 ± 189

[a] mean ± standard deviation; (n = 4).

[b] Radioactivity (cpm) was corrected for that of the cell control.

[c] Radioactivity (cpm) was corrected for that of the corresponding R control and α-mm for this and all following cpm in this column.

The supernatants raised in culture flasks were divided into several portions, each of which was processed in a different way and then assayed for HLMF activity. One portion was frozen to -80° C for one week. Upon subsequent analysis, the HLMF activity was low in all samples except number 1124 and ranged from 0-30% of what one expected based on the activity in the microculture supernatants (Table 6). After storage at -80° C for six weeks or three months, however, the activity was recovered to the level found in the microcultures in 75% of the samples (Table 6).

Some supernatants were concentrated 30-fold by diafiltration and then frozen to -80° C. When assayed, HLMF activity was not detected in supernatants 924 and 1124, and the activity in the other samples was only 19-50% of that in the corresponding microculture supernatants (Table 6).

Another portion was lyophilized without prior dialysis and the powder was stored at -80° C. When these samples were rehydrated to one-tenth their original volumes, precipitation occurred. This evidently was a salting-out effect. After removing the precipitates by centrifugation, the supernatants were assayed. The HLMF activity again was less than expected, and ranged from 11-54% of that expected.

Others supernatants were dialyzed in NH_4HCO_3 solution before lyophilization. These samples were rehydrated to one-tenth their original volume with medium and precipitation did not occur. Additional medium was then added to bring the samples to their original volumes. They were dialyzed in supplemented medium and assayed. Only the activity in sample 1248 was less than that of the corresponding microcultures. The activities in the other samples ranged from the same to 30% greater than those of the corresponding microculture supernatants, and were as good or better than those in supernatants stored at -80° C for six weeks of three months.

Sephadex Affinity Chromatography. Sephadex G-100 was tested for its ability to remove con A from RPMI-1640 medium. In optical density and trace label experiments (Table 8), con A was removed with 97% effectiveness as measured by $OD_{280\ nm}$ or scintillation counting. In the bioassay experiments, the eluate was assayed for its ability to induce primary blastogenesis in target lymphocytes. In these experiments, a 4 μg or 10 μg con A/ml stock suspension showed no significant change over the AB control experiment which indicated complete removal of the lectin.

However, when serum was included to 10% ($^{v}/_{v}$) in the con A suspension applied to the column, the bioassay results of the eluates showed minimal removal of con A and, in fact, the eluates stimulated greater incorporation of ^{3}H-thymidine than did a con A control of 10 μg con A/ml.

Molecular Sieve-Affinity Chromatography with Sephadex G-100. Control experiments with con A in PBS proved the ability of Sephadex G-100 to remove all the contaminating con A (Table 9). The inclusion of 0.1 M glucose had no apparent effect on the binding of con A to the Sephadex, even though glucose is a known competitive inhibitor of the con A-Sephadex interaction. When the con A at 150 μg/ml in 30 X-N-T medium was fractionated, significant con A contamination was noted near the column volume (Fractions 43-46) corresponding to the less than 7000 weight range. Fractionation of con A-containing, HLMF-active 30 X P supernatants showed unbound con A to be in similar

TABLE 8

Concanavalin A Removal by Sephadex
G-100 Affinity Chromatography

Exp	Load (mg)	Elute (mg)	% Removal
		Optical Density$_{280\ nm}$	
1	29.07	1.20	96
2	33.85	0.87	97
3	25.64	0.78	97
		^{3}H - Con A (cpm)	
1	48,825	1,460	97
2	86,300	2,524	97

Bioassay, ^{3}H-thymidine Uptake (cpm; absence of serum)

	Test	% Removal
AB Control	1,351	
Con A Control	110,151	
4 μg Stock (Sephadex Eluate)	1,079	99
10 μg Stock (Sephadex Eluate)	1,602	99

Bioassay, ^{3}H-thymidine Uptake (cpm; in presence of serum)

	Test	α-mm	% Removal
AB Control	1,193	792	
Con A Control	50,370	1,515	
Exp. 1	137,397	2,247	0
Exp. 2	146,205	2,167	0

TABLE 9

Molecular Seive-Affinity Chromatography with Sephadex G-100

		% Unbound Con A Activity				
Fraction	Corres-ponding Mol wt	Con A/ PBS	Con A + Glu	Con A + NT	Con A in MF Sup	α-mm Resistant activity in MF Sup
19	V_o	0	0	3.0[a]	0	0
22		0	0	1.9	1.1	0
25	63,400	0	0	2.5	0.9	0
28	12,200	0	0	1.4	1.5	0
31	28,200	0	0	1.5	1.1	0
34	19,000	0	0	3.4	2.2	58.5
37	13,300	0	0	24.4	23.8	36.4
40		0	0	20.5	30.3	5.1
43		0	0	21.0	27.3	0
46	V_T	0	0	20.4	11.7	0

[a] $$\frac{\text{CPM fraction} - \text{CPM AB Control}}{\Sigma_{46}^{19}\ (\text{CPM fraction} - \text{CPM AB Control})} \times 100 - \%\ \text{unbound con A activity}$$

TABLE 10

Bio-Gel P-100 Column Chromatography of Con A from Sephadex Affinity Chromatography

Molecular Weight	% Total by Category
V_0-45K	51%
45K-35K	47%
35K-25K	2%
25K-15K	0%

positions (Fractions 43-46).

The majority of HLMF activity was noted, however, in fractions (34-40) which eluted just prior to the con A contaminated fractions, and there was significant overlap between the activities.

To evaluate the molecular weight characteristics of the eluting con A, fractions exhibiting con A activity were pooled and rechromatographed over Bio-Gel P-100 (Table 10). The results showed greater than 51% of the contaminating con A was in excess of 45,000 in molecular weight, and 98% was greater than 35,000.

Affigel-Ovalbumin Affinity Chromatography. Con A appeared to be removed from HLMF-active supernatants in substantial quantities by binding to the ligand ovalbumin. Affigel-ovalbumin was tested also for its ability to remove con A from either HLMF-active supernatants generated in Neuman-Tytell medium, or from a 20 μg con A/ml stock suspension. In both cases, substantial amounts of contaminating con A were removed, but because of product expense and variability of effective binding between lots, this was not considered the most favorable alternative (Table 11).

Bio-Gel P-100 Column Chromatography. Molecular sieve chromatography was used to investigate the apparent molecular weight of con A and the mitogenic activity in the supernatants. The elution profile of con A from several suppliers is shown in Figure 14. All three samples were tested in PBS, pH 7.2, or in supplemented medium. Each sample eluted in the molecular weight range of 35,000-60,000 with a major peak of activity at 45,000. There were, however, significant amounts of biologically active con A in all fractions between 15,000 and 60,000.

TABLE 11

Removal of Con A by Affigel-Ovalbumin Affinity Chromatography

Sample	Test	α-mm Resistant
AB	1,193	792
Con A (10 μg/ml)	50,370	1,515
MF Supernatant	60,634	4,000
Adsorbed Supernatant	4,435	2,720
Adsorbed con A	1,472	1,447

Figure 14. Elution profile of commercially obtained concanavalin A from Bio-Gel P-100. ●, Pharmacia; ■, Miles Laboratories; ▲, Sigma.

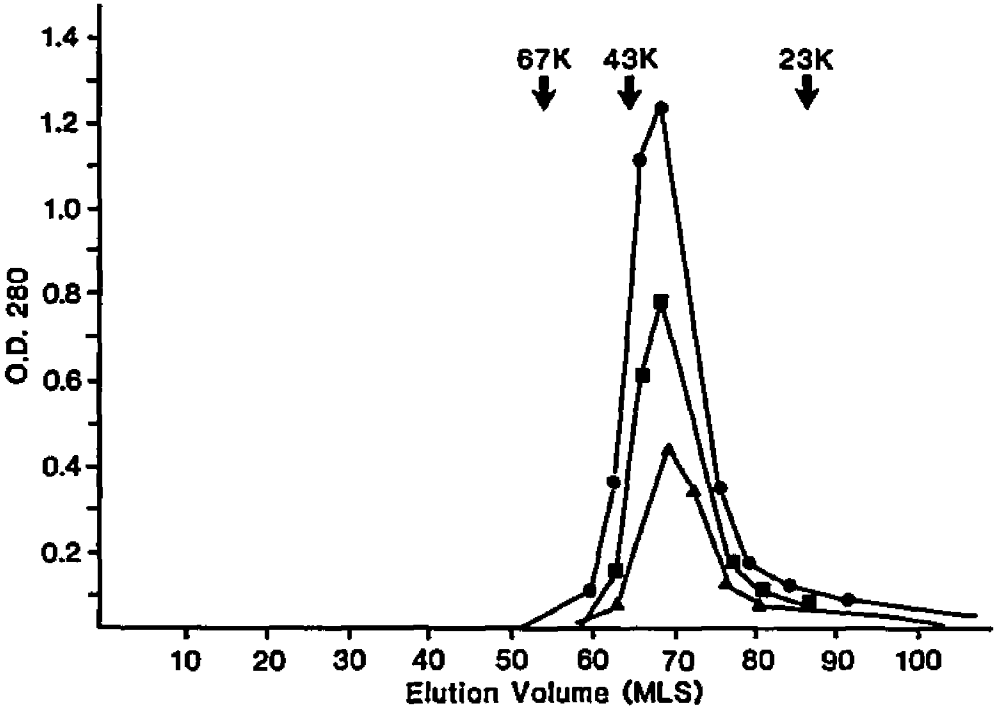
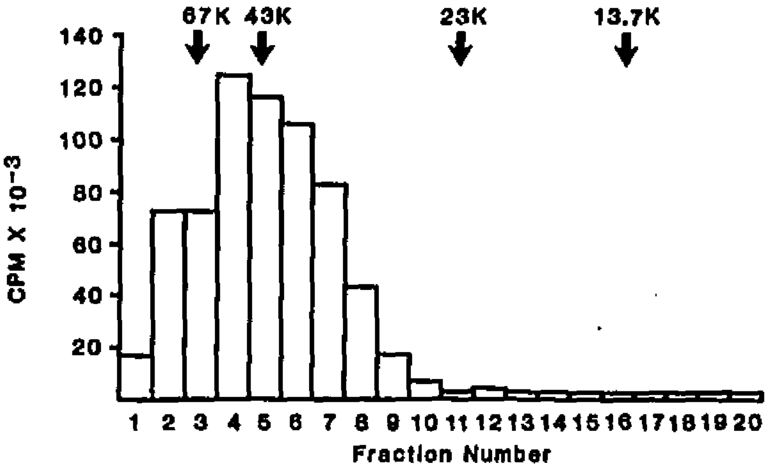
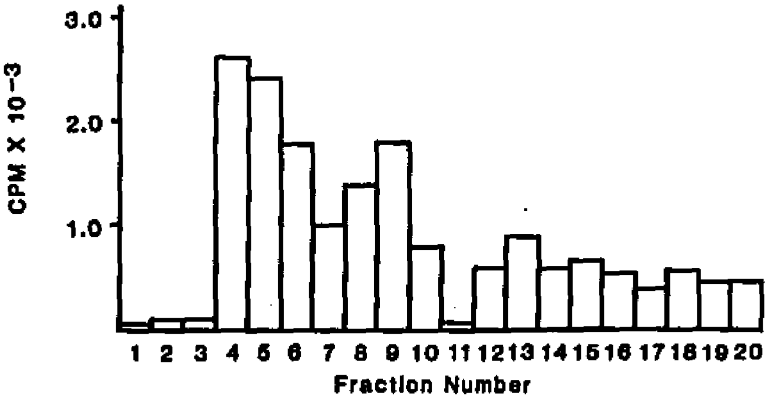

67K
43K
23K
1.4
1.2
1.0
0.8
0.6
0.4
0.2
O.D. 280
10
20
30
40
50
60
70
80
90
100
Elution Volume (MLS)

Figure 15. Elution profile of the biological activity of a 30-X P supernatant fractionated on Bio-Gel P-100. Proliferation of target cells in the absence (A) and presence (B) of 0.1 M α-mm.


67K
43K
23K
13.7K
140
120
100
80
60
40
20
CPM X 10⁻³
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Fraction Number

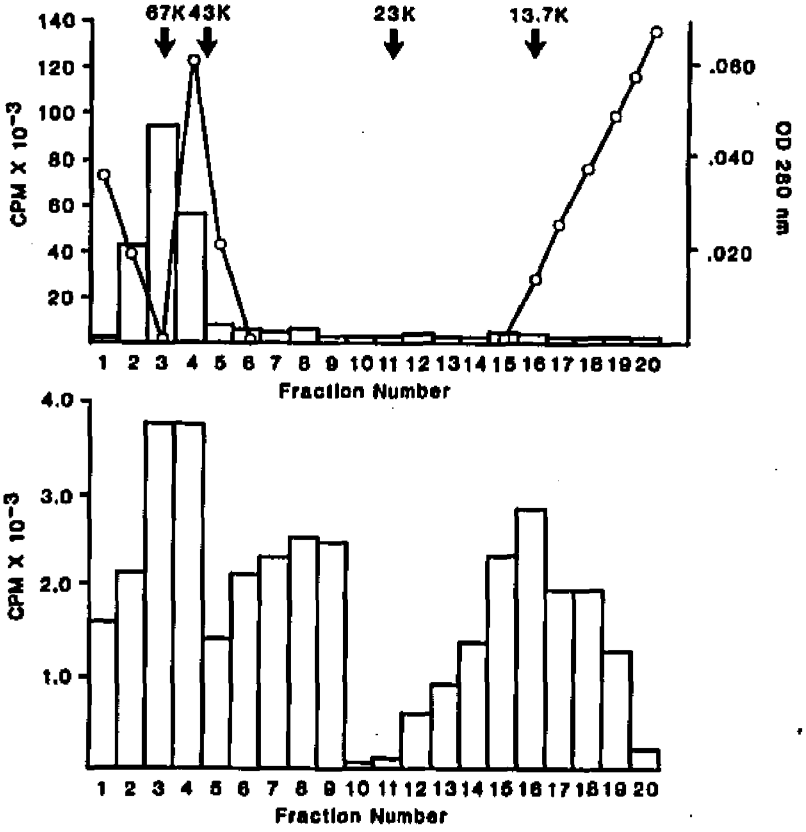

3.0
2.0
1.0
CPM X 10⁻³
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Fraction Number

When 30-fold concentrated P supernatants were fractionated on Bio-Gel P-100 the elution profile was a broad area of biological activity ranging between 20,000 d and the exclusion volume (Fig 15A). There were, however, two peaks of α-mm-resistant activity (Fig 15B). The smaller 30,000-45,000 d peak was observed in fractions where con A activity was found to be decreasing progressively. The larger 50,000-65,000 d peak corresponded to fractions showing the greatest amount of con A activity in test samples. Essentially no optical activity was observed in the eluted samples. α-mm caused at least partial ablation of counts with all samples tested.

Since con A is found in significant quantities as a contaminant in the expected size ranges of HLMF activity, a method was devised increase the apparent molecular weight of the lectin and thereby facilitating its separation from HLMF by molecular sieving. Ovalbumin, the same glycoprotein-binding moiety as on the affigel matrix, was added to P supernatants to a final concentration of 33 μg/ml. After dialysis and concentration to 1/30 the original volume, the active supernatant was chromatographed. The elution profile of biological activity in the absence of the competitive inhibitor α-mm was a single band of activity which peaked at greater than 65,000 d (Fig 16A). Two peaks of light absorption were noted. The smaller peak was at the exclusion volume and the larger peak corresponded to the free ovalbumin position. Although the molecular weight of ovalbumin is 43,000, it consistently eluted in the 50,000-60,000 range on Bio-Gel P-100.

Three district peaks of α-mm resistant activity were observed. The lower molecular weight peak was observed in the 12,000-20,000 range. The intermediate peak was between 30,000 d and 50,000 d and

Figure 16. Elution profile of the biological activity of a 30-X P supernatant containing 1 mg/ml ovalbumin fractionated on Bio-Gel P-100. Proliferation of target cells in the absence (A) and presence (B) of 0.1 M α-mm. OD_{280} (o).


67K
43K
23K
13.7K
CPM X 10^{-3}
140
120
100
80
60
40
20
OD 280 nm
.060
.040
.020
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Fraction Number
CPM X 10^{-3}
4.0
3.0
2.0
1.0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Fraction Number

corresponded to a similar peak observed previously (Fig 15). The larger molecular weight peak again corresponded positionally to the primary peak of blastogenic activity attributed to con A in the assay of fractions derived from supernatants which had no ovalbumin added prior to chromatography.

In the >65,000 d peak, significant reduction of con A counts was noted when assayed with α-mm. However, in fraction 9 of the intermediate peak, and fractions 14-18 of the lower molecular weight band, the α-mm moderately boosted cpm by 250-1400 cpm above those observed in the absence of the inhibitor.

Isoelectric Focusing of HLMF-Active Supernatant Fluid. Concentrated HLMF-active supernatants from which some con A was removed by Affigel affinity chromatography showed two broad areas of activity (Fig 17). The larger area encompassed a pH range of 5.3-8.2 with the peak occurring at pH 6.7. The smaller area spanned pH 3.5 to 4.5 with the peak occurring at pH 4.2.

When concentrated HLMF-active supernatants were fractionated over Bio-Gel P-100, at least two distinct peaks of activity were observed. The smaller 12,000-20,000 d and intermediate 30,000 to 50,000 d activities were focused separately and presented different profiles. The lower molecular weight material showed three minor areas of activity, the largest of which was a broad band between pH 3.4 and 4.6 (Fig 18). Minor peaks of activity were noted at pH 5.2, 5.7, 8.7, and 9.5.

The larger molecular weight fraction showed two distinct peaks of activity (Fig 19). The larger peak was broad, ranging from pH 3.25 to 5.11 with a defined peak at pH 4.14. A lesser peak of activity

Figure 17. Concentrated HLMF Active 30-X P supernatant fluid from Affigel-ovalbumin affinity column was focused in a pH gradient of 3.5-10 and assayed for HLMF activity. The pH of each fraction was measured and plotted (o). HLMF activity in each fraction is illustrated by the bars.

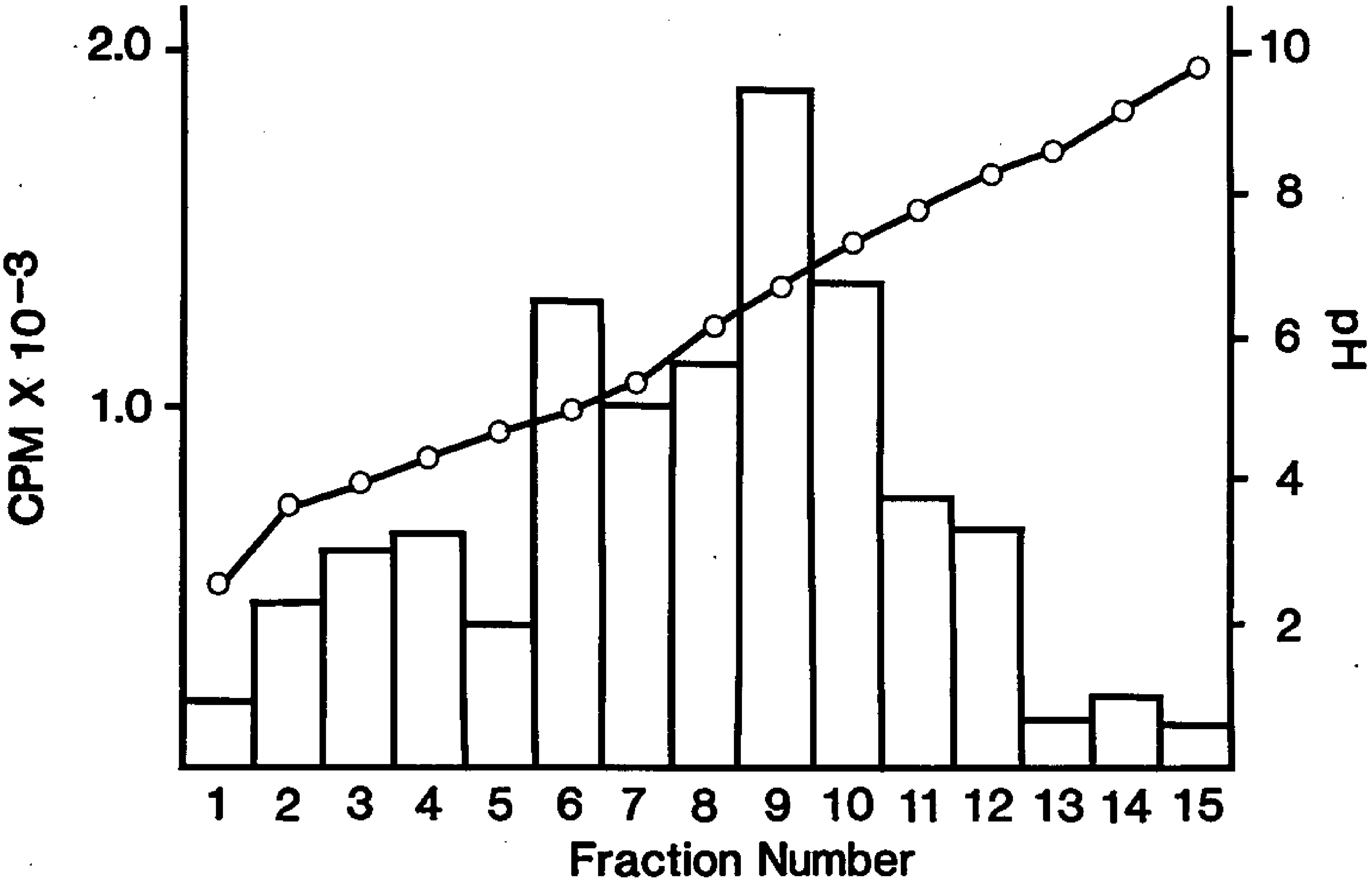

2.0
1.0
CPM X 10−3
10
8
6
4
2
pH
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
Fraction Number

Figure 18. HLMF Active 12,000-20,000 molecular weight fractions from Bio-Gel P-100 molecular sieve chromatography of 30 X P supernatants containing 1 mg/ml ovalbumin were pooled and reconcentrated. The concentrated material was focused in a pH gradient of 3.5-10 and assayed for HLMF activity. The pH of each fraction was measured and the gradient plotted (o). HLMF activity in each fraction is illustrated by the bars.

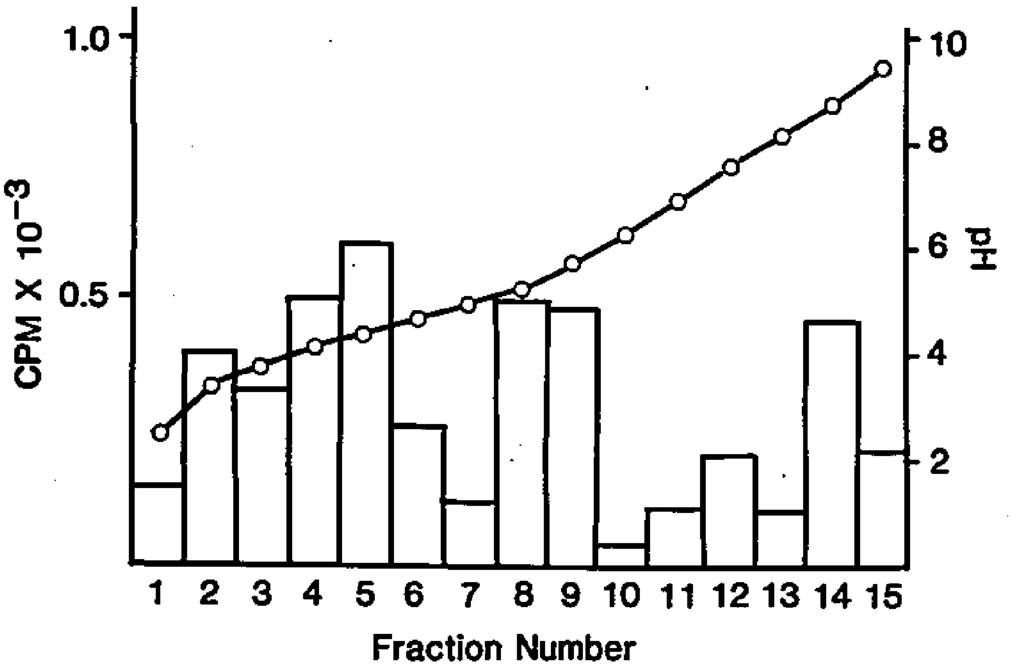
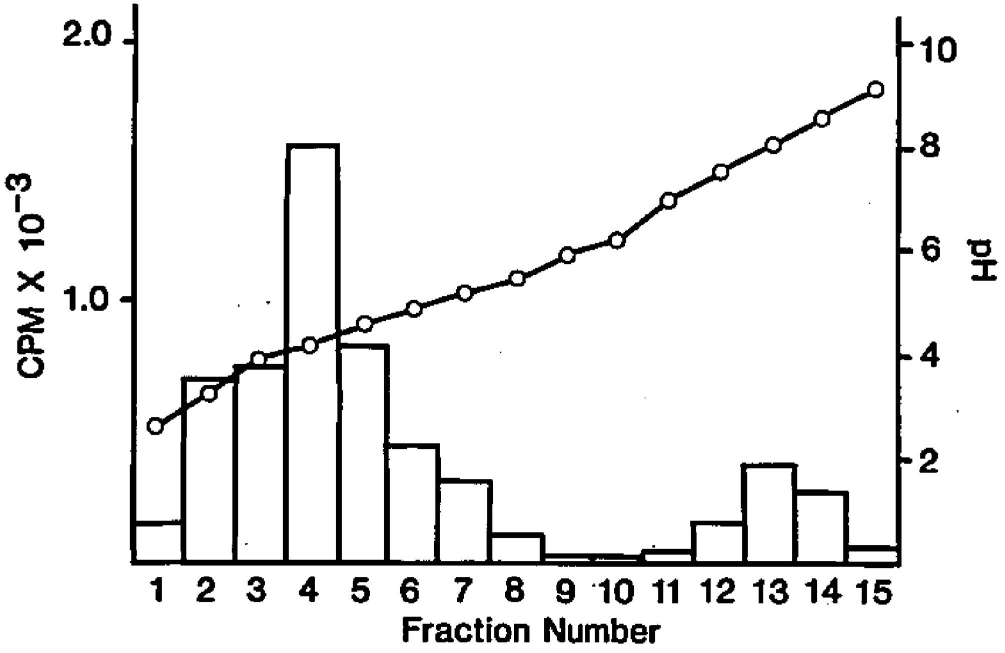

CPM X 10^{-3}
1.0
0.5
pH
10
8
6
4
2
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
Fraction Number

Figure 19. HLMF-active 30,000-50,000 molecular weight from Bio-Gel P-100 molecular sieve chromatography of 30 X P supernatants containing 1 mg/ml ovalbumin were pooled and reconcentrated. The concentrated material was focused in a pH gradient of 3.5-10 and assayed for HLMF activity. The pH of each fraction was measured and the gradient plotted (o). HLMF activity in each fraction is illustrated by the bars.



2.0
1.0
CPM X 10^{-3}
10
8
6
4
2
pH
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
Fraction Number

was observed in the pH range 8.07-8.5

pH Stability of HLMF-Active Supernatants. HLMF-active supernatants were tested for their stability within the pH range 2-7. All supernatants tested showed at least 83% activity remaining after all treatments (Table 12). Three of the five supernatants tested were significantly enhanced by exposure to the pH 7 buffer, and one of these was persistently augmented after all pH exposures.

Thermal Stability of HLMF-Active Supernatant. HLMF-active supernatants were tested for thermal stability at 56° C, 80° C, and 100° C. The results are shown in Table 13. The HLMF-activity was partially lost in two of three experiments at 56° C for 30 min. At 80° C for 10 min, all three supernatants retained between 21% and 45% of the control activity. Treatment at 100° C for 5 min completely irradicated the activity. No precipitation was noted in any of the experiments, after any treatment.

Trypsin Sensitivity of HLMF-Active Supernatants. HLMF-active supernatants were tested for their sensitivity to immobilized trypsin (Table 14). Five of the ten supernatants showed essentially no loss of activity after a one hour exposure to trypsin at room temperature. The remaining five supernatants had activities of 58, 65, 71, 75, and 82%. The data suggested that HLMF activity was resistant to trypsin digestion.

Optimum Bioassay Parameters. Cells at varying densities were assayed for optimum blastogenic response to concentrations of con A ranging from 5-15 μg (Fig. 20). In the absence of stimulation, the highest concentration of cells gave the highest ^{3}H-thymidine incorporation. At the lowest concentration of con A tested, however, the

TABLE 12

pH Stability of HLMF-Active Supernatants

Supernatant Number	Control[a] MI	pH-7[b] MI	% Control[c] MI	pH-5 MI	% Control MI	pH-3 MI	% Control MI	pH-2 MI	% Control MI
1	2.12	2.46	116	2.00	94	1.80	85	1.79	84
2	1.95	2.37	121	2.10	107	2.05	105	2.48	127
3	2.96	3.37	114	2.52	85	3.04	102	2.99	100
4	2.58	2.32	90	2.38	92	2.39	93	2.35	91
5	2.30	2.08	90	2.37	103	2.33	101	1.91	83

[a] $\text{Control MI} = \dfrac{\alpha\text{-mm resistant supernatant cpm}}{\alpha\text{-mm resistant AB control cpm}}$; Control material was maintained at pH 7.2 in supplemented N-T continuously until assay.

[b] $\text{Test MI} = \dfrac{\alpha\text{-mm resistant supernatant cpm after trypsin exposure}}{\alpha\text{-mm resist AB control cpm}}$

[c] $\dfrac{\text{Test MI}}{\text{Control MI}} \times 100 = \%\text{ control MI}$

TABLE 13

Thermal Stability of HLMF-Active Supernatant

Supernatant		% Activity[b]			
Number	MI[a]	Untreated	56° C x 30 min	80° C x 10 min	100° C x 5 min
1	1.95	100	96	21	0
2	2.96	100	40	36	0
3	2.30	100	20	45	0

$$^{a}\text{MI} = \frac{\text{Supernatant } \alpha\text{-mm-resistant cpm}}{\text{AB control } \alpha\text{-mm-resistant cpm}}$$

$$^{b}\%\text{ activity} = \frac{\text{Treated Supernatant } \alpha\text{-mm-resistant cpm} - \text{AB control } \alpha\text{-mm-resistant cpm}}{\text{Untreated Supernatant } \alpha\text{-mm-resistant cpm} - \text{AB control } \alpha\text{-mm-resistant cpm}}$$

TABLE 14

Trypsin Sensitivity of HLMF-Active Supernatant

Supernatant Number	Control MI[a]	Test MI[b]	% of Control MI[c]
1	2.38	2.23	94
2	3.68	3.58	97
3	1.55	1.75	100
4	4.24	3.01	71
5	2.87	1.87	65
6	1.74	2.75	100
7	2.29	1.88	82
8	3.26	1.90	58
9	2.27	2.39	100
10	2.53	1.86	74

[a] Control MI $= \dfrac{\alpha\text{-mm-resistant supernatant cpm}}{\alpha\text{-mm-resistant AB control cpm}}$

[b] Test MI $= \dfrac{\alpha\text{-mm-resistant supernatant cpm after trypsin exposure}}{\alpha\text{-mm-resistant AB content cpm}}$

[c] $\dfrac{\text{Test MI}}{\text{Control MI}} \times 100 = \%\ \text{Control MI}$

order reversed and the lowest density of cells incorporated the most ^{3}H-thymidine. Although this remained true at the higher dose, there was no statistical difference in the number of cpm of the 1×10^6 and 2×10^6 densities Incorporation of ^{3}H-thymidine by cells at the highest density was significantly lower than the incorporation at intermediate or lower densities at all concentrations of con A.

The intermediate density of cells was selected for use in the experiments designed to determine optimum serum concentrations. Three concentrations of HIABS were tested for their ability to support cell growth in the presence of varying concentrations of con A in two media (Fig 21). Supplemented RPMI-1640 proved to be a significantly inferior to supplemented N-T in its cell support capability at all serum concentrations tested. At the lowest con A concentration tested, the higher concentration of serum resulted in the lowest level of ^{3}H-thymidine incorporation. At the intermediate concentration of con A there was no appreciable difference in the ^{3}H-thymidine incorporation at any of the serum concentrations in the supplemented RPMI, and these values were not generally higher than for the other dose of con A.

For cells suspended in the supplemented N-T, the number of cpm at the intermediate dose of con A was significantly less for the 10% HIABS than for the lower concentration tested. At 15 µg con A/ml, there was an appreciable drop in the ability of 2.5% serum to support ^{3}H-thymidine incorporation in both media, and in both media, the effect of 5.0% and 2.5% HIABS were statistically indistinguishable.

When media containing HIABS or heat inactivated serum autologous to donor cells were tested for their relative support of incorporation of ^{3}H-thymidine by the PBL, no appreciable differences were noted at

Figure 20. The incorporation of ^{3}H-thymidine by PBL stimulated with 0, 5.0, 10.0, or 15.0 μg con A/ml culture fluid. Single representative experiment selected from a group of ten; S.D. ≤ ± 10% mean. ●, 10^6 PBL/ml; ■, 2 x 10^6 PBL/ml; ▲, 4 x 10^6 PBL/ml.

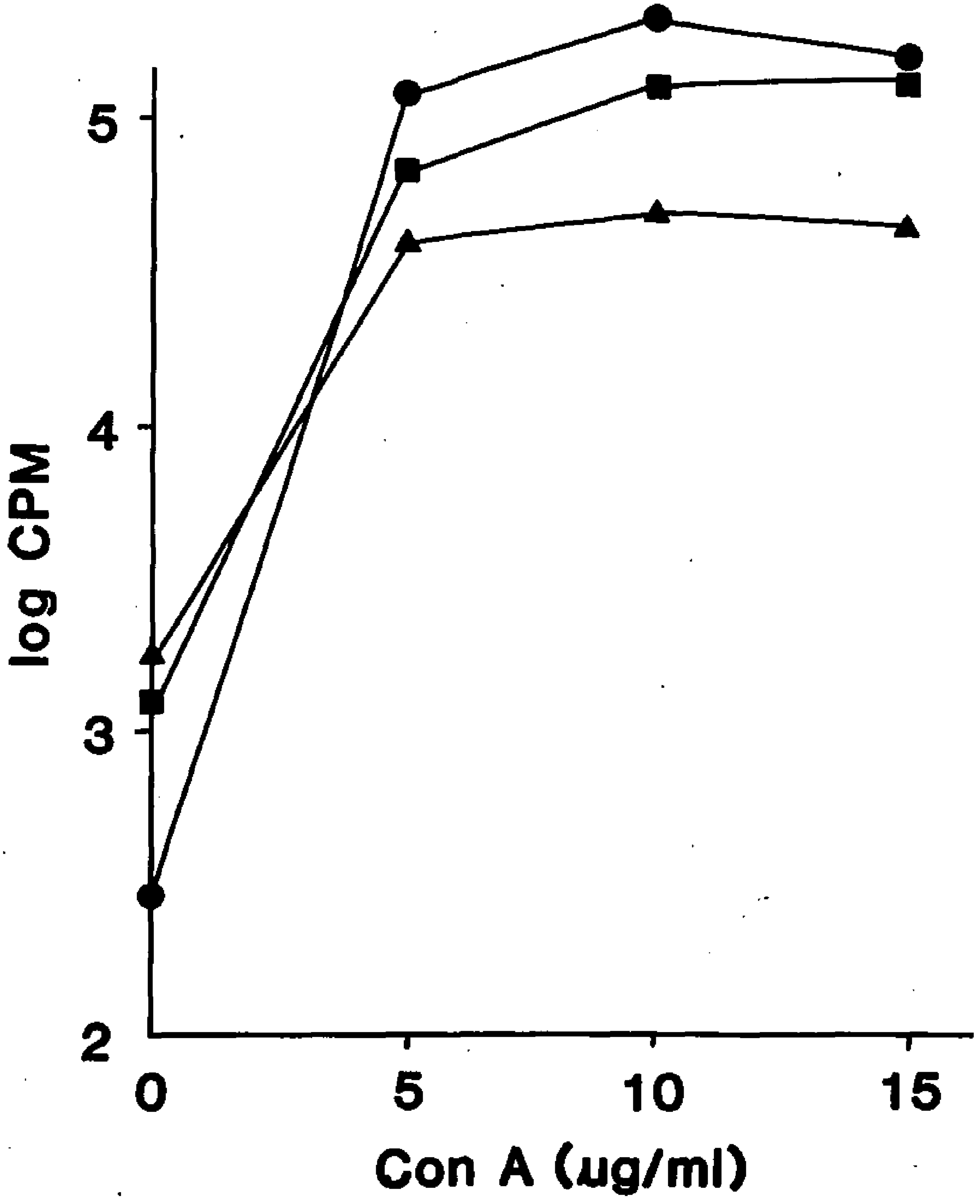
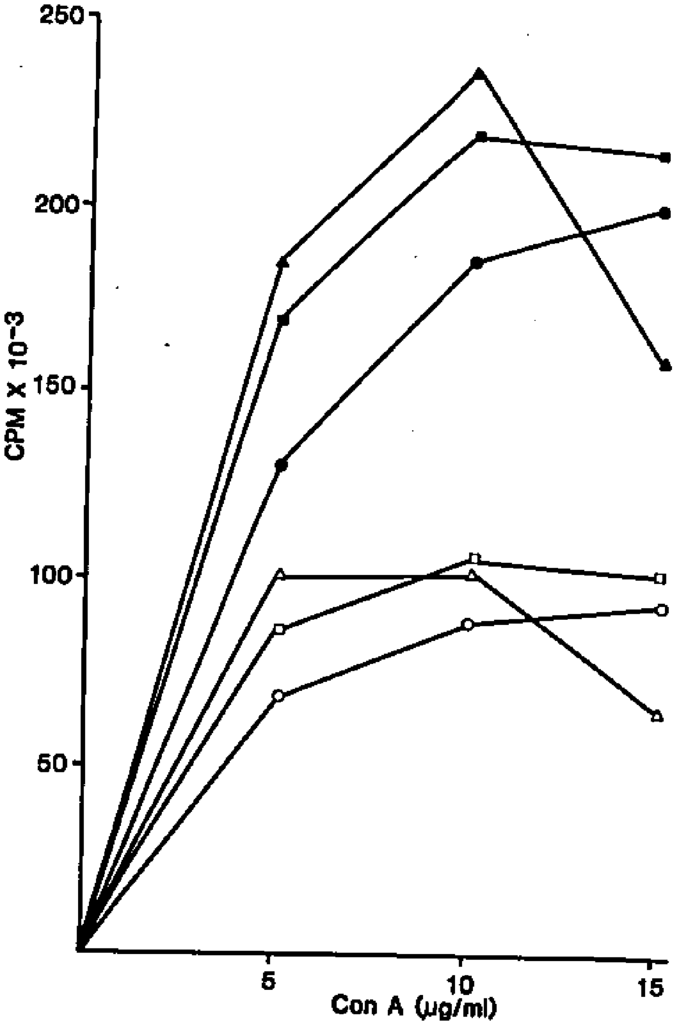

log CPM
5
4
3
2
0
5
10
15
Con A (µg/ml)

Figure 21. The proliferative response of 2 x 10^6 PBL/ml in supplemented media to varying concentrations of con A.
○, RPMI-1640 + 10% HIABS; □, RPMI-1640 + 5% HIABS;
△, RPMI-1640 ± 2.5 HIABS; ●, N-T + 10% HIABS;
■, N-T + 5% HIABS; ▲, N-T + 2.5% HIABS.


250
200
150
100
50
CPM X 10-3
5
10
15
Con A (μg/ml)

any of the con A values tested (Fig. 22).

For evaluation of the filter media performance, standard statistical analyses were used. A two-way analysis of variance of all the log transformed raw data showed statistically significant differences in performance between the filter media ($F = 34.66$; $V1 = 3$, $V2 = 80$). The internal data were very good (error mean square = 0.0048). Multiple comparisons identified the GF/F medium as being statistically inferior to each of the other three media at $P = < 0.05$ (Table 15). The GF/F medium compared favorably with the others in collection of radioactivity in unstimulated cells, and somewhat less favorably with cells treated with con A at 5 µg/ml (Table 15) at higher doses of the mitogen, however, the GF/F filter medium clearly was unacceptable. There were no statistical differences in the performance of 934-AH, GF/A and GF/C media (Table 15), but the radioactivity recovered on GF/A and GF/C media always was equal to or greater than that recovered on 934-AH medium.

HLMF Dose Response. HLMF-active fractions from the Bio-Gel P-100 column in the molecular weight ranges of 30,000-50,000 and 12,000-20,000 were serially diluted and assayed for HLMF, and TCGF activity (Fig. 23). The 30,000-50,000 molecular weight peak demonstrated significant HLMF activity, which behaved in a dose dependent fashion. The pooled proteins of lower molecular weight had essentially no HLMF activity, although the individual fractions used to form the pool did have activity.

No TCGF activity was observed in either peak at any concentration. Commercially prepared TCGF controls, however, demonstrated significant activity, the magnitude of which was not

Figure 22. The proliferative response of 2×10^6 PBL/ml to varying concentrations of con A in 10% HIABS (o), and 10% heat-inactivated serum autologous to donor cells (●).

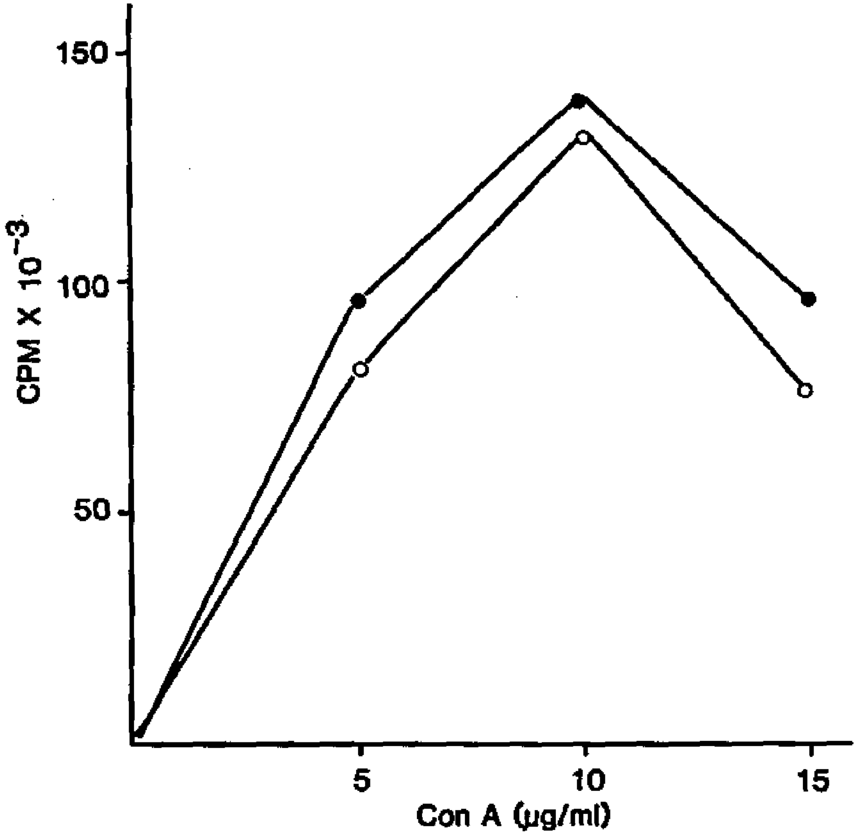

150
100
50
CPM X 10^{-3}
5
10
15
Con A (µg/ml)

TABLE 15

95% Confidence Limits Comparison of Filter Media[a]

Con A (μg/ml)	934-AH	GF/A	GF/C	GF/F
0	2.6251[b]	2.7051	2.6489	2.7287
5	5.1498	5.1469	5.1441	5,0431
10	5,1364	5.1895	5.1969	4.9615
15	5,1094	5.1747	5.2001	4.8836
	4.5033[c]	4.5541	4.5475	4.4042

df = 80　　$S^2 = 0.0048$　　N = 6

Comparisons

934-AH vs GF/A	4.5033 - 4.5541	=	-0.0508	N.S.[d]
934-AH vs GF/C	4.5033 - 4.5475	=	-0.0442	N.S.
934-AH vs GF/F	4.5033 - 4.4042	=	0.0991	Sig.[e]
GF/A vs GF/C	4.5541 - 4.5475	=	0.0066	N.S.
GF/A vs GF/F	4.5541 - 4.4042	=	0.1499	Sig.
GF/C vs GF/F	4.5475 - 4.4042	=	0.1433	Sig.

95% confidence level = ± 0.0602

---

[a] Characteristics of filter media is given in Table I; page 46.

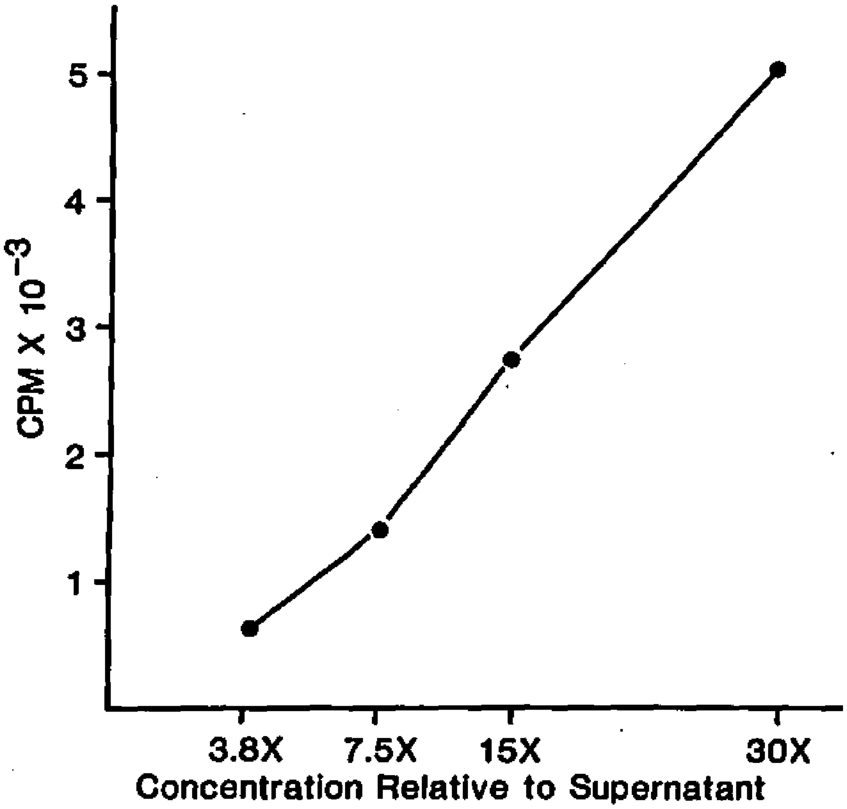
[b] ± 0.0857 (for all means)

[c] ± 0.0426 (for all average means)

[d] Not significant

[e] Significant

Figure 23. Activation of PBL by serial dilutions of pooled active fractions obtained from Bio-Gel P-100 chromatography of 30 X concentrated P supernatants. Points are expressed as X-fold concentrations of the original supernatant volume.


CPM X 10^{-3}
5
4
3
2
1
3.8X
7.5X
15X
30X
Concentration Relative to Supernatant

diminished even at 1/8 dilutions of the original material.

Subpopulation Target Identification. Standard HLMF assays were performed using as targets unfractionated PBL, T-cell, and B-cell enriched populations obtained as previously described. Samples of HLMF active unfractionated supernatants, pooled 30,000 molecular weight active fractions from Sephadex G-100 column chromatography, and the pooled 30,000-50,000 active region from the Bio-Gel P-100 column. Mitogenic indices of all samples were greater than 1.0 when assayed against unfractionated PBL (Table 16). The MIs of the T-cell enriched population ranged from 1.53 to greater than 9.48. The mitogenic indices of the B-cell enriched population were generally less than for either the unfractionated or T-cell enriched population, but were positive in all but one case.

TABLE 16

Proliferative Response of Human Lymphocytes to HLMF Active Supernatants

Stimulant	Human Mononuclear Cells Unfractionated PBL MI[c]	T-cell enriched MI	B-cell enriched MI
Whole Supernatant[a]			
1	6.11	3.24	1.80
2	4.44	9.48	1.92
3	7.24	6.07	2.04
MF Active Fraction[b]			
F_1	3.30	2.38	1.31
F_2	4.06	2.21	0.97
F_3	3.68	5.80	2.58
MF Active Fraction[d]			
F_1	1.05	1.99	2.06
F_2	1.82	1.53	2.54

[a] Unfractionated MF active supernatants

[b] Pooled 30,000 molecular weight active region from Sephadex G-100

[c] $\text{MI} = \dfrac{\text{cpm test}}{\text{cpm control}}$

[d] Pooled 30,000-50,000 molecular weight active region from Bio-Gel P-100

CHAPTER 4

DISCUSSION

It has been known for many years that the humoral and cellular immune systems function synergistically in response to antigen stimulation. The mechanism of communication, and regulation of their response, however, has been poorly understood. More recently, it has been observed that certain soluble products of the activated cells have the capacity to regulate the behavior of populations of cells not directly involved in the original stimulation. The responsible factors which function in a hormonal manner to regulate the magnitude and duration of an integrated immune response are termed lymphokines or monokines (2). In the last several years lymphokines have been partially characterized biologically and physiochemically. The Interleukins 1, 2, and 3 appear to have many common physiochemical features with regard to molecular size, charge heterogeneity and lability. They each, however, demonstrate quite specific functions in controlling cellular response to stimulation.

The major problem with obtaining definitive biological and physiochemical information on all lymphokines is the inherent difficulty created by the small initial amount of lymphokine material. This difficulty is compounded by poorly defined handling and assay procedures which result in substantial losses before isolation and purification. In many cases, the presence of serum proteins added to stabilize responding cells during activation significantly interferes with the purification to homogeneity of an active, *de novo* product of the activated cells.

In investigating the lymphokine, HLMF, it was necessary to examine the methods of procuring larger and more active supernatants by screening alternative sources of cells, defining the culture conditions for optimum yield and probing methods of handling which resulted in minimal losses. The biological assay was examined in the areas that could increase sensitivity, thereby leaving greater quantities of the partially pure factor for physiochemical characterization. The original source of HLMF was human PBL, but only limited quantities of cells are obtainable from single donors. Alternate cell sources are tonsils which are routinely removed as a result of recurrent tonsillitis.

Tonsils are lymphatic organs which contain essentially equivalent numbers of T and B lymphocytes (201, 202) and very low numbers of monocytes (202). The cells from tonsils have been reported to produce several lymphokines including Interleukin 2 (87), colony stimulating factor (203), and lymphotoxin (204). As a prerequisite to the use of tonsil cells as a source of HLMF, it was necessary to directly compare PBL and tonsil lymphocytes with respect to their initial states of activation, response to the stimulating mitogen, and any modifying effects which might occur due to required additives.

Because the tonsils are assaulted continually by microorganisms due to their location in the oral cavity, it is necessary to reduce the possibility of microorganism growth during transportation to the laboratory and during processing and culturing of the tonsillar cells. This is accomplished in part by transporting the tonsils, and later culturing their cells in media fortified with various antibiotics. Mycological organisms are frequently found in the oral cavity and the use of specific antimycotic agents is recommended to avoid fungal contamination. Amphotericin B (Am B) was used in these studies for this purpose, but it was necessary to specifically investigate its effect on peripheral blood and tonsillar lymphocyte activation because of the following literary citations regarding its effects on various immune responses.

Amphotericin B (Am B) is a polyene antibiotic used not only for treatment of systemic and deep-seated mycotic infections, but also to protect against mycotic contaminations in cell cultures during incubation and storage (205). Some investigators use Am B as a combination drug during cancer therapy (206, 207). There are reports of the drug causing immunopotentiation (208-210), augmentation and adjuvant effects (211, 212), mitogenicity (213) and suppression (214-216) with varying normal systems. The drug also causes various *in vitro* effects on cell lines (217-220). This brief literature review warranted an evaluation of the Am B effect on tonsillar and peripheral blood lymphocytes. Experiments were undertaken to determine the normal response of these cells to con A. This was followed by experiments to detect any effect the presence of Am B might have on the normal cellular response to the mitogen.

It has been known for a long time that con A stimulates proliferation of lymphocytes. In my experiments, the shape of the dose response curves indicated that the human PBL underwent a typical proliferative response to con A. It also was noted that when the concentration of the PBL was increased while maintaining constant con A levels, the uptake of ^{3}H-thymidine decreased, and the radioactivity in PBL cultured at 4 x 10^6/ml was less than half that in PBL cultured at 1 x 10^6/ml or 2 x 10^6/ml. Previously, other investigators demonstrated that increasing the concentration of con A while maintaining the cell concentration constant resulted in increased ^{3}H-thymidine incorporation (221, 222). My results suggested that the response of PBL to con A under the conditions described depended on several variables including the extent of binding of con A by serum proteins, the ratio of number of cells to concentration of con A and the ability of the cells themselves to respond to a lectin. In general, however, the magnitude of the incorporation of ^{3}H-thymidine was directly dependent on the availability of con A on a per cell basis.

Although the responses of PBL to 10 μg con A/ml usually were enhanced if Am B was present at 2.5-5.0 μg/ml during culture, some cases of negligible effect or even suppression were noted at the intermediate doses. When the concentration of the drug was raised to 10 μg/ml, however, the PBL responses to con A always were decreased significantly. Am B has been reported both to enhance and to suppress lymphocyte responses. Contact sensitivity of 2,4-dinitrofluorobenzene was augmented in mice by Am B (209), and the drug reversed a form of tolerance to 2,4-dinitrobenzene-1-sulfonate (223). Humoral immunity in mice also was enhanced by Am B (212). As a result of their studies, however, Ferrante et al. (224) concluded that Am B suppressed the delayed type hypersensitivity response to sheep red blood cells in mice, and had no effect on the antibody response. The results are just the opposite of those reported by Blanke et al. (212) and by Shirley et al. (210). The time of administration of Am B to the mice as well as the dose of the drug may have been influential on the results. Ferrante et al. (224) reported that inhibition occurred if Am B was administered after the day of priming or on the same day as challenge, but administration on the day of priming had no effect. On the other hand, Blanke et al. (224) noted enhancement of 2,4-dinitrofluorobenzene responses when Am B was administered on the day of priming, and Shirley et al. (209) reported enhancement of the response when Am B was administered with priming and one day after priming.

In vivo sensitization of mice with 2,4-dinitrofluorobenzene plus Am B led to enhancement of the *in vitro* proliferative responses of spleen and lymph node cells to 2,4-dinitrobenzene-1-sulfonate. Augmented proliferation of lymph node cells was found in the nylon-adherent cell fraction which comprised T and B cells. It was concluded that the immunopotentiating effect of Am B may be the result of its selective interaction with a subset of T cells which results in impairment of the normally induced suppressor regulation that limits the magnitude and duration of immune responses (210). Similarly, the enhanced responsiveness of human PBL to a mitogen, noted here, may reflect a depressing effect of Am B at intermediate concentrations on suppressor cells.

There have been several reports that Am B inhibited the activation of lymphocytes by con A, PHA, and certain antigens. DNA synthesis in human PBL cultured with PHA, con A, pokeweed mitogen (PWM), purified protein derivative (PPD) and in mixed lymphocyte culture was inhibited progressively by Am B at levels of 2-10 μg/ml (215). That Am B was a potent inhibitor of activation of PBL by PHA and PWM also was reported by others (216). The response of mouse spleen lymphocytes to PHA or lipopolysaccharide W was inhibited by Am B at 1, 5, or 25 μg drug/ml (224). The data indicated that Am B had a greater effect on the T-cell than on the B-cell response. The results reported here demonstrated that con A activation of PBL was strongly inhibited by

Am B at 10 μg/ml. At this concentration of the drug, the conclusion drawn substantiated those of others (215, 216, 224) who also reported inhibition.

Tonsil cells were active in the absence of deliberate *in vitro* stimulation. This preactivation phenomenon occurred repeatedly, and it has been reported by others (225). It was assumed that it was the result of *in vivo* stimulation by microorganisms. Am B reduced the spontaneous uptake of ^{3}H-thymidine by TL to PBL background levels at 5-10 μg Am B/ml. The data are consistent with the suggestion that Am B acts on T-blast cells (224).

The tonsil lymphocyte population responded well to con A even though some of the cells were preactivated. Tonsils comprised 30-55% T cells and 38-60% B cells. This was considerably different from the distribution in peripheral blood which comprised 60-80% T cells and 15% B cells (201, 202). Although there was a difference in the percentage of T cells in tonsils and blood, the magnitude of the response of con A-stimulated tonsil cells was as great as that of con A-stimulated PBL at the same cell concentration. Unlike PBL, Am B markedly reduced the proliferative response of tonsil cells at all concentrations of the drug tested. The reduction in radioactivity was greater than could be accounted for by the diminished proliferation of preactivated cells alone caused by Am B. Further, the percentage reduction of proliferation of tonsil cells to con A in the presence of 10 μg Am B/ml was greater than the reduction seen in PBL at that level. It can be concluded that tonsil cells were much more sensitive to inhibition of proliferation by Am B than were PBL.

An apparent inhibition of the proliferative response could occur if the cells were killed by the drug. The attempts to determine cell viability after culture with Am B and con A suggested viability was good, but technical problems related to cell clumping made the experiments unsatisfactory. Moreover, Ferrante et al. (224) reported that Am B at 5 μg/ml was not toxic to mouse spleen cells after 48 hr culture (224).

Higher densities of tonsil cells responded better to con A in the presence of 5-10 μg Am B/ml than lower densities. For example, when the concentration of Am B was 5 μg/ml, the peak responses of cells at a density of 2 x 10^6/ml or 4 x 10^6/ml were 2.5 and 3.4 times respectively greater than the response at a density of 10^6/ml. When the concentration of Am B was raised to 10 μg/ml the 2 x 10^6/ml or 4 x 10^6/ml to 10^6/ml ratios ranged from 34 to 79. This was primarily due to the greater effect of 10 μg Am B/ml on the lower cell densities, thereby creating significantly higher ratios by lowering the denominator function of the SI formula.

It was concluded that Am B at 2.5-5.0 μg/ml enhanced the response of PBL to con A, but at 10 μg/ml the drug suppressed the response. All doses of Am B tested inhibited both spontaneous proliferation and con A-induced proliferation in TL. In spite of this inhibition, the TL population responded to various doses of con A in the presence of Am B.

After reviewing the available data for tonsillar lymphocytes, it was surmised that they were unsuitable for use as HLMF producer because of their pre-activated state, and the effect of Am B on the con A-stimulated population. After electing to continue use of human PBL for HLMF production, experiments were undertaken to determine the conditions necessary for optimum factor production.

The density of lymphocytes responsible for the greatest amount of HLMF activity in supernatants when cultured in Neuman-Tytell serumless medium was 10^7/ml. The addition of 2-mercaptoethanol to the culture resulted in increased HLMF activity over that generated in its absence. Other investigators also noticed the beneficial effect of mercaptan on other lymphokine production (73, 226, 227). The failure to find HLMF activity in supernatants from cells cultured at the lowest density probably was due to there being so very little produced that it was not sufficient to activate target cells. A similar situation occurred when less than 10^6 CBA thymocytes/ml were cultured with con A. So little costimulator, now referred to as Il-2 (73), was secreted that blastogenesis could not occur (67, 73).

When the stimulating dose of con A was 5 or 10 μg/ml, HLMF production peaked within 24 hr culture. It appeared that whatever cells were capable of producing HLMF when stimulated with these doses of con A did so within 24 hr but did not continue to produce lymphokine thereafter. At doses of 15-35 μg con A/ml, HLMF production also occurred during the first 24 hr culture, and continued in the absence of replenished con A for the next 24 hr. It was possible, however, that in the latter cases, the increased incorporation of ^{3}H-thymidine might have been partially attributable to the effects of Il-1 and Il-2. HLMF was produced by non-proliferating cells at all doses of con A. Proliferation in the producer-cell population began at the lowest dose of con A and gradually progressed with time toward the higher doses. During the time period that was studied, proliferation did not occur at the highest doses. Others have shown that there were optimal doses of the lectin to induce proliferation, and when the dose became too great, suppression of proliferation occurred (96, 97).

Il-1 is produced by monocytes and it augments lectin-induced lymphocyte proliferation (72, 228). This probably is an indirect effect with proliferation really being the result of Il-2 whose production is induced by the Il-1 (94, 119-122, 228). Il-2 is a T-lymphocyte product which induces proliferation of activated T lymphocytes (18-20). This lymphokine has no mitogenic effect on unstimulated lymphocytes (75, 76, 85, 229-231). The supernatants used in the experiments reported here contained con A, and it was necessary to inactivate the lectin to distinguish its activity from that of HLMF, and to prevent the development of mitogen-activated lymphocytes which might respond to Il-1 or Il-2. α-mm completely inhibited the action of con A when the inhibitor concentration was 0.1 M. This inhibition resulted from α-mm occupanying the glucose-binding site on the lectin. It was noted that spontaneous low level proliferation of PBL also was reduced. This probably was related to the high osmolarity imposed on the cells. At any rate, the mitogenic activity in the supernatant seemed to be induced by a soluble mediator which probably was not Il-1 or Il-2.

It was previously demonstrated that amphotericin B at 2.5 or 5 μg/ml usually enhanced the proliferative response of PBL to con A, but when the drug was used at 10 μg/ml, the response of the PBL always was significantly below that of the cells treated with con A in the absence of amphotericin B.

Experiments were undertaken to determine if HLMF production could be augmented in the same manner as the previously observed con A-induced PBL blastogenesis. The results were somewhat inconclusive in that expression of HLMF was reduced or unchanged in supernatants of cultures which received doses of Am B that previously had stimulated proliferation, and at higher doses that had inhibited proliferation. The data trends were consistent with a given set of experiments, however. If profileration of con A-treated PBL was due to Il-2, then amphotericin B as used in the previous work (232) must influence the production or the effect of that lymphokine, depending on the dose of the drug. Since there was inhibition or no effect, but never a stimulation of the expression of HLMF, the data suggested that amphotericin B may affect HLMF differently from the way it affects Il-2.

The induction of HLMF production by mitogens appears to be temporary. HLMF production usually dropped in cases where no additional con A was added after the first 24 hr incubation. When con A was added to the culture with fresh medium after 24 hr incubation, equivalent or greater amounts of HLMF were produced. The mechanism which abrogates the HLMF production in the absence of subsequent con A addition is not known; however, the possibility of suppressor action against HLMF-producing cells as suggested by Gullberg et al. (96, 97) is possible.

The biological action of soluble mediators occurs at very low concentrations on the order of 10^{-10}-10^{-15} M (228). To minimize losses on limited amounts of partially purified factor it was necessary to determine the optimum procedures for handling and storage. Assay of samples stored for short periods at -80° C showed only minimal activity. After prolonged freezing at -80° C, however, the activity usually was not reduced below that in untreated samples stored at -10° C. Perhaps some kind of suppressor was gradually destroyed while the sample was frozen. In contrast, Mier and Gallo (231) reported that partially purified TCGF was unstable at -70° C. When supernatant volumes in my experiments were reduced by diafiltration using a YM-10 membrane and the retentates were frozen at -80° C, HLMF activities were poor after thawing. They were about the same as those demonstrated in supernatants which simply were stored at -80° C for a short time. The YM-10 membrane was selected because it was designed to bind less protein (233) than other diafilter membranes. The conclusion, however, is that the membrane bound the HLMF leading to a loss of activity. When samples were dialyzed in NH_4HCO_3 solution before lyophilization, they were active when rehydrated. If the loss of activity following diafiltration was attributed to an inhibitor being in the retentate, then it should also have been retained within the dialysis sack, making the HLMF inactive.

Although the mechanism of blastogenesis induction by con A requires the intermediate action of Interleukin 2, the end result is target cell proliferation, just as is the case for HLMF. α-mm can be used to block the con A effect, but any purification scheme for HLMF eventually requires the removal of the mitogen. Several methods of HLMF-con A separation were examined, based on the literary documentation of the affinity interactions and molecular size of the mitogen. As suggested by Northoff et al. (37) removal of con A from solutions or media in the absence of serum proteins could be accomplished without difficulty. The inclusion of serum, as was required for HLMF production in RPMI-1640, interferred with the con A removal such that Sephadex G-100 affinity chromatography was not acceptable. Other investigators have reported similar difficulties with con A removal when serum was included in the medium prior to lymphokine production. In an attempt to avoid the necessity for serum, the N-T medium was employed, since unlike RPMI-1640, it requires no protein additives to support lymphocyte viability and growth.

Attempts to fractionate 30-fold concentrated HLMF-active supernatants in N-T medium over Sephadex G-100 resulted in significant contamination of con A in the fractions eluting near the total column volume, even though HLMF activity eluted earlier in the 25,000-30,000 d range. Fractionation of 30-fold concentrated N-T medium containing 150 μg con A/ml led to similar elution profiles which suggested that an inhibitor of the con A-dextran interaction was present in the N-T medium. Subsequent fractionation on Bio-Gel P-100 of the fraction taken from Sephadex G-100 which were contaminated with the low molecular weight con A suggested that the actual size of the apparently low molecular weight mitogen was greater than 35,000. This also supports the notion that the apparent low molecular weight of contaminating con A in the Sephadex G-100 fractionation experiments was artifactual. This artifact was probably due to an endogenous inhibitor in the N-T medium whose molecular weight corresponded to the position where con A was observed.

Commercially-obtained Affigel-ovalbumin was used to attempt removal of con A from HLMF-active supernatants and experimental controls. Affigel, which has covalently-bound ovalbumin as the affinity ligand, successfully removed contaminating con A from active supernatants, but was far too expensive for the large scale operation required to obtain the significant amounts of HLMF needed for subsequent physiochemical and biological characterization.

Literature sources site the molecular weight of con A to be 55,000 at pH values less than 6, and greater than 100,000 d in pH ranges greater than 7.0 (165). Molecular sieve chromatography of con A on Bio-Gel P-100 repeatedly demonstrated its molecular weight under the given experimental conditions to be 45,000 d. When active concentrated supernatants were fractionated, the con A and HLMF activities were found to overlap significantly. However, when ovalbumin was added to HLMF-active supernatants at a concentration of 33 μg/ml prior to concentration before fractionating the supernatants, the elution profile of con A was shifted significantly toward the exclusion volume. This presumably occurred because of binding of con A to ovalbumin, a glycoprotein. The absorbance of the eluted material suggested there was some unbound ovalbumin, because a maximum of light absorption was noted coincident to the elution position of the control (ovalbumin) sample, and was not observed in the previously described 30-fold concentrated supernatants which did not contain ovalbumin. This appears to be a promising method for rapid separation of residual con A from HLMF in concentrated supernatants.

In the ovalbumin-fractionated supernatants, there appeared to be greater recovery of active material in the 30,000-50,000 molecular weight range, but there was significant HLMF activity in the 12,000-20,000 range, too. The bioactivity associated with the lower molecular weight entity did not appear in chromatographs done in the absence of ovalbumin. This presumably was attributable to stabilization by ovalbumin of smaller molecular weight forms of HLMF. Previously, the smaller sized HLMF may have been lost during handling prior to assay. Certainly, other lymphokines such as LAF, have been reported to bind to glassware in the absence of other proteins. The observation of a 12,000-20,000 d and 30,000-50,000 d form suggests the possibility of self-association, although two distinct forms may occur. The notable absence of the lower molecular weight form in those supernatants fractionated in the absence of ovalbumin could suggest extreme lability of the smaller molecular weight activity in the absence of exogenous protein.

The largest molecular weight form eluted very close to the exclusion volume, but was so contaminated with con A that further characterization was not attempted. Although the possibility existed that the larger sized α-mm-resistant activity may actually represent a concentration of con A in excess of that which can be inhibited by 0.1 α-mm, equally possible is the association of HLMF with either *de novo*-produced lymphocyte proteins or the exogeneous ovalbumin. McDaniel (234) reported the association of another lymphokine, macrophage activation factor, with albumin and α2-macroglobulin when the factor was generated in a serum-free culture system.

Isoelectric focusing of the Affigel-ovalbumin-treated HLMF-active supernatants resulted in two peaks of activity. The activity at pH 6-7 is certainly suspiciously close to the pI reported for Il-2 (100). However, the inability of Il-2 to directly activate target cells in the absence of other stimulation drastically reduces this possibility (75, 76). Lower pI's for other lymphokines have but not for HLMF.

Isoelectric focusing of the 50,000-70,000 d and 12,000-20,000 d active fractions from the Bio-Gel P-100 chromatography resulted in profiles decidedly different from one another. The lower molecular weight fraction contained three areas of activity, with the largest occurring in the pH range 3.4 to 4.6, but with significant activity at 5.2, 5.7, 8.7, and 9.5. The larger molecular weight chromatographic fraction had greater activity at pH 3.25 and 5.11, but there was no other significant activity until the pH values exceeded 8.0. These data were in contrast to those presented by Wood et al. (60). They recovered a B cell-LMF which had a molecular weight of 20,000-50,000 d when sieved through Sephadex G-100. The fractions with molecular weights less than 26,000 d focused isoelectrically between pH 4.5-7.5 and those whose weights were greater than 26,000 d had a pI of 7.6-8.4.

Aside from the possibility of multiple factors being represented by the different peaks, the breadth of the activity suggested significant charge heterogeneity, not unlike that reported for other lymphokines (74, 101). There are reports of post-translational glycosylation among lymphokines (101) and charge heterogeneity could certainly result from such intracellular modifications.

My experiments demonstrated that HLMF retained biological activity after exposure to pH values from 2-7. Similar experiments by others demonstrated some lymphokines remain active after exposure to pH values ranging from 2-10 (98). Although this information was of extreme value in designing handling procedures, it did not discriminate between HLMF, and other lymphoproliferative factors such as the interleukins.

In thermal stability experiments, HLMF activity was only partially destroyed by exposure to 56° C for 30 min. Higher temperature for shorter periods destroyed essentially all the activity but without obvious visible denaturation. This was in agreement with the reports of Sundar et al. (49) who reported complete loss of activity after treatment at 100° C for 10 min, and with Rutenberg et al. (47) who reported inactivation after exposure at 70° C for 5 min. It contradicted, however, others who reported HLMF activity after exposure to 56° C for 30 min (13, 14, 59).

HLMF proved to be resistant to trypsin digestion. This did not suggest however, that HLMF was or was not protein in nature. Others have reported loss of HLMF activity after trypsin exposure, but found HLMF to be resistant to treatment with RNase, neuraminidase, and α-amylase (13, 14, 59). Il-2, however, was found to be sensitive to trypsin under similar conditions (98, 100). This provides another distinguishing piece of evidence between the interleukins and HLMF.

Assay of HLMF generally requires significant amounts of the partially purified material. It was decided that if biological assay parameters could be modified to increase sensitivity, then smaller quantities of material could be used for assay leaving more partially purified HLMF for subsequent physiochemical analyses or purification steps.

In experiments measuring the effect of cell density on blastogenic activation, the greatest density of lymphocytes incorporated significantly less ^{3}H-thymidine than lower densities (1 or 2 x 10^6 cell/ml). Of the lower cell concentrations, 1 x 10^6 cells/ml usually incorporated slightly more radioactive material, but the 2 x 10^6 cell/ml was not significantly less. A density of 2×10^6 cell/ml was selected for use based on the consideration that should HLMF only activate a limited subpopulation of cells, it would provide greater numbers of reacting cells than the lowest density tested.

Further experimentation using 2×10^6 cells/ml suspended in RPMI-1640 and N-T, and three different concentrations of HIABS were undertaken to determine the best conditions for assay. In every case N-T medium supported blastogenesis significantly better than RPMI-1640. When HIABS was added to N-T medium to 2.5% (v/v), ^{3}H-thymidine incorporation by the cells was at its best. In contrast, a serum concentration of 10% appeared to be optimum level when assaying for HLMF. These conclusions were founded on information obtained from the con A dose response experiments. In those experiments, con A concentrations resulting in maximum blastogenesis were higher in the presence of 10% serum than in the presence of lower serum concentrations. This was probably a consequence of the binding of con A to serum glycoproteins which may have reduced the con A available to activate cells. For con A-containing HLMF-active supernatant assays, those conditions which offered maximum blastogenesis and with maximum protection of cells from con A were considered optimum. Since no significant differences were noted between HIABS and autologous serum, the former was chosen because it offered internal consistency among reagents.

The cells used in bioassays are most frequently harvested using multiple automated sample harvesters (MASH). These units offer rapid collection of microsupernatants, and with simultaneous deposition of cells on a filter media. Since viable cells which were activated in an assay have assimilated the radiolabel, it is important to select the filter medium which offers the highest retention of these cells. The automated harvester (MASH II) was utilized extensively in this laboratory. An unexpected and serious deterioration in recovery on 934-AH filter medium of expected radioactivity from human lymphocytes stimulated with con A or with HLMF-rich supernatants occurred. A considerable amount of time and effort was expended trying to identify and correct the situation. Several small problems were associated with the MASH unit, the most serious of which were tiny leaks in the pooling chamber. These problems were easily rectified. The major problem and the most difficult one to identify, however, had to do with the 934-AH filter medium. Eventually, it was learned that the medium had been manufactured outside of control limits. The data reported here evolved from extensive efforts to correct the problem and to determine if that grade of filter medium actually was the most suitable for the work.

The characteristics of the filter media manufactured by Whatman Inc. which were tested are given in Table 1. They were made of borosilicate glass microfibers without a binder. The wet strengths of the GF media were low but suitable for low suction filtration (235). The 934-AH medium had a wet strength greater than 150 gm/inch width (236). All of the media tested had high loading capacity. The data show that GF/F filter medium was unsuitable for use in the MASH II. Aspiration of the cultures and washes was difficult. This grade filter medium was the thickest and was a finer particle retention than the others.

Although there were no significant differences in the performance of 934-AH, GF/A, and GF/C media, the radioactivity collected on the latter two was usually greater than that collected on 934-AH. In lymphocyte stimulation assays, the filter medium which is most efficient in collecting the radioactivity incorporated into DNA should be used. Often investigators must deal with relatively low incorporation which manifests itself as only a few thousand counts per minute. GF/A or GF/C media are better choices than 934-AH medium. The cost of the GF/C medium, however, is considerably greater than that of GF/A. This is probably a more important factor than differences in performance in selecting GF/A or GF/C filters for investigations of lymphocyte transformation when using a multiple automated sample harvester.

Dose response assays were performed on the 12,000-20,000 d and 30,000-50,000 d pooled active fractions, resulting from the Bio-Gel P-100 chromatography. The larger molecular weight material demonstrated significant HLMF activity which behaved in a dose dependent fashion. The lower molecular weight material, however, essentially had no HLMF activity, even though the composing individual fractions had contained varying, but significant amounts of α-mm-resistant activity. The lower molecular weight forms had only appeared during chromatography of active supernatants which contained exogeneous ovalbumin. Subsequent loss of the activity from individually active fractions which were pooled following molecular sieving certainly tended to support the notion that extreme lability or glassware tenacity are characteristics of the lower molecular weight forms in the absence of exogeneous and presumably stabilizing proteins.

The absence of TCGF from either fraction was unexpected. Although Il-2 has a defined molecular weight of 15,000, Duncan et al. (55) demonstrated minimal TCGF activity in the pooled 27,000-35,000 d HLMF-active material. This TCGF activity could be adsorbed by TCGF-dependent cytotoxic T-cell transformed lines. In those experiments, however, no exogeneous protein was added to the HLMF-active materials which had been generated in N-T medium, and neither HLMF or TCGF activity was observed in the lower molecular weight areas. This also may reflect the instability or adherence qualities of HLMF and TCGF in that system as well.

Target cell subpopulation studies failed to clearly demonstrate complete containment of the HLMF-responding cells in either the T-cell-enriched or B-cell-enriched subpopulation. The methods of subpopulation enrichment used in these experiments were well established, and controls demonstrated adequate separation of E-rosette positive and negative cells into the appropriate subpopulations. Reinherz et al. (17) separated cells based on their surface markers using a fluorescent activated cell sorter. That group demonstrated significant activation of all three subpopulations obtained (T-cells, B-cells, and Null cells), with the null cell population responding to a much greater extent than either T-cells or B-cells. Since the methods employed in this research would allow for Null cell contamination of both T-cell-enriched and B-cell-enriched subpopulations, the data could be used to indirectly support the report of Reinherz et al. (17) of Null cell activation by HLMF. Alternatively, Duncan et al. (55) procured B-cell enriched populations using similar T-cell depleting isolation techniques and noted a selective B-cell-enriched population activation by the Bio-Gel P-100 HLMF active fractions. That group, however, also used T-cells for target cells even after cold hypotonic saline exposure. The hypotonic saline treatment was used to lyse remaining SRBC after Ficol-Paque density separation.

The use of T-cells procured in this manner was deemed unacceptable at this time, since the possibility remained that the lysis treatment may have impaired the T-enriched populations HLMF response capability.

With regard to activation of both major subpopulations the distinct possibility also exists that multiple factors remain, as yet, unseparated even in the pooled column fractions, and indeed reactive cells of T-cell or B-cell specificity may be responding to distinct activating entities. It also cannot be ruled out at this time whether HLMF as a single entity may possess the capability of activating cells of several population specificities.

A number of alternate but less widely used PBL fractionation procedures remain viable and should be tried. Until such time as the specific subpopulation of HLMF responder cells can be identified via fluorescent antibodies or radio- or fluorescent-labeled homogeneous HLMF preparations, the biological separation procedures should be surveyed.

As a matter of convenience to the reader, a composite of the reviewed literature is provided in Table 17. References for particular quotations are keyed to the bibliography. Lastly, HLMF data presented in the dissertation have been collated into the table for ease of comparison.

In summary, the initial objectives were achieved in so much as it was demonstrated that HLMF productivity was dramatically increased, and handling and isolation procedures which allowed significantly higher yields of partially purified material were defined. The new assay procedures allowed detection of HLMF at lower concentrations of the partially purified material. Isolation and partial characteriza-

TABLE 17

Literature and Experimental Result Comparisons

Factor	Synonyms	Cell Source	Molecular Weight	pH Proteins/ Thermo Sensitivity	Isoelectric Point	Target Cell
Il-1	LAF (112) Mitogenic Protein (114) Helper Peak (115) T-cell-replacing factor III (116) T-cell-replacing factor Mø (61) B-cell-activating factor (117) B-cell differentiation factor (118)	Macrophage (126-131)	11,000 (113) on SDS-PAGE 12,000-15,000 (139) on Ultrafiltration 12,000-22,000 and 50,000-70,000 on Bio-Gel P-100 (141)		7.0 (140) 5.2, 6.0, 6.9 (74)	Il-2 producing T-lymphocytes (119-122)

TABLE 17 (cont)

Literature and Experimental Result Comparisons

Factor	Synonyms	Cell Source	Molecular Weight	pH Proteins/ Thermo Sensitivity	Isoelectric Point	Target Cell
Il-2	Thymocyte mitogenic factor (68) Co-stimulator (73) Killer cell helper factor (68) Secondary cytotoxic T-cell Inducing factor (69)	T-lymphotytes (88, 89 90, 91)	15,000 ACA 54 and Sephadex (98) 27,000-35,000 (55) on Bio-Gel P-100	Stable pH 2.7-10.0 (98) Stable 70° C x 15 min (98) trypsin, chymotrypsin (100) and subtilisin sensitive (98)	6.5 (98) 6.8, 8.0 (100)	Cytotoxic and helper T cells (85, 103, 107, 108)

TABLE 17 (cont)

Literature and Experimental Result Comparisons

Factor	Synonyms	Cell Source	Molecular Weight	pH Proteins/ Thermo Sensitivity	Isoelectric Point	Target Cell
HLMF (Literature)	Mitogenic factor (55) MF (17)	T-cells (13-22)	15,000-31,000 on (11-14) Sephadex G-100 29,000-56,000 (11) and post-albumin on Sephadex G-200 (47) 40,000-45,000 (30) and 27,000-35,000 (55) on Bio-Gel P-100	Stable 56° C (13, 14, 59) Sensitive 70° C x 5 min (47) Sensitive 100° C x 10 min (49)	8.0-8.5 (50) 7.6-8.4 (60) 4.5-7.5 (60)	T cells (13, 14, 17, 19) B-cells (11, 13, 14, 17, 19, 55) Null cells (17)

TABLE 17 (cont)

Literature and Experimental Result Comparisons

Factor	Synonyms	Cell Source	Molecular Weight	pH Proteins/ Thermo Sensitivity	Isoelectric Point	Target Cell
HLMF (Experimental)		T-cells	12,000-20,000 on Bio-Gel P-100 30,000-50,000 on Bio-Gel P-100 18,000-25,000 on Sephadex G-100	Stable 2.0-7.0 Stable-Trypsin Partial Loss 56° C x 30 min Sensitive 70° C x 10 min 100° C x 5 min	4.2, 6.7	

tion of the factor was significantly accelerated because a serumless culture medium was employed, and this reduced the number of contaminating proteins. The ultimate goal of purification to homogeneity was not achieved, since even the increase in yield of partially purified material would not preclude the eventual need for amounts of supernatant far in excess of that obtainable through single donor phlebotomies. Although HLMF was clearly shown to possess *in vitro* characteristics decidedly different from the other lymphoproliferative factors, an unequivocal immunoregulatory function *in vivo* was not defined.

The immediate direction of this research certainly should have several facets. Initially, the relative purity of the 30,000-50,000 d active fraction from Bio-Gel P-100 column chromatography should be determined. This can be determined by the use of analytical isoelectric focusing or polyacrylamide gel electrophoresis followed by silver stain. This should be followed by development of monoclonal or natural antibodies to HLMF. This would facilitate not only the use of radioimmunoassay for quantitating HLMF, but also would allow selective immunoaffinity chromatography techniques to be developed. The latter have proven invaluable in procuring quantities sufficient for purification to homogeneity of other lymphokines. Alternatively, discovery of an established transformed cell line which endogeneously or inducibly produces HLMF would allow significantly greater availability of starting material for purification through current physiochemical technology. Identification of the specific HLMF-reactive cell population would allow definition of any *in vivo* immunoregulatory function which the factor may possess. Eventual purification of the HLMF-receptor protein would provide the avenue for investigation into potential points of selective clinical intervention for correction of HLMF producer-responder anomalies.

Information derived from the above mentioned experimental pathways will aid ultimately in defining the apparently complicated mechanism of immunoregulation. Understanding the products and activities of activated lymphocytes is critical if immunologists and clinicians are to use these regulatory molecules to selectively intervene in altering, amplifying or suppressing specific components of the immune responses (153).

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