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Tonsil Cell Products which Modify in Vitro Proliferation of Blood Lymphocytes

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TONSIL CELL PRODUCTS WHICH MODIFY IN VITRO PROLIFERATION OF BLOOD LYMPHOCYTES

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TONSIL CELL PRODUCTS WHICH MODIFY *IN VITRO* PROLIFERATION OF BLOOD LYMPHOCYTES

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

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by

Thomas Weston Hodge

May, 1982

DISSERTATION APPROVAL

THOMAS WESTON HODGE

Tonsil Cell Products Which Modify *In Vitro* Proliferation of Blood Lymphocytes

This is to certify that the Graduate Committee of Thomas Weston Hodge has reviewed and evaluated his dissertation and conducted his defense of it in an open oral examination. The committee recommends to the Graduate Council and the Dean of the School of Graduate Studies acceptance of the dissertation in partial fulfillment of the requirements for the degree Doctor of Philosophy in Biomedical Science.

April 22, 1982

Chairman, Graduate Committee

Signed on behalf of the Graduate Council

Dean of Graduate Studies

Abstract

TONSIL CELL PRODUCTS WHICH MODIFY *IN VITRO*

PROLIFERATION OF BLOOD LYMPHOCYTES

by

Thomas Weston Hodge

Human palatine tonsil lymphocytes, when compared to peripheral blood lymphocytes (PBL), were in an activated state even though there was no *in vitro* stimulation. When these tonsil lymphocytes were cultured in the absence of serum and polyclonal mitogens or antigens, the supernatant fluid often inhibited the proliferative response of target PBL to con A. The extent of this suppression ranged from 22% to 84%, and target cell viability was 90% or greater. There was no evidence for the presence of immunoglobulins or α2-macroglobulin in whole supernatant fluids. The suppressor was partially denatured at 80° C and was rendered completely inactive upon exposure to 100° C for 5 min. It was trypsin sensitive, and had an apparent molecular weight of 100,000 or greater. The protein adhered strongly to DE-52 cellulose, and the most active material eluted with 0.4-0.6 M NaCl. The suppressor was active in the pH range 5.0 ± 0.6 as demonstrated by isoelectric focusing.

Occasionally, supernatant fluids comprised material which augmented the expected response of con A stimulated PBL. The augmentor was 30,000 in molecular weight and was eluted from DE-52 cellulose in the 0.15-0.25 M NaCl range.

Nearly all supernatant preparations tested contained a mitogenic substance which stimulated naive allogeneic human PBL without the necessity of co-stimulation by a mitogen. The mitogenic factor (MF) behaved in a dose dependent fashion and was evidently different from the augmentor since the MF stimulated PBL independently of lectin co-stimulation.

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I also would like to thank Steve Hamm, Tom Seay and the other graduate students for making my graduate years memorable.

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ABBREVIATIONS

Con A	Concanavalin A
DEAE	diethyl-amino-ethyl
DNA	deoxyribonucleic acid
DNAase	deoxyribonuclease
DNCB	dinitrochlorobenzene
DPT	Diptheria-Pertusis-Typhoid
EAC	Erythrocyte-antibody-complement rosetting
HLMF	human lymphocyte mitogenic factor
IDS	inhibitor of DNA synthesis
IRSF	immune response suppressor factor
LAF	lymphocyte activating factor
LDL	low density lipoprotein
LPS	lipopolysaccharide
MAF	macrophage activating factor
MIF	migration inhibitory factor
MLC	mixed lymphocyte culture
NIP	normal immunosuppressive protein
PAS	periodic acid-Schiff's reagent
PBL	peripheral blood lymphocyte
PBS	phosphate buffered saline
Pg	prostaglandin
PHA	phytohemagglutinin
pI	isoelectric point
PIF	proliferation inhibitory factor
PPD	purified protein derivative of tuberculin

PWM	pokeweed mitogen
RNAase	ribonuclease
RPMI	Roswell-Park Memorial Institute
SISS	soluble immune suppressor substance
SSF	soluble suppressor factor
TCGF	T cell growth factor
TMED	N,N,N',N'-tetramethylethylenediamine
Tris	Tris (hydroxy methyl) amino methane

CHAPTER 1

INTRODUCTION

In recent times there has been exponential growth in the understanding of the mechanisms of the immune response. One aspect of immunity which has gained particular attention is the area of lymphokine (1) research.

Lymphokines are soluble mediators which can be generated with antigens or mitogens, either *in vivo* or *in vitro*. They are produced by normal thymus-derived T lymphocytes or bursal-equivalent lymphocytes and by transformed T cell lines. They function by suppressing or helping immune responses, and they may or may not possess antigenic determinants encoded by the major histocompatibility complex.

Lymphokines affect a wide variety of cell types *in vitro* including lymphocytes, macrophages, neutrophils, basophils, eosinophils, polymorphonuclear leukocytes, thymocytes, bone marrow cells, fibroblasts, lymphoid cell lines, osteoclasts, and endothelial cells. *In vivo*, the role of lymphokines remains unclear, but they are assumed to be involved in delayed type hypersensitivity, particularly in the reticuloendothelial system, in the circulation, and at mucosal sites.

Presently there are 90 such lymphokines which have been identified according to function and shown to have distinct molecular characteristics apart from immunoglobulins. All lymphokines appear to be proteins or glycoproteins of a molecular weight greater than 10,000. They behave similarly to glycoprotein hormones in that they both affect distal targets and are recognized by specific membrane receptors.

Lymphokines may function as monomeric subunits, polymerize to larger molecular weight forms, or non-specifically aggregate in the

presence or absence of serum components (115). They may also be transported by larger molecular weight proteins such as α2-macroglobulin (32).

Prior to the advent of monoclonal antibodies, it was difficult to develop strictly specific antibodies to lymphokines due to the lack of substantial amounts of purified material. However, hybridoma technology has been instrumental in producing antibodies specific for certain lymphokines because this technique requires only partially purified material. It is reasonable then to assume that further exploitation of hybridoma monoclonal antibodies will facilitate purification and biochemical characterization of the various lymphokines.

A comprehensive review of all the lymphokines is a somewhat daunting task and is beyond the scope of this dissertation. Instead, the reader is referred to other sources for further discussion of this expansive topic (2-6). This literature review will focus primarily on selected factors which are found in the human and pertinent to the work described herein.

The existence of mitogenic factor in humans was first noted by Kaskura and Lowenstein (7) in the supernatant fluids of mixed leukocyte cultures. Using peripheral blood from allogeneic donors, cells were mixed and incubated. The supernatant fluids were added to non-sensitized cells which then were examined for blastogenesis by their ability to incorporate radiolabelled thymidine. Since then, mitogenic factors have been redefined operationally as activities that appear in supernatant fluids of leukocyte cultures and cause lymphocytes to divide and/or synergistically augment cell division induced by other lymphocyte stimulants.

Mitogenic factors potentially may be produced by one or more

cell types in leukocyte cultures, e.g. lymphocytes, monocytes, and neutrophils. The present discussion will concern human lymphocyte mitogenic factor (HLMF) and T cell growth factor (TCGF), which are T cell products, and lymphocyte activating factor (LAF) which is a product of macrophages.

A number of investigators, using a variety of antigens and mitogens as stimulatory agents, have successfully implicated a sub-population of thymus-derived cells which, during activated conditions, produce human lymphocyte mitogenic factor (8, 9, 10). Peripheral lymphocytes from Mantoux positive individuals, were separated into T and B cell populations according to their banding patterns on sucrose density gradients (11). By adding purified protein derivative of Tuberculin (PPD) (the antigen in this case) to cultures of each of these cell types, it was shown that only the culture supernatant fluids from the T cell population (devoid of PPD) ellicited any blastogenic effect on non-sensitized target cells in a subsequent assay.

By far the most common antigen system used to stimulate secretion of HLMF *in vitro* is the mixed lymphocyte culture (MLC) (7, 12, 13, 14). This technique involves mixing allogeneic lymphocytes from two donors which then stimulate cells in each population to produce a variety of lymphokines (13). Treating one of the cell populations with mitomycin C prior to mixing, however, inhibits its DNA synthesis and permits only the untreated population to respond to the treated cells' surface antigens (12, 13, 14). This technique is known as the one way MLC and is helpful in reducing the number of lymphokine contaminants in the supernatant.

In pioneering studies, Chess et al. (9, 15, 16, 17) separated lymphocyte populations by immunoaffinity column chromatography and discovered that only T cells proliferated in response to cell surface antigens in an MLC and consequently produced HLMF (9).

Other evidence indicating T cell production of HLMF lies in the fact that patients with X-linked agammaglobulinemia show normal levels of HLMF (8). Since the disease is characterized by a deficiency in plasmacytes, it may be assumed the B cell plays a minor role in HLMF production. In addition, athymic mice exhibit little or no LMF activity while normal mice produce detectable quantities of the lymphokine (18).

Findings by Mackler and Altman (18) showed lymphocytes that spontaneously rosetted with sheep red cells were capable of inducing, via a soluble mediator, a mitogenic effect on autologous cells.

Bacterial extracts (19), and products (14) have also been used to induced HLMF synthesis. Other antigens which have been used for HLMF *in vivo* production studies include PPD (15, 20, 21, 22), dinitrochlorobenzene (DNCB) (8, 10, 23) and lymphocytes from donors sensitized to tetanus toxoid (8, 17, 23).

Earlier experiments designed to prove the existence of HLMF explored the use of individuals sensitive to PPD. Valentine and Lawrence (20) observed that (a) when leukocytes from a donor sensitive to both PPD and Diptheria-Pertussus-Typhoid (DPT) were incubated with PPD, the resultant supernatant material could transfer delayed hypersensitivity only for TB and not DPT, and (b) incubating cells with PPD and then adding the activated supernatant fluid to naive lymphocytes caused a 4-25 fold increase in radiolabelled thymidine uptake.

A prerequisite for HLMF production in an antigen system is that the producing cells must be obtained from positive individuals i.e. persons previously sensitized to the particular antigen used (13).

A large portion of the information on HLMF comes from studies of mitogen-stimulated lymphocytes; pokeweed mitogen (PWM) (15), phytohemagglutinin (PHA) (15, 18), and concanavalin A (con A) (15, 18, 24, 25, 26) have all been used with moderate success to generate HLMF.

The relative ease with which con A can be removed from culture fluids by absorption to cross-linked dextran (Sephadex G-50) makes the mitogen useful for stimulating lymphokine synthesis. The lectin, con A, is a metallo-protein (29) containing manganese and calcium ions. With a basic subunit of 23,000, con A forms dimers predominant at pH 5-6 and tetramers at alkaline pH in solutions of 1 M NaCl (30). The binding site of con A to lymphocyte cell membranes is specific for α-D-manopyranose, α-D-glucopyranose or sterically similar residues (31) and consequently is specifically inhibited by these sugars.

Reports concerning the partial purification of HLMF are difficult to compare because of the divergent conditions utilized for the production of this lymphokine. These differences raise the possibility that the various investigators are not examining the same factor and may account for some of the discrepancies in the biochemical parameters of this factor.

Valantine et al. (20) and Maini (21) reported that HLMF was heat stable at 56° C for 30 minutes, was not removed by centrifugation at 100,000 x g and could stimulate both autologous and allogeneic lymphocytes. Kashura (7) noted that there was no HLMF activity released from dead cells.

Using actinomycin D, an inhibitor of protein synthesis, it was shown (14) that HLMF was not produced in treated cells stimulated with mitogens. This indicated mitogenic factor was evidently endogenous. Treatment with trypsin or chymotrypsin resulted in loss of lymphokine activity while neuraminidase had no effect (8).

The molecular weight of HLMF is generally accepted to be around 25,000 (9, 25) as judged by Sephadex chromatography. Other investigators (8), however, have noted that HLMF activity was excluded by Sephadex G-200 indicating a molecular weight in excess of 100,000. This may be due to polymeric forms of HLMF or complexes of HLMF with a large carrier protein such as α2-macroglobulin (32).

The isoelectric point of HLMF is the only other molecular parameter measured thus far (19). Seravalli reported that the pI of Group A Streptococcal filtrate-stimulated HLMF was 8.0-8.5 as evidenced by the fraction's high mitogenic activity on target cells.

Recently, Northroff et al. (33) reported that incubating mononuclear cells i.e. T and B lymphocytes and monocytes, 2 to 5 days before the addition of con A dramatically increased the production of HLMF. The active material was eluted from a Sephadex G-150 column in two peaks at 12,000 to 27,000 and 50,000 to 70,000. The effect of preincubation was abolished by the addition of fresh mononuclear cells leading to the conclusion that suppressor T cells may regulate production of HLMF when stimulated with con A.

In addition to studies focusing on HLMF, mouse (28, 34, 38), chicken (39), and guinea pig (38, 40-49) mitogenic factors have been similarly investigated.

In the guinea pig, ovalbumin-induced MF had an apparent molecular

weight of 20,000, a D_{20W} of 9.2 ± 0.2 x 10^7 cm^2/sec, and S_{20W} of 2.4 ± 0.25, and a partial specific volume of 0.71. It was heat stable at 60° C for 20 minutes or at 56° C for one hour (47).

Geczy (43) reported that MLC-induced and soluble-antigen-induced MF were separate molecular entities in the guinea pig. The antigen-stimulated MF, possessing a molecular weight of 20,000-25,000 and an isoelectric point of 7.0-7.5, was unaffected by L-fucose whereas, the MLC-induced MF, weighing 15,000-18,000 with a pI of 6.0-6.5, was inhibited by L-fucose.

Antibodies prepared against MLC-MF inhibited the MLC reaction. This antibody did not affect the response of lymphocytes to PHA or PPD and had no suppressive effect on PPD-MF. Antibodies could not be absorbed out with lymphocytes; thus, the antibodies probably were directed against the MF rather than membrane antigens (43).

Soon after the observation that lymphocytes were capable of self renewal came the realization that purified T cells could be maintained in continuous exponential proliferative culture provided that conditioned medium from PHA-stimulated lymphocytes was present (50, 51).

This discovery was confirmed and expanded by Gillis et al (52) who concluded that a soluble T cell product was the active moiety responsible for this effect and suggested the name T cell growth factor (TCGF). More recent studies showed that the mitogenic stimulus was delivered to the cell by TCGF rather than the lectin or antigen. Furthermore, it was found that the presence of a macrophage-derived factor was required for the biological expression of TCGF (53).

This adherent cell product was termed lymphocyte activating factor (LAF). LAF alone is not mitogenic for T lymphocytes nor does it support continuous T cell proliferation. Instead, macrophages, when activated by antigens or mitogens, secrete LAF which in turn stimulates T cells to produce TCGF and augments the effect of the mitogen (54).

The biological assay for LAF relies heavily on using high densities of purified mouse thymocytes as target cells. In the presence of a suboptimal concentration of a T cell mitogen, i.e. PHA or con A, and LAF, the thymocytes exhibit a marked proliferative response that is dependent on LAF concentration (54).

Since LAF is a macrophage-derived product, mitogens specific for these cell types, particularly lipopolysaccharide (LPS), enhance its production (55). Other inducers include phagocytic stimuli such as phorbol myristate acetate, antigen-antibody complexes, and latex particles. LAF is also produced in mixed lymphocyte cultures which contain monocytes.

Although LAF may appear in an aggregated form, the apparent molecular weight of the monomer was determined to be 13,000-15,000 by gel filtration (56-59). That the molecule was protein was shown by its sensitivity to chymotrypsin.

LAF was apparently heterogeneous with respect to charge since more than one charged species was resolved by DEAE-ion-exchange chromatography and isoelectric focusing (58, 61). Lachman et al. (58) reported three isoelectric points at pH 5.2, 5.9, and 7.0, with the major component of activity at pH 7.0. The observed heterogeneity probably was not the result of variation in sugar moieties since LAF was unaffected by treatment with sodium periodate (60).

The most sensitive assay for TCGF is simply a ^{3}H-thymidine uptake experiment using T blast cells which are transformed with PHA (55). After transformation, these cells are no longer responsive to PHA. They are, however, sensitive to TCGF, and are used to detect its presence.

Other workers employ a co-stimulator assay in which the mitogen is added (at suboptimal concentrations) simultaneously with purported TCGF-containing supernatant fluids to PBL (62). This results in greater incorporation of the radiolabeled thymidine than with the mitogen alone.

TCGF was readily isolated from supernatant liquids of PHA-stimulated human lymphocytes by ammonium sulfate precipitation (63). The concentrate was then applied to a DEAE-Sepharose column and eluted with sodium chloride. The active fractions were pooled, concentrated by ultrafiltration and then subjected to molecular sieve chromatography using Ultragel AcA54. TCGF eluted from the anion-exchanger in 0.07 M NaCl and had an apparent molecular weight of 23,000. The active moiety was sensitive to trypsin digestion while deoxyubonuclease (DNAase) and ribonuclease (RNAase) had negligible effects.

It is apparent then that lymphokines in all likelihood perform an immunoregulatory function during an immune response. Indirect evidence for this phenomenon stems from investigations of soluble suppressor substances of immune origin.

Using supernatant fluids derived from transformed lines, Vesole et al. (64) noted that both mitogenic and suppressor activities were present in the same supernatant preparation but that separation of

the two molecules by gel filtration was required to detect mitogenic activity in the bioassay. This was apparently due to the masking effect of the suppressor. Similarly, it was suggested by Wolf et al. (65), and later confirmed by Warren et al. (66) that tonsil lymphocyte culture fluids contained both suppressive and T cell growth factor activity.

The remainder of this discussion will consider various suppressor factors and their roles in the immune response. A rather broad spectrum of molecules has been implicated as suppressors of cellular proliferation (DNA synthesis) of immunocompetent cells. These include prostaglandins, serum derived immunosuppressive factors, leukocyte extracts, complement-related factors, low density lipoproteins, α-1-acid glycoprotein, and lymphokines.

Prostaglandins, particularly the E_1 and E_2 series, were shown to inhibit the expected response of PHA-stimulated mononuclear cells (67). The suppression was noted in cultures containing glass-adherent cells and was ablated upon treatment with indomethacin, an inhibitor of prostaglandin synthetase, or by dialysis of the supernatant fluids.

Rice (66) reported a prostaglandin suppressor system which suppressed T lymphocyte proliferation. The factor was resistant to corticosteroids and did not depend on cellular division for its production. Baker et al. (69) showed that PGE_1, and PGE_2 reduced proliferation of differentiated cytotoxic T cells harvested from T cell growth factor-dependent cultures by 43%.

Human leukocyte extracts obtained by freeze-thaw lysis were growth inhibitory for a transformed B cell line and mitogen-treated

peripheral blood lymphocytes. The suppressor was dialyzable through 3500 molecular weight cut-off tubing and may have been similar to prostaglandins (70).

There have been several reports of serum components which inhibited proliferation of activated lymphocytes. Low density lipoprotein (LDL) extracted from human serum was shown to inhibit PHA- or poke weed mitogen (PWM)-stimulated PBL and PBL in mixed lymphocyte cultures (71). The LDL had an apparent molecular weight of 2-3 x 10^6 and contained 20-55% protein and 75-80% lipid. The molecule migrated electrophoretically in the β range and had a buoyant density of 1.055 g/ml in potassium bromide. There was no loss of viability, and the suppressive effect was evident irrespective of mitogen concentration.

Normal human plasma was shown to inhibit lymphocyte stimulation by PHA at suboptimal (>1 μg/ml) concentrations (72). The suppressor was non-dialyzable and stable when heated at 56° C for 30 min. It was further shown that the plasma bound directly to PHA since delay in adding the plasma to the PHA cultures reversed the effect.

Habict et al. (73) found that pooled human α-globulin fractions (Cohn IV and V) suppressed the plaque-forming-cell (B lymphocyte) response in mice. The material was partially sensitive to trypsin and contained lipid and/or carbohydrate moieties. The molecular weight was determined to be 10,000-50,000. Isoelectric focusing revealed four peaks of suppressive activity and three peaks of enhancing activity. This was confirmed by the dose response in which both activities were revealed in a single preparation.

Nelken et al. (74, 75) isolated normal immunosuppressive protein

(NIP) from the α-globulin fraction of serum of 24 hr whole blood. This glycoprotein was shown to inhibit T cell and B cell DNA synthesis, EL-4 tumor growth, and antibody-dependent cell-mediated cytotoxicity. The material was 10,000-25,000 in molecular weight and was resistant to boiling. The factor was not species specific, had no effect on fibroblasts, and acted by a non-cytotoxic mechanism.

Benson et al. (76) also identified a serum protein which suppressed a B cell response, the antibody response, to sheep red blood cells. The suppressor was labile at 56° C for 30 min and was absorbed by immune complexes. The factor was shown to be C_1 or C_4 complement components but not C_2, C_5, or C_8.

α-1-Acid glycoprotein (orosomucoid), a serum protein produced by the liver, was shown to be elevated during stress and pregnancy, concentrated around sites of inflammation, and found in individuals with neoplasia. Orosomucoid suppressed the response of mouse spleen cells to PHA, con A, LPS, the antibody response to sheep red blood cells, and cell-mediated cytotoxicity (77). The carbohydrate content of the protein was 45% of the weight of the intact molecule and was necessary for expression of biological activity.

The concentration of C-reactive protein (CRP) was found to be increased in the acute stages of inflammation, particularly in pleural effusions (78). CRP inhibited ^{3}H-thymidine incorporation by lymphocytes stimulated in mixed lymphocyte culture, diminished the response of PPD, and caused a decrease in the production of migration inhibitory factor (MIF). It was postulated that CRP may modulate the cell-mediated immune response and may contribute to anergy i.e. unresponsiveness to antigen insults associated with inflammation.

By far the most prevalent suppressors are proteins derived from

cells of the immune response. Larsson et al. (79, 80) demonstrated the existence of a factor produced by PHA-stimulated PBL which induced macrophages to become suppressive to PPD-stimulated PBL.

Green et al. (81) using PHA-stimulated lymphocytes found a factor which inhibited the growth of a variety of transformed cell lines. Proliferation inhibitory factor (PIF) was non-dialyzable, non-sedimentable at 90,000 x g for one hr, sensitive to trypsin, and stable at 85° C for 30 min. The suppressive effect behaved in a dose-dependent manner, caused no loss of viability of target cells, and was reversible when the target cells were removed from PIF-containing media before 24 hrs.

A factor produced by T cell-enriched lymphocytes stimulated with insoluble con A was described by Williams et al. (82). The factor suppressed the mixed lymphocyte culture response without loss of viability. The activity was sensitive to chymotrypsin treatment, non-dialyzable, partially labile at 56° C for 30 min, and sensitive to pH 2.0 after 24 hrs. Treatment with neuraminidase resulted in 15% loss of biological activity. It was probable that this factor was an α-lymphotoxin since antibodies directed against this family of lymphotoxins ablated the suppressive effect.

Other workers (83) have shown that treatment of PBL with con A resulted in the elaboration of a substance suppressive for the mixed lymphocyte culture and for cell-mediated lympholysis. It was also demonstrated that the suppressive effect was mediated by macrophages. It was non-cytotoxic, and did not require cellular division for the expression of activity.

Shou et al. (84) used PBL grown four days in culture without

serum or any stimulatory agent to produce a suppressor of con A-stimulated PBL and mixed lymphocyte cultures. The suppressive material was stable at 56° C for 30 minutes, partially denatured at 70° C for 30 minutes and destroyed at 80° C for 30 minutes. The active moiety was non-cytotoxic.

Two soluble immune suppressor substances (SISS) were identified in PBL cultures stimulated with PHA or con A (85, 86). SISS-T was shown to inhibit T cell mitogen and antigen responsiveness non-cytotoxically. The material was 30,000-45,000 d in size and was blocked i.e. activity by N-acetyl-D-glucosamine. SISS-B inhibited polyclonal B cell activation (i.e. immunoglobulin production) noncytotoxically. The suppressor was inhibited by L-rhamnose. The apparent molecular weight of SISS-B was 60,000-80,000 and was stable at pH 2.5. Both factors were heat labile at 56° C for 30 minutes.

Another soluble suppressor factor (SSF) produced by pokeweed mitogen-stimulated PBL was found by Hoffman et al. (87). The factor was dependent on the presence of the macrophage for production. SSF was shown to inhibit MLC, T cell cytotoxic activity, and the generation of killer cells. It was further noted that the suppressor had to be present at the initiation of culture for activity to be expressed.

An inhibitor of DNA synthesis (IDS) was suggested by Jegasothy et al. (88). IDS was produced by con A-stimulated PBL in 48 hrs and inhibited PHA stimulation of PBL. The factor was glycoprotein in nature with the requirement of intact carbohydrate moieties for biological activity. The suppressor appeared as a 20,000 d monomer, but it was also found as a dimer, trimer, and tetramer in solution.

IDS was sensitive to trypsin and periodate. It was heat stable at 60° C for 30 min, had an apparent pI of 3.4, and was non-cytotoxic for target cells.

Another soluble immune response suppressor factor (IRSF) produced by non-adherent, unstimulated PBL was reported (Lederman et al.). IRSF suppressed PHA, PWM, and con A stimulation of PBL. The activity was partially dialyzable, stable at 56° C for one hr, and its production was unaffected by indomethacin.

Mixed lymphocyte cultures also were shown to produce a soluble factor which suppressed cytotoxic lympholysis and mixed lymphocyte culture responses (90). The production of the active moiety was dependent upon the presence of antigen-sensitized lymphocytes and required restimulation of those primed lymphocytes in the primary MLC.

Tibbetts et al. (91) used a nitrogen bomb to lyse PBL and found that the cell extract comprised suppressive material which inhibited PHA stimulation of PBL noncytotoxically, mixed lymphocyte cultures, and growth of the transformed B cell line PGLC-33H. The suppressor was DNAase resistant, trypsin sensitive, stable at pH 5.4-8.4, and had an apparent molecular weight greater than 100,000.

By separating tonsil lymphocytes on an albumin gradient, Wolf et al. (65) demonstrated that a subpopulation of T lymphocytes stimulated with PHA could suppress PBL mitogen responsiveness. Furthermore, it was noted that the suppressive substance was produced constituitively in tonsil T cells. The factor eluted predominantly in the exclusion volume of Sephadex G-100 and was resistant to heating at 60° C for 15 min.

Several investigators used lymphoblastoid cells as a source of soluble inhibitors of mitogen-stimulated PBL. Green et al. (92) generated crowded lymphoblast culture supernatant fluids and subjected them to ion exchange chromatography. The suppressive fraction was non-dialyzable with an apparent molecular weight of 40,000-70,000. The material was stable at 56° C for 30 min but thermolabile at 80° C for 30 min. It was resistant to neuraminidase, and sensitive to pronase and to pH below 2.4. The suppression occurred without cytotoxic effects on target cells.

Hersch et al. (93) identified a suppressor which possessed the same characteristics as the factor described above. It was shown to be DNAase, RNAase, and trypsin resistant in addition to being pronase sensitive. Production of the inhibitor was blocked by puromycin, cyclohexamide, actinomycin D, arabinosyl-6-mercaptopurine, and arabinosyl cytosine. This indicated cellular proliferation and protein synthesis was required for its expression. It was postulated that crowding of the lymphoblasts resulted in contact inhibition by production of an inhibitor via protein synthesis.

Other investigators have found transformed cell culture supernatants to comprise suppressor molecules. Using human B cell lines (SWB-16, RPMI-1788, RPMI-6410) and one T cell line (CCRF-CEM), Vesole et al. (94) identified a soluble inhibitor of PHA-stimulated lymphocytes. The active moiety co-eluted with albumin on Sephadex G-100 and was reported to have two active isoelectricfocusing fractions at pH 2.99-3.55 and 4.35-4.55.

An inhibitor produced by transformed cell lines derived from non-lymphoid tissue was noted by Werkmeister (95). The soluble

material was shown to inhibit PHA, PWM, MLC responses as well as decrease immunoglobulin production in a dose dependent manner.

As documented by the preceding literature review, there are a myriad of mitogenic proteins and suppressor proteins which are produced by and/or affect lymphocytes. Until the advent of monoclonal antibodies specific for lymphocyte cell surface antigens, it was impossible to determine precisely the cell population responsible for producing the various lymphokine activities. This discovery coupled with fluorescent activated cell sorting has lead to the identification of at least two distinct T lymphocyte subpopulations, TH_4^+ and TH_4^-. It has been shown that the T helper subpopulation (TH_4^+) produces TCGF. Although there is no direct evidence available, it is presumed that the suppressor cell subpopulation (TH_4^-) produces soluble suppressor factor(s). Furthermore, it is known that tonsil lymphocytes contain the suppressor subpopulation in question and, therefore, it is probable that these cells produce a soluble factor capable of inhibiting the proliferative response of PBL to mitogens or antigens.

This report presents evidence which suggests that tonsil cell culture supernatant fluids comprise substances which are suppressive and stimulatory for allogeneic PBL.

OBJECTIVES

A major obstacle in lymphokine research is obtaining enough homogeneous material for biochemical analysis. Initially, it was thought that tonsil lymphocytes would provide a large and readily available source for human lymphocytes and that, when stimulated with mitogens, these cells would produce substantial quantities of HLMF. It was found, however, that tonsil cells were not only undergoing blastogenesis without *in vitro* stimulation, but that these cells, grown without serum or *in vitro* stimulatory agents, constituitively produced three discreet soluble factors which affected the proliferation of allogeneic PBL in different ways.

Since there were different activities present in the same supernatant fluids, was it possible that the factors were involved in regulating cellular proliferation of immunocompetent cells? In order to address this problem, it was necessary to obtain purified material of each active moiety for the purpose of biochemical characterization. This report describes our observations and presents biochemical data defining some molecular parameters of a suppressor factor, and two mitogenic factors.

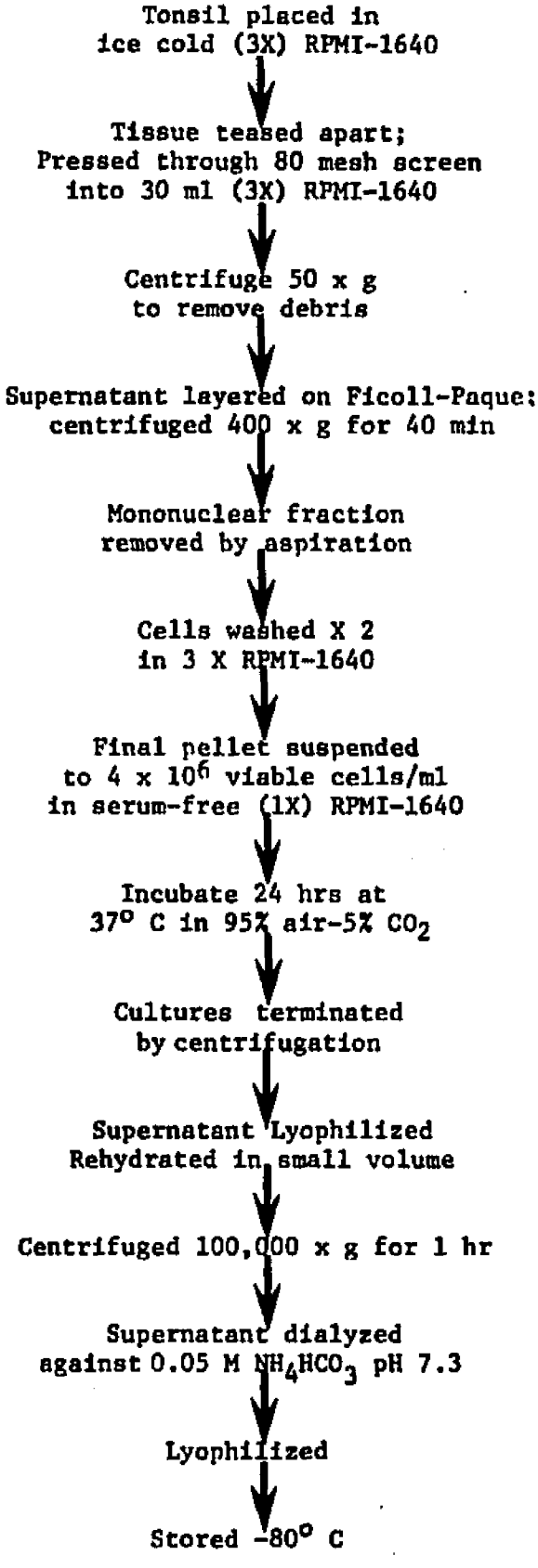
CHAPTER 2

MATERIALS AND METHODS

Preparation of Tonsil Cell Culture Supernatants. Palatine tonsils were removed from 3-16 yr old patients undergoing elective surgery for recurrent tonsillitis. The surgery was performed at least two weeks post-infection. The organs were placed immediately in ice cold RPMI-1640 medium (Flow Laboratories) supplemented to triple strength (3X) with penicillin (300 U/ml), streptomycin (300 µg/ml) (Grand Island Biological Co.), gentamycin 100 µg/ml) (Sigma Corp.), fungisome (2 µg/ml) (Grand Island Biological Co.), and HEPES (25 mM) (Fig. 1) for transport from the hospital to the laboratory. Within two hr, the tissue was sliced in the laboratory into small sections and gently pressed through a fixed 80-mesh screen (Cellector: E. C. Apparatus Co). The cells were collected in a petri dish containing 30 ml of supplemented RPMI-1640. After centrifugation at 50 x g for 10 min to remove the large debris, the mononuclear cells were separated by flotation on Ficoll-Paque (Pharmacia Fine Chemicals) and counted on a hemacytometer. Tonsil cell preparations with less than 85% viability, as determined by trypan blue exclusion (96), were not used. The cells were adjusted to yield a final concentration of 4-5 x 10^6 viable cells/ml in RPMI-1640 supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), and gentamycin (40 µg/ml) (1X). These primary cultures were incubated in Corning 150 cm^2 tissue culture flasks (Corning Glass Works) for 24 hr in 95% air-5% CO_2 at 37° C.

Culture samples were examined for the presence of bacteria by the gram stain, and by growth on blood agar plates or in thioglycollate solution. Sabouraud's agar plates were used to test for the presence of yeasts. Contaminated cultures, which were rare, were discarded.

Figure 1. Scheme for preparation of tonsil cell supernatant cultures.

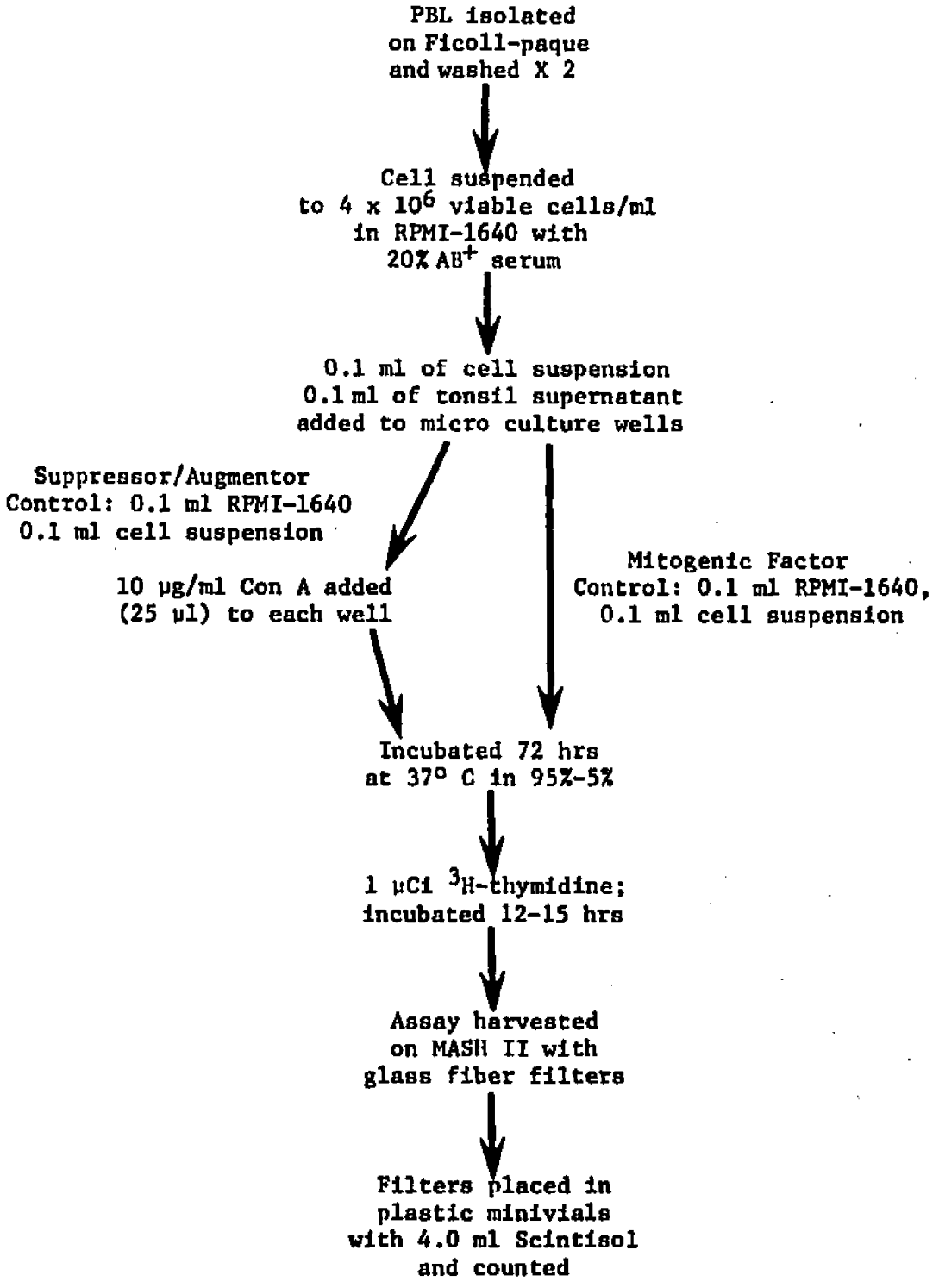

Tonsil placed in
ice cold (3X) RPMI-1640
Tissue teased apart;
Pressed through 80 mesh screen
into 30 ml (3X) RPMI-1640
Centrifuge 50 x g
to remove debris
Supernatant layered on Ficoll-Paque;
centrifuged 400 x g for 40 min
Mononuclear fraction
removed by aspiration
Cells washed X 2
in 3 X RPMI-1640
Final pellet suspended
to 4 x 10^6 viable cells/ml
in serum-free (1X) RPMI-1640
Incubate 24 hrs at
37° C in 95% air-5% CO2
Cultures terminated
by centrifugation
Supernatant Lyophilized
Rehydrated in small volume
Centrifuged 100,000 x g for 1 hr
Supernatant dialyzed
against 0.05 M NH4HCO3 pH 7.3
Lyophilized
Stored -80° C

The cultures were terminated by centrifugation at 400 x g for 10 min. The supernatant fluids were then concentrated by lyophilization and centrifuged at 100,000 x g for 1 hr at 10° C in a 35 TI (Sorvall) rotor to remove insoluble matter. These supernatants were frozen, or dialyzed against 0.05 M NH_4HCO_3 solution, pH 7.3, and lyophilized in both instances, the samples were stored at -85° C. Prior to testing for activity in the bioassay, lyophilized supernatants were rehydrated in 5.0 ml of phosphate-buffered saline (PBS: 0.15 M NaCl, 0.0076 M Na_2HPO_4, 0.0024 M NaH_2PO_4; pH 7.2) and dialyzed against the same buffer to remove residual ammonia. They were finally dialyzed in supplemented (1X) RPMI-1640.

Bioassays. Blood was obtained in heparinized (10 U/ml) syringes by phlebotomy of normal adult humans. The lymphocytes were prepared by flotation on Ficoll-Paque (Fig. 2). The mononuclear cell layer was removed by aspiration, and the cells were washed three times with Hank's balanced salt solution. Recovery of washed cells was accomplished by centrifugation at 400 x g for 10 min. The washed cells were suspended in a small volume of RPMI-1640, and viability was determined by trypan blue exclusion.

The PBL were adjusted to 2 x 10^6 viable cells/ml in supplemented RPMI-1640 made 20% with human AB^+ serum (Flow Laboratories). Assays were done in sextuplet (unless otherwise noted) in flat-bottomed 96-well Falcon Micro Test II sterile tissue culture plates (Becton-Dickenson and Co). Each well received 0.1 of the cell suspension, and 0.1 ml of tonsil cell culture supernatant which had been dialyzed against supplemented RPMI-1640 and filter-sterilized with a 0.22 μm

Figure 2. Suppressor/augmentor and mitogenic factor bioassay protocols.


PBL isolated
on Ficoll-paque
and washed X 2
Cell suspended
to 4 x 10^6 viable cells/ml
in RPMI-1640 with
20% AB^+ serum
0.1 ml of cell suspension
0.1 ml of tonsil supernatant
added to micro culture wells
Suppressor/Augmentor
Control: 0.1 ml RPMI-1640
0.1 ml cell suspension
10 µg/ml Con A added
(25 µl) to each well
Mitogenic Factor
Control: 0.1 ml RPMI-1640,
0.1 ml cell suspension
Incubated 72 hrs
at 37° C in 95%-5%
1 µCi ^3H-thymidine;
incubated 12-15 hrs
Assay harvested
on MASH II with
glass fiber filters
Filters placed in
plastic minivials
with 4.0 ml Scintisol
and counted

filter (Gelman Sciences) before being used in the bioassay.

Suppression and augmentation were determined by the ability of the tonsil cell supernatants to reduce or augment the incorporation of ^{3}H-thymidine by the PBL upon addition of con A to the wells. Con A (Miles-Yeda) was suspended in a solution of 1 M NaCl and $10^{-3}M$ $MgCl_2$, $CaCl_2$, and $MnCl_2$. The concentration of the con A in the stock solution was 750-800 µg/ml as measured by its optical density ($E^{1\%}_{280\,nm} = 13$). The stock was diluted to 80 µg/ml with supplemented RPMI and 25 µl were added to each well for a final concentration of 10 µg/ml. The plates were incubated three days at 37° C in 95% air-5% CO_2 before the addition of 1 µCi (6 Ci/mMole). The cultures were incubated another 12 hr, and then harvested with a MASH II (Microbiological Associates). The glass fiber filters (Whatman 934 AH or GF-C) were dried, placed in plastic mini-vials containing 4.0 ml of Scintisol (Isolab), and radioactivity was determined in a Beckman 3155T Scintillation Counter pre-set at 1.0% error. Background counts per minute with vials containing Scintisol were 25 ± 5. The controls were cells cultured with medium made 20% in AB^{+} serum, with and without con A, in the absence of supernatant fluid. Counts per minute and standard deviations (n = 6) were recorded.

After determining radioactivity, suppression was calculated using the formula:

$$\% \text{ Suppression} = \left(1 - \frac{\text{cpm suppression supernatant}}{\text{cpm con A-stimulated control}}\right) \times 100$$

Target cell viability was examined by the trypan blue exclusion method (96). In some cases, trypan blue was added directly to the

microculture wells just prior to termination of the bioassay.

Augmentation was calculated using the formula:

$$\% \text{ Augmentation} = \left(\frac{\text{cpm augmentation supernatant}}{\text{cpm con A-stimulated control}} - 1 \right) \times 100$$

In experiments measuring mitogenic activity, the same assay was employed but con A was absent from the wells. The mitogenic index (M.I.) was then calculated by the formula:

$$\text{M. I.} = \frac{\text{cpm of tonsil supernatant}}{\text{cpm of } AB^{+} \text{ serum control}}$$

Primary Culture Blastogenesis. PBL and tonsil lymphocytes were tested for their ability to incorporate ^{3}H-thymidine in the absence of *in vitro* stimulation. Lymphocytes isolated by flotation on Ficoll-Paque were suspended in supplemented (1X) RPMI-1640. Concentrations of 4×10^{6} cells/ml were used. Aliquots of 0.2 ml of the cell suspension were added to flat bottom microculture plates. The cells were incubated for 24 or 48 hr at 37° C in 95% air-5% CO_2. Twelve hr prior to harvesting the assay 1 µCi of ^{3}H-thymidine (6 Ci/mmole) was added. The assays were done in sextuplet. The cells were harvested and the incorporation of radioactivity was done as described earlier. Protein was determined by the modified Lowry method (97).

Dose Response. Lyophilized tonsil lymphocyte supernatant fluids were rehydrated with PBS to give a 16-fold concentration of the original supernatant volume. Serial dilutions were made in supplemented RPMI-1640 which then were assayed for suppression as described.

Supernatants which had augmenting and mitogenic activity were rehydrated in PBS to a concentration of 60-fold. Serial dilutions

were made in supplemented RPMI-1640 and then assayed. Protein was determined using a modified Lowry method (97).

Statistical Analysis. To determine the statistical significance of suppression or augmentation, the difference between means of test supernatant and control supernatant ± 95% confidence limits about the difference was calculated. The confidence limits were constructed using Dunnett's Q' (98) statistic and the standard error of the difference used to establish the limits was computed from the error mean square of a one-way analysis of variance (ANOVA).

The fit of dose response data for mitogenicity and augmentation to a straight line was estimated by least square linear regression.

When individual comparisons were made between experimental and control values, Dunnett's test was used (PL.05).

Trypsin Sensitivity. Immobilized trypsin (1238 U/gi Millipore Corporation) was washed repeatedly in several volumes of 0.1 M Tris-HCl solution, pH 8.0. Concentrated supernatants with biological activity were dialyzed against the same buffer. A small aliquot of each dialyzed supernatant was reserved to serve as control, and the remainder was treated with 50 µg of the washed enzyme for 1 hr at room temperature. The reaction was terminated by the addition of 10 µl of diisopropylphosphofluoridate (DFP) (.054 M). Each sample then was centrifuged at 100 x g to remove the immobilized trypsin and the supernatants were dialyzed against PBS to remove unreacted DFP.

Gel Filtration Chromatography. Concentrated crude suppressor-active supernatant fluids were fractionated using columns (1.5 x 90 cm) of Sephadex G-100 (Pharmacia Fine Chemicals). The gel

matrix was equilibrated with NH_4HCO_3 (0.05 M, pH 7.3). Aliquots of 100 µg of 60 X concentrated supernatant material were weighed out and rehydrated in 2 ml of PBS, dialyzed against the ammonium bicarbonate buffer and applied to the column. Fractions of 2 ml were collected and their protein content determined by OD_{280}. The fractions were pooled as shown in Fig. 5, lyophilized, rehydrated in PBS and dialyzed against supplemented RPMI-1640 in preparation for bioassay. The column was calibrated with the following molecular weight standards: bovine serum albumin (mol wt 68,000), carbonic anhydrase (mol wt 29,500) and myoglobin (mol wt 17,200). Blue dextran (0.1%) was used to determine the exclusion volume.

Isoelectric Focusing. Preparative isoelectric focusing was done using Ampholytes 3-10 (LKB) or Pharmalytes 3-10, 4-7, and 5-8 (Pharmacia Fine Chemicals) with Ultradex (LKB) serving as the support medium.

Supernatant fluids exhibiting suppressive activity in the bioassay were concentrated by lyophilization, rehydrated in PBS to a final concentration of 60 X and then dialyzed against 1% glycine solution pH 7.2. After dialysis the experimental sample was mixed with the gel slurry. Plates 3 mm in depth were prepared and allowed to dry to the evaporation limit at room temperature. The gels were then focused at 10° C using an LKB multiphor apparatus. Electrophoresis was at 8 watts constant power until no further drop in milliamperage could be detected. A control gel incorporating an identical mixture of Pharmalytes and Ultradex but containing no protein was treated under the same conditions.

The pH gradient was determined using an LKB Multiphore electrode.

The gel bed then was cut into 12 equal sized sections. Each was placed in a plastic funnel and eluted with 3-5 ml of deionized water. To remove residual Pharmalytes, the eluates were dialyzed against PBS. Polyethylene glycol 6000 (Eastman Kodak Co) was included in the dialysis buffer to help stabilize protein. The protein content of each fraction was determined by OD_{280} on a Carey 219 Spectrophotometer (Varian) or by a modified Lowry technique (97). Nucleic acid was measured by OD_{260}. The eluates finally were dialyzed against supplemented RPMI-1640 and subjected to the bioassay.

Ion-Exchange Chromatography. Anion exchange resin DE-52 (Whatman) was equilibrated in tris-HCl buffer (40 mM, pH 7.2; start buffer) and columns 0.9 x 15 cm (packed gel) were prepared with the pressure head set at 30 cm. Aliquots of 1.0 ml (200 µg) of concentrated supernatants having suppressive or augmenting activity were dialyzed against the starting buffer and then layered on the gel. The column was washed with two volumes (20 ml) of starting buffer before beginning the linear gradient of 1 M NaCl. The elution rate was 20 $ml/cm^2/hr$.

Conductivity of the 2-ml fractions was determined using a conductivity meter (Radiometer CDM 2) and each fraction was examined for total protein OD_{220} or nucleic acid by OD_{260}. A small aliquot of the crude preparation was used for comparison purposes. Fractions were pooled as indicated in RESULTS, dialyzed against PBS, and lyophilized. The dried material was rehydrated with deionized H_2O in a small volume and dialyzed first against PBS, to remove salt and then against supplemented RPMI-1640 in preparation for bioassay.

To determine specific activity of the suppressive material, several supernatants were separated by anion exchange chromatography.

Preliminary data indicated the 0.4 M to 0.6 M fraction to contain suppressive material; therefore, this fraction was used in the bioassay. An aliquot of the crude preparation was tested simultaneously with the partially purified material.

Protein was measured by OD_{280}. Specific activity (S. A.) was then calculated by:

$$\text{S. A.} = \frac{\text{\% Suppression}}{\text{O.D.}_{280}\text{ units}}$$

Immunoelectrophoresis. Commercially prepared agarose immunoelectrophoresis plates equilibrated with sodium barbitol (pH 8.6, ionic strength 0.05) were used to examine culture supernatant for the presence of human immunoglobulins and α2-macroglobulin. Tonsil cell supernatants that demonstrated a suppressive effect in the bioassay were lyophilized and rehydrated in PBS to give a 120-fold concentration with respect to the original supernatant volume. A small aliquot was dialyzed against the barbitol buffer just prior to use. Volumes of 50, 100, 150, or 200 µg were added to the wells and electrophoresis was carried out using a Corning 700 electrophoresis Power Unit for 30 min. Pooled human AB^+ serum served as a control.

Pooled goat antisera to human IgG, IgA, IgE, IgM, and commercially prepared (Cappel Laboratories) α2-macroglobulin antisera were added to appropriate troughs and allowed to diffuse overnight. Residual antibody was removed by soaking the plate in PBS. The plates were stained with thiazine red (1%), destained in acetic acid (5%), and photographed.

Temperature Sensitivity. Suppressor-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 0.5 ml of concentrated (60 X) material rehydrated in PBS were placed in 1-ml pyrex test tubes with stoppers. Each sample was placed in water bath set at the appropriate temperature. After the sample had reached the appropriate temperature, timing was begun. The samples were removed, dialyzed against supplemented RPMI-1640 and assayed for suppressor activity.

Polyacrylamide Gel Electrophoresis. Standard alkaline gel electrophoresis was done as described by Brewer et al. (99). Acrylamide (Bio-Rad Laboratories) gels 6.5 mm containing 7.5% TEMED-polymerized acrylamide were cast in 0.7 x 8 mm glass tubes. A solution of 3.75% acrylamide was layered on top and photopolymerized with riboflavin (Bio-Rad Laboratories) to form stacking gels.

Aliquots of 50-100 µl of the 60 X concentrated crude suppressor preparation or the 0.5 M-0.6 M anion exchange fractions were dialyzed against the electrophoresis buffer (tris, 5 mM; glycine, 38 mM; pH 8.9) and applied to the gels with an Adams suction apparatus (VWR Scientific, Inc). Sucrose was included in each sample to final concentration of 10%. Human serum albumin (100 µl of 500 µg/ml), pooled AB^+ serum (10 µl of 100%) and ovalbumin (100 µl of 500 µg/ml) were used as controls.

The gels were electrophoresed in tris-glycine buffer at 1.0 mA per tube until the dye exited the stacking gel. Then the current was increased to 1.5 mA for the remainder of the electrophoresis.

Gels were stained for protein by one of the following methods: (1) Formaldehyde-Coomassie Blue (100). Proteins were fixed and stained

for 3 hrs in 700 ml of an aqueous solution of ethanol (24% *v/v*) and formaldehyde (5% *v/v*) containing 0.8 gm of Coomassie Brilliant blue R-250 (Eastman Kodak Co). Destaining was affected with an aqueous solution of ethanol (77% *v/v*) and formaldehyde (1% *v/v*) overnight in a gel electrophoresis diffusion destainer (Bio-Rad Laboratories). (2) Periodic acid-Schiff reagent (PAS). Glycoproteins were sought by a PAS technique (101). The protein was fixed in 12.5% trichloroacetic acid solution for 1 hr, and oxidized with 1% periodic acid solution for 2 hr. The gels were washed in 15% acetic acid for 2 hr and then reacted with Schiffs Reagent (Fisher Scientific) for 2 hr at 4° C in the dark. Destaining was with 15% acetic acid. Ovalbumin served as a control for the PAS method.

Stained gels were photographed with a Minolta X D 35 mm camera (Minolta Corp) containing Plus X Pan 125 black and white film (Eastman Kodak, Co). A light orange filter (Tiffen Inc) was placed over the lens to help reproduce the lighter staining bands.

Chromatofocusing. Suppressor active supernatants were fractionated by chromatofocusing. The concentrated crude preparation was dialyzed against a start buffer of 0.025 MM imidazole-HCl, pH 7.4 overnight at 4° C.

Chromatographic columns (9 mm x 15 cm) were fully packed with PBE-94 (Pharmacia Fine Chemicals) and the gel beds equilibrated with several column volumes of start buffer until the eluted buffer registered pH 7.4.

Polybuffer 74 (Pharmacia Fine Chemicals), was titrated to pH 4.0 with 0.5 N HCl, diluted (1:8) to the appropriate volume, and the pH readjusted to 4.0. Five ml of the eluent buffer was run onto the

column and then 0.5 ml of the dialyzed supernatant was applied. The elution was continued with the polybuffer using a pressure head of 30 cm.

Fractions of 2.0 ml were collected until no further drop in the pH could be detected. The fractions then were combined into six pools of equal volume. The pools were placed in dialysis tubing with a 14,000 molecular weight exclusion limit (Spectraphor) and dialyzed against two, four liter volumes of 0.05 M NH_4HCO_3, pH 7.2 overnight at 4^{o} C. The material was lyophilized, rehydrated with PBS to 0.5 ml, dialyzed against PBS and then supplemented (1X) RPMI-1640 before bioassay.

In one experiment, the pools were concentrated by Amicon filtration in a step wise fashion. The pools were first concentrated on a YM 50 membrane (Amicon Corp) followed by reconcentration of the eluate on a YM 10 membrane. The retentates of each filtration were washed repeatedly with PBS until the pH of the filtrate was equivalent to the buffer. Both the 50,000 and 10,000 retentate fractions of each pool were assayed following dialysis against (1X) RPMI-1640.

To determine the effects of non-dialyzable polybuffer constituents on the bioassay, a small aliquot of Polybuffer 74 was titrated to pH 4.0 and diluted (1:8) to the appropriate volume. The buffer solution was dialyzed as described above and assayed.

Separation of Adherent and Non-adherent Cells. To determine the cell population responsible for producing the augmentor, tonsil cells were separated into plastic-non-adherent and plastic-adherent populations in the following manner. Tonsil cultures were incubated for 2 hr in plastic tissue flasks containing supplemented RPMI-1640 (1X)

under normal conditions. The non-adherent cell population was then decanted into another flask and incubated for an additional 22 hr. The adherent cells were reconstituted in fresh RPMI-1640 and incubated for 22 hr. The control cultures were treated in the usual manner.

CHAPTER 3

RESULTS

The Proliferative Activity of the Tonsil Cells. As shown in Table 1, the amount of radioactivity incorporated by PBL in culture for 24 hr or 48 hr was low, and typical of what was routinely accepted as "background" level for unstimulated PBL. Conversely, some tonsil lymphocytes showed greater than a tenfold higher incorporation of ^{3}H-thymidine compared to that taken up by PBL at 24 hr (Table 1). In addition, a significant decrease in actual cpm was observed at 48 hr. The proliferative activity in a 24 hr period of tonsil lymphocytes from among the many tonsils studied varied widely, but was usually greater than that of PBL. The number of proliferating cells in the population was not determined.

Effect of Tonsil Cell Culture Fluids on the Con A-Induced Proliferation of Normal PBL. As demonstrated above the tonsil cell population was in an activated state when compared to PBL. It was also found that supernatant culture fluids generated in the absence of *in vitro* stimulation of the tonsil cells often inhibited the proliferative response of PBL to con A.

Tonsil cells were cultured in the absence of serum for 24 hr. After recovery of the supernatant fluids, they were centrifuged at 100,000 x g, to remove insoluble particulate matter, and then tested for their effect on the incorporation of ^{3}H-thymidine by PBL cultured with con A. The data are presented in Table 2. Of 29 different tonsil cell supernatants assayed, 24 significantly suppressed the incorporation of ^{3}H-thymidine by PBL cultured with con A. The extent of suppression ranged from approximately 22% to 84% with the average being 43%. Five of the tonsil fluids had little or no effect on the

TABLE 1

Unstimulated Proliferative Activity *in vitro*

of PBL and Tonsil Cells

Sample Number	Incorporation of ^{3}H-thymidine[a] 24 hr	48 hr
	PBL[b]	
1	4,574 ± 714	1,350 ± 524
2	1,177 ± 130	1,235 ± 88
3	1,279 ± 284	1,350 ± 1,314
4	1,156 ± 371	1,463 ± 240
5	1,264 ± 427	
	Tonsil Cells[b]	
1	13,940 ± 1,184	7,340 ± 604
2	7,631 ± 494	4,330 ± 286
3	12,093 ± 1,227	10,545 ± 780
4	14,705 ± 1,196	
5	11,399 ± 1,639	
6	12,253 ± 1,272	
7	18,085 ± 2,481	
8	9,265 ± 2,230	
9	11,187 ± 1,640	
10	9,964 ± 1,010	
11	5,262 ± 535	
12	21,596 ± 1,991	
13	9,064 ± 642	
14	9,383 ± 483	

TABLE 1

Unstimulated Proliferative Activity *in vitro*

of PBL and Tonsil Cells (Cont)

Sample Number	Incorporation of ^{3}H-thymidine[a] 24 hr	48 hr
	Tonsil Cells[b]	
15	4,516 ± 411	
16	2,445 ± 390	
17	5,120 ± 759	
18	3,029 ± 668	
19	9,288 ± 1,415	
20	10,597 ± 1,100	
21	1,937 ± 368	
22	2,717 ± 297	
23	5,714 ± 960	
24	3,656 ± 450	

[a]Cells were cultured in microculture trays without stimulation for 24 hr or 48 hr. Twelve hr prior to harvest, 1 μCi ^{3}H-thymidine was added to the wells.

[b]Mean cpm ± S.D. n = 6.

TABLE 2

Effect of Tonsil Cell Culture Fluids on Transformation of Normal PBL Stimulated with Con A[a]

Experiment Number	Sample Number[b]	CPM[d]	% Suppression[e]	Δ Mean[f]	Signif.[g]
1	Con A[c]	107,021 ± 12,022			
	1	74,023 ± 22,846	31	-32,988 ± 18,806	+
	2	99,405 ± 7,327	7	- 7,616 ± 18,806	-
	3	95,483 ± 14,103	11	-11,538 ± 18,806	-
	4	95,803 ± 8,818	12	-11,218 ± 18,806	-
	5	95,128 ± 9,405	11	-11,893 ± 18,806	-
2	Con A	79,793 ± 14,494			
	6	61,909 ± 6,940	23	-17,884 ± 20,098	-
3	Con A	54,660 ± 7,515			
	7	35,578 ± 4,875	45	-19,082 ± 8,108	+
	8	41,441 ± 2,901	24	-13,219 ± 7,606	+
	9	11,394 ± 858	79	-43,266 ± 8,108	+
	10	8,495 ± 1,397	84	-46,165 ± 8,108	+
4	Con A	100,067 ± 6,745			
	11	60,765 ± 8,570	39	-39,302 ± 14,712	+
5	Con A	88,953 ± 1,631			
	12	34,251 ± 3,484	61	-54,702 ± 6,589	+
	13	52,713 ± 4,370	40	-36,240 ± 6,589	+

TABLE 2

Effect of Tonsil Cell Culture Fluids on Transformation of Normal PBL Stimulated with Con A[a] (Cont)

Experiment Number	Sample Number[b]	CPM[d]	Suppression[e]	Δ Mean[f]	Signif.[g]
6	Con A[c]	72,382 ± 3,982			
	14	39,783 ± 2,971	45	-32,599 ± 6,735	+
	15	41,412 ± 4,531	42	-30,970 ± 6,735	+
	16	43,049 ± 6,523	40	-29,333 ± 6,735	+
	17	47,665 ± 4,406	34	-24,717 ± 6,735	+
7	Con A	47,249 ± 21,180			
	18	22,802 ± 2,855	52	-24,447 ± 5,169	+
	18	29,108 ± 3,517	38	-18,141 ± 5,421	+
8	Con A	132,621 ± 16,834			
	20	48,084 ± 11,442	64	-84,537 ± 24,745	+
	21	39,236 ± 6,200	70	-93,385 ± 24,745	+
	22	83,574 ± 15,289	37	-49,047 ± 24,745	+
	23	89,868 ± 14,854	32	-42,753 ± 24,745	+
	24	104,069 ± 18,105	22	-28,552 ± 24,745	+
	25	71,734 ± 5,267	46	-60,887 ± 24,745	+
	26	103,933 ± 9,847	22	-28,688 ± 24,745	+
	27	92,531 ± 17,785	30	-40,090 ± 24,745	+
9	Con A	64,118 ± 13,646			
	28	48,828 ± 5,694	24	-25,289 ± 11,936	+
	29	46,195 ± 6,381	28	-17,923 ± 11,936	+

TABLE 2

Effect of Tonsil Cell Culture Fluids on Transformation of Normal PBL Stimulated with Con A[a] (Cont)

[a]Tonsil cells were cultured in the absence of stimulation. The supernatants were assayed by their effect on PBL cultured in the presence of con A.

[b]Numerals refer to individual patients' tonsils.

[c]Con A refers to radioactivity incorporated by PBL stimulated with con A in the absence of tonsil cell culture fluid.

[d]Mean cpm ± S. D. n = 6.

[e]Percent suppression of radioactivity incorporated by PBL stimulated with con A in the presence of tonsil cell culture fluid compared to that incorporated in the absence of tonsil cell culture fluids as described in text.

[f]The difference between means of test supernatant and control ± 95% confidence limits about the difference.

[g]The error used to calculate limits was from analysis of variance. (+) indicates significant suppression.

PBL target cells. In all experiments target cell viability was 90% or greater, regardless of the suppressive effect of the tonsil cell supernatant.

Suppressor supernatant fluids were examined for protein according to Lowry (97) and for primary culture blastogenesis (Table 3). From this, one might predict that an increase in total protein would be accompanied by an increase in the primary culture cpm. However, when the measured parameters of supernatant fluids 9 and 10 were compared it was evident that, although they comprised approximately equal amounts of protein (Table 3), there was considerable difference in the degree of non-induced blastogenesis. Similarly, one would expect a concomitant increase in total protein and suppressor activity or in the amount of blastogenesis in the primary culture and suppressor activity. This, however, was also disputed by the data. It was concluded, that there was no apparent relationship between total protein in the crude supernatant preparation and blastogenesis nor between protein and blastogenesis and suppressor activity.

Absence of Immunoglobulins and α2-Macroglobulin in Suppressor Supernatants. The tonsil cell supernatants were tested by immunoelectrophoresis for immunoglobulins (Fig. 3). There was no evidence for the presence of immunoglobulins G, A, M, E or α2-macroglobulin in suppressive supernatants. The visible patterns of the control indicated the antiserum used was reactive towards the AB^+ serum antigens.

Dose Response of Suppressor-Active Supernatant. The suppressive effect of various concentrations (8 - 1 mg) of supernatant fluid is illustrated in Fig. 4. The amount of suppression increased as the concentration of the supernatant was raised. In several experiments of this kind,

TABLE 3

Comparison of Percent Suppression with Total Protein and Non-Induced Blastogenesis of Primary Culture

Primary Culture Parameters				Bioassay of Primary Culture
Supernatant Number	Protein Concentration (µg/ml)[a]	Volume (ml)[b]	Blastogenesis (cpm)[c]	Percent Suppression (cpm)[d]
1	50	50	4,516 ± 411	11
2	90	250	1,937 ± 368	23
3	100	550	9,383 ± 483	12
4	115	150	9,064 ± 642	11
5	130	300	21,596 ± 1,997	7
6	150	167	29,285 ± 1,951	45
7	265	550	14,985 ± 1,755	36
8	300	550	7,967 ± 843	83
9	330	300	5,262 ± 535	31
10	330	600	7,373 ± 1,188	40
11	340	600	7,193 ± 871	44
12	380	200	9,993 ± 499	30
13	400	200	9,522 ± 466	18
14	420	350	11,629 ± 227	87
15	580	1,000	12,430 ± 1,433	64

TABLE 3

Comparison of Percent Suppression with Total Protein and Non-Induced Blastogenesis of Primary Culture (Cont)

---

[a]Protein concentration (µg/ml) in primary culture supernatant preparation as determined by Lowry technique (97).

[b]Volume of original supernatant fluid (ml).

[c]cpm ± S. D. (n = 6) of non-induced blastogenesis of primary culture.

[d]Percent suppression in bioassay of primary supernatant culture fluids.

Figure 3. Immunoelectrophoresis of suppressor active supernatant preparations. Aligots of 5, 10, 15, or 20 µl of pooled human AB^+ serum (A, C, E, G) or 100, 150, 200 µg of concentrated crude suppressor supernatant fluids (B, D, F) were added to the wells. Pooled goat antisera to human IgG, A, M, E, and α2-macroglobulin were added to each troughs. The plate was developed as described in text.

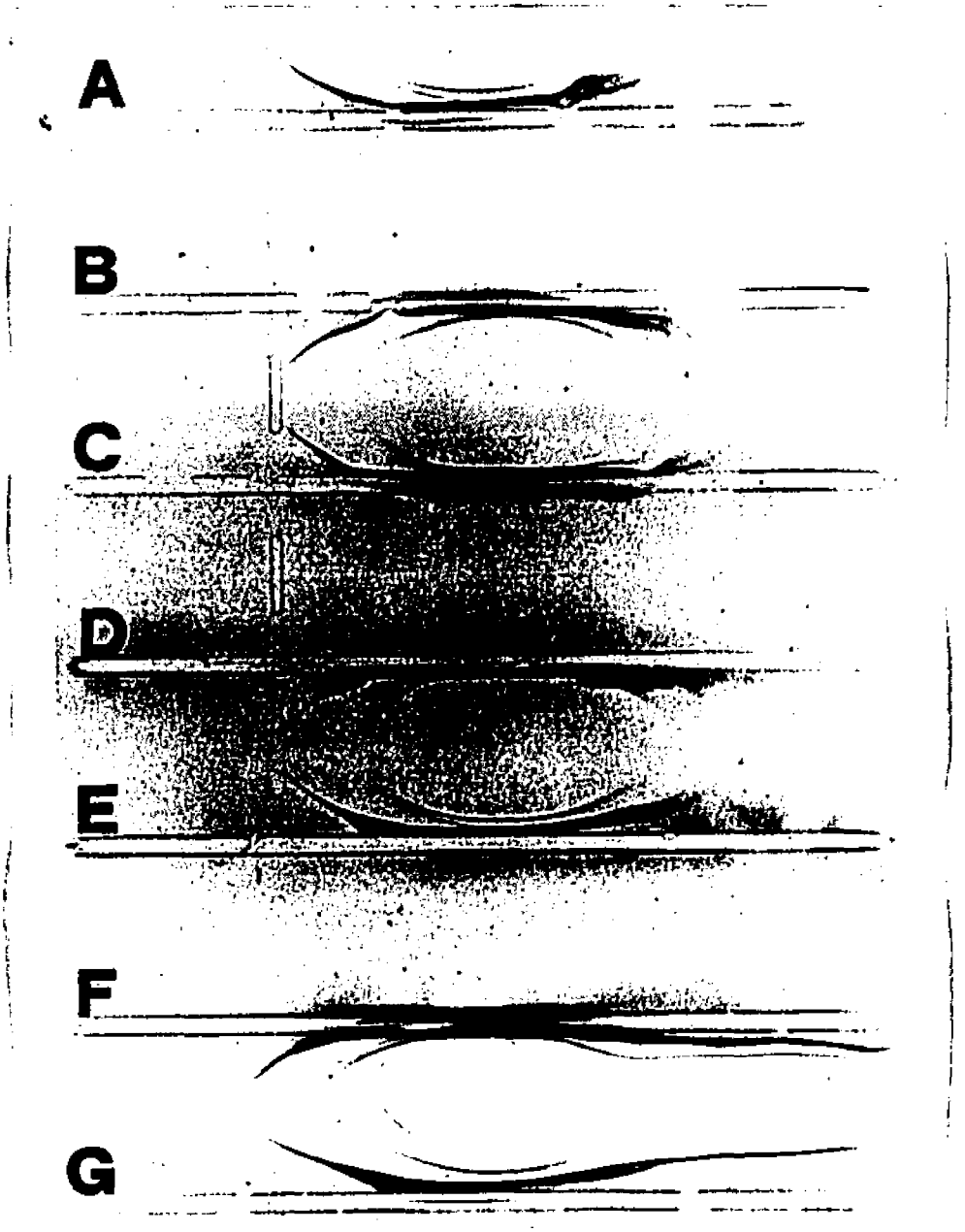
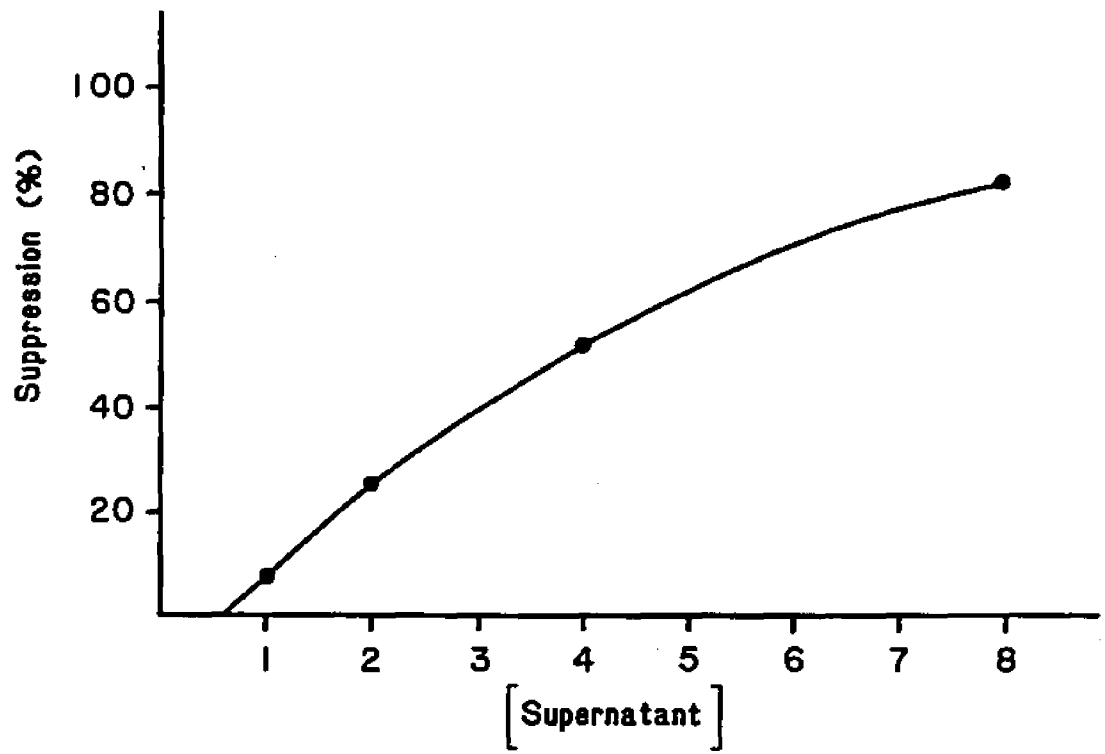

A
B
C
D
E
F
G

Figure 4. Suppression of con-A activated PBL by different concentrations of tonsil cell supernatant. Concentrated crude preparations were serially diluted in RPMI 1640 (8 = 1 mg crude protein) and submitted to the suppressor/augmentor assay.


100
80
60
40
20
Suppression (%)
1
2
3
4
5
6
7
8
[Supernatant]

it became apparent that a two-fold concentration usually produced significant suppression.

Thermal Stability of Suppressor-Active Supernatants. Suppressor-active supernatants were tested for thermal stability at 56° C, 80° C, and 100° C. The results are shown in Table 4. The suppressor activity was partially denatured at 80° C for 10 min while 100° C completely eradicated the activity. No loss of activity was noted at 56° C for 30 min (data not shown).

Effect of Trypsin. Immobilized trypsin completely destroyed the suppressive activity in four of the six active supernatants (Table 5), and about 67% of the activity in another supernatant (No. 2) was destroyed. Only 26% of the activity of sample six was destroyed, however. These data suggested that suppressor activity was attributable to protein.

Molecular Sieve Chromatography of Suppressive Material. Molecular sieve chromatography was used to investigate the apparent molecular weight of the suppressor substance. The elution profile of concentrated suppressor culture fluid on Sephadex G-100 is shown in Fig. 5. The majority of suppressor activity eluted in the exclusion volume and corresponded to an apparent molecular weight >100,000 d (Pool 1). A small amount of detectable activity was also noted in the 68,000 d range (Pool 2). Pool 5, as shown by hatching, contained augmenting activity and will be discussed below.

Ion Exchange Chromatography of Suppressor-Active Supernatant. Concentrated suppressor-active supernatant fluids were fractionated on columns of DE-52 equilibrated with 50 mM tris-HCl at pH 7.2. A salt gradient of NaCl was used to elute the suppressor-active material.

TABLE 4

Thermal Stability of Suppressor-Active Supernatants

% Suppression	% Suppression after heating
Untreated	80^{o} x 10 min
87	55
40	15
64	42
44	31
	100^{o} x 5 min
38	0
35	0
29	0

TABLE 5

The Effect of Trypsin on Suppressor Activity

Supernatant Number	CPM[a]	% Suppression[b]
Con A Control	76,538 ± 6,199	-
Con A + Trypsin + DFP[c]	75,116 ± 2,038	-
1	31,967 ± 3,548	58
1 T[d]	77,113 ± 9,017	NS[e]
2	43,318 ± 6,522	43
2 T	65,721 ± 8,011	13
3	46,840 ± 5,264	38
3 T	79,292 ± 2,664	NS
4	65,183 ± 8,991	13
4 T	76,012 ± 9,728	NS
5	40,734 ± 1,288	46
5 T	76,453 ± 2,851	NS
6	31,942 ± 3,030	58
6 T	43,627 ± 7,444	42

[a]Counts per minute ± standard deviation. n = 3.

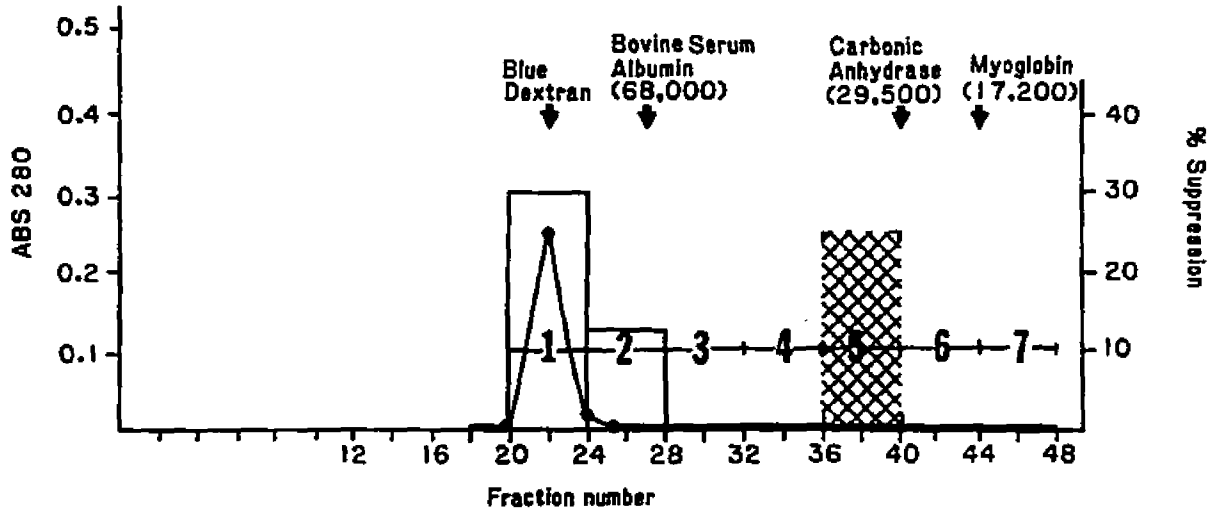
[b]% Suppression calculated using con A + trypsin + DFP control value.

[c]DFP added to trypsin prior to addition of trypsin to con A control.

[d]Trypsin-treated supernatant.

[e]Not significant.

Figure 5. Elution profile of tonsil supernatant fluids on Sephadex G-100. After equilibrating the gel in .05 M NH_4HCO_3 pH 7.2, a 1.5 x 90 cm column was poured. An aliquot of 200 µg of the crude preparation was rehydrated in 1.5 ml of buffer and applied to the column. Fractions of two ml were collected, pooled as shown and concentrated by lyophilization. The concentrated pools were rehydrated in RPMI-1640 and submitted to the suppressor/augmentor assay. Percent suppression (pools 1 and 2), percent augmentation (pool 5), and OD_{280} nm (●——●).


0.5
0.4
0.3
0.2
0.1
ABS 280
Blue Dextran
Bovine Serum Albumin (68,000)
Carbonic Anhydrase (29,500)
Myoglobin (17,200)
40
30
20
10
% Suppression
1
2
3
4
5
6
7
12
16
20
24
28
32
36
40
44
48
Fraction number

Representative data from one of several experiments are shown in Fig. 6. They revealed that the significantly suppressive substance (Pool 6) adhered strongly to the anion exchanger and eluted in fractions of conductivity corresponding to 0.4 M to 0.6 M NaCl. Less than one-fourth of the total protein (as determined by OD_{220}) was found in the active fraction (Pool 6), but the activity present in this pool comprised about one-half of the total bioactivity.

Five supernatants were examined for protein (OD_{280}) and suppression before and after fractionation on the anion exchanger. Specific activity was then determined as described. As shown in Table 6, four of the five supernatants tested had improved specific activity after elution compared to unfractionated preparations. The amount of actual increase in units of activity varied greatly with a range of 6.3 to 459.

Polyacrylamide Gel Electrophoresis of Suppressor Active Supernatant Fluids. Polyacrylamide gel electrophoresis was performed on the crude suppressor supernatant preparations and the bioactive anion exchange fraction. Control proteins were human serum albumin and pooled AB^+ serum. There was considerable protein heterogeneity within each crude supernatant preparation. The observed pattern (Fig. 7) was noted repeatedly in several active supernatant fluids. There consistently were 7 or 8 stainable bands visible in each gel.

Electrophoresis of the anion exchange fraction (Pool 6) revealed a single band lightly stained with Coomassie Blue. The protein migrated slightly faster than human serum albumin in a control gel. Its mobility corresponded to that of a protein present in the unfractionated supernatant.

Figure 6. Ion exchange (DE-52) chromatography of a suppressor active tonsil supernatant. The gel was equilibrated in with 50 mm tris-HCl pH 8.2. 100 µg of lyophilized supernatant preparation was applied to the column and eluted with a linear salt (NaCl) gradient of 0 to 1 M. Fractions of 2.0 ml were collected and prepared for bioassay as described. Fractions were pooled as shown by shaded areas. $O.D._{220\ nm}$ (●——●), conductivity (mMHO's ■ ··· ■), shaded area is percent suppression in bioassay.

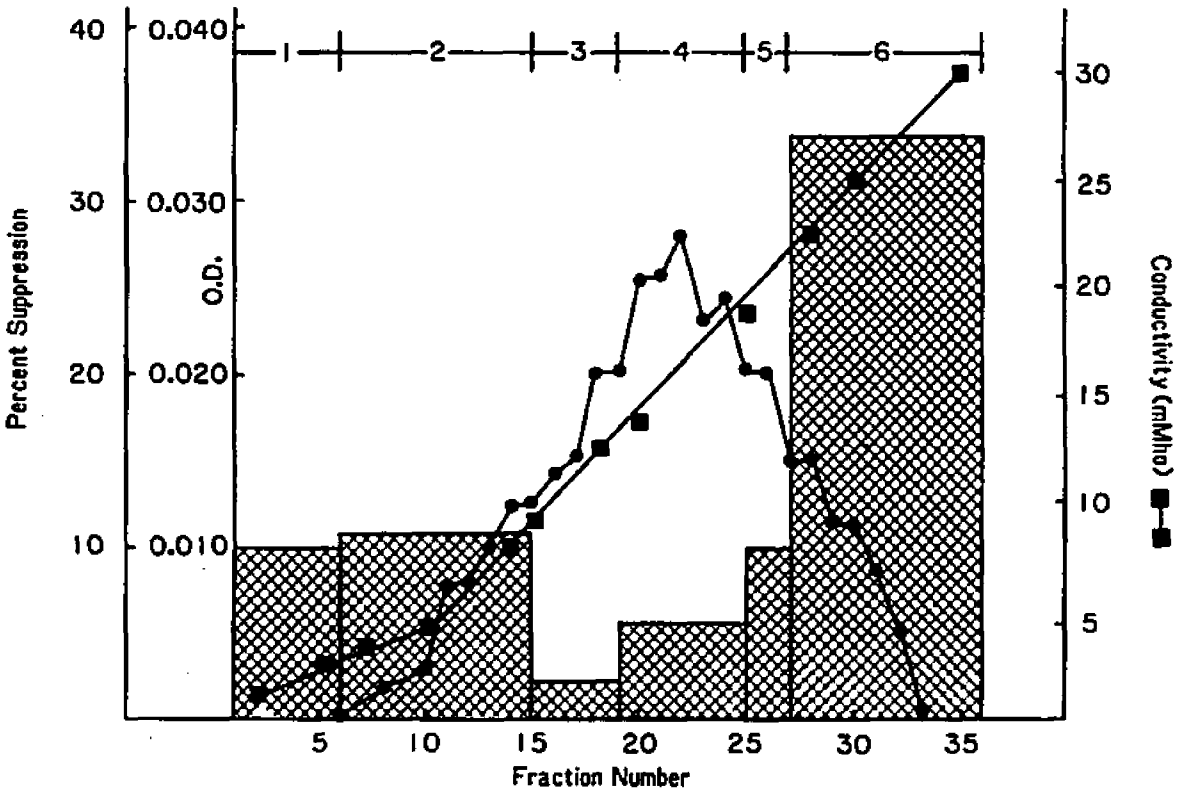

Percent Suppression
40
30
20
10
0.040
0.030
0.020
0.010
O.D.
1
2
3
4
5
6
30
25
20
15
10
5
Conductivity (mMho)
5
10
15
20
25
30
35
Fraction Number

TABLE 6

Specific Activities of Unfractionated and Fractionated Suppressor Supernatants

Unfractionated Supernatant		Specific	Ion Exchange Fraction 6		Specific
OD_{280}[a]	Percent Suppression[b]	Activity[c]	OD_{280}[a]	Percent Suppression	Activity
1.79	21	12	.604	24	40
1.43	38	27	.608	20	33
1.43	44	31	.588	15	26
0.409	16	39	.186	30	161
0.117	58	496	.073	70	959

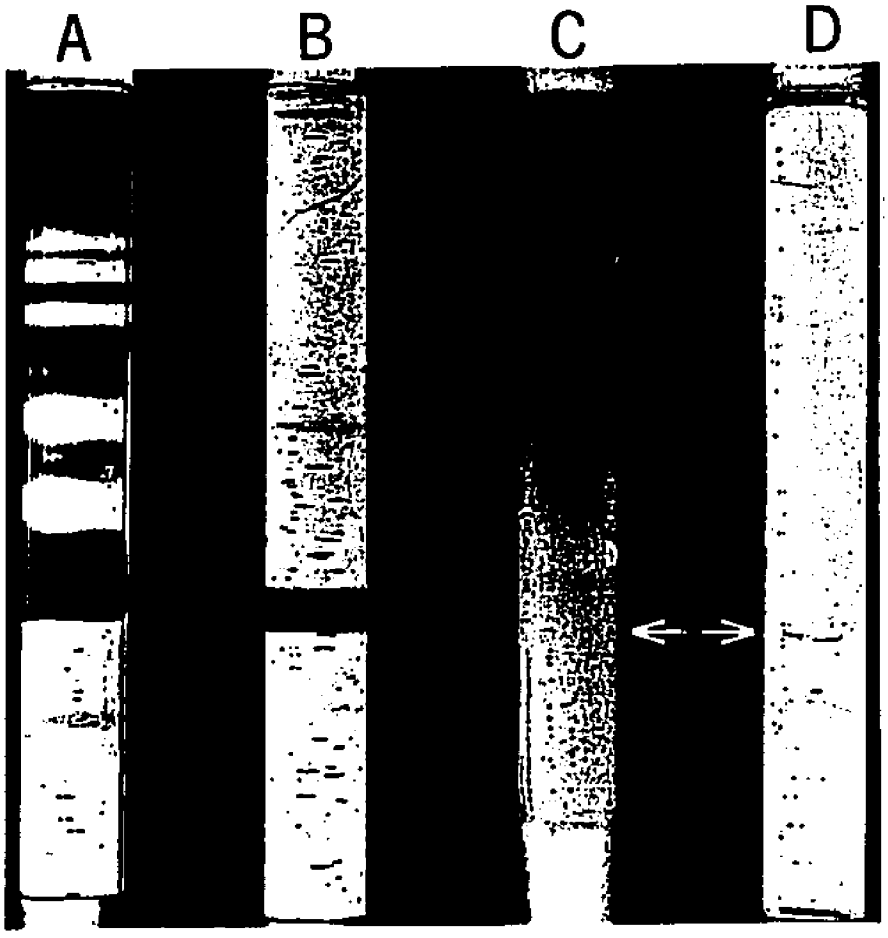
[a] OD_{280} spectrophotometric measurements before and after ion-exchange fractionation.

[b] Percent suppression calculated as described in text.

[c] Specific activity calculated by: $S.A. = \frac{\% \text{ Suppression}}{OD_{280}}$

Figure 7. Polyacrylamide Gel Electrophoresis of Active Supernatant Fluids

Polyacrylamide gel electrophoresis of pooled human AB^+ serum (A), human serum albumin (B), crude supernatant preparation (C), and pool 6 of anion exchange fractionation (D) are shown. Arrows indicate lightly staining bands of Coomassie Blue.


A
B
C
D

Gels loaded with a sample of Pool 6 were stained also with PAS to detect glycoproteins. The protein which took the Coomassie Blue did not stain with PAS (data not shown). The control protein, ovalbumin, (20 µg) accepted the stain and was visible approximately two thirds of way down the gel.

Isoelectric Focusing of Suppressive Supernatant Fluids. Suppressor-active preparations were focused in gradients of pH 3-10, 4-7, or 5-8. The result of isoelectric focusing through a pH gradient of 3-10 is shown in Fig. 8. Signfiicant activity focused diffusely beginning at pH 4.4 and extending to pH 5.6. The most active fraction (No. 9) produced 70% suppression in the bioassay. In several experiments, conducted in gradients of pH 3-10, 4-7, or 5-8, the pH range of significant activity was 4.3-6.3, and the mean pI was 5.0 ± 0.6 (Table 7).

A control plate containing no protein was also focused (data not shown). Fractions were treated in a manner identical to the experimental. There was no suppressor activity noted in the bioassays of control fractions.

Chromatofocusing of Suppressor Supernatant Fluids. Suppressor active supernatant preparations were fractionated on chromatofocusing columns using PB 74 as the eluent buffer. Fractions of 2.0 ml were collected and the pH of each fraction determined. The fractions were combined into six equal pools. The first 5 fractions served as a column control in the bioassay. In two separate experiments, there was no demonstrable biological activity in any of the six pools.

A control experiment was done to determine if the polybuffer interfered with con A stimulation of PBL. From this experiment (data

Figure 8. Isoelectric Focusing of suppressor active tonsil supernatant using Pharmalytes (range: pH 3-10). Shaded area is percent suppression of individual fractions and pH (●——●).

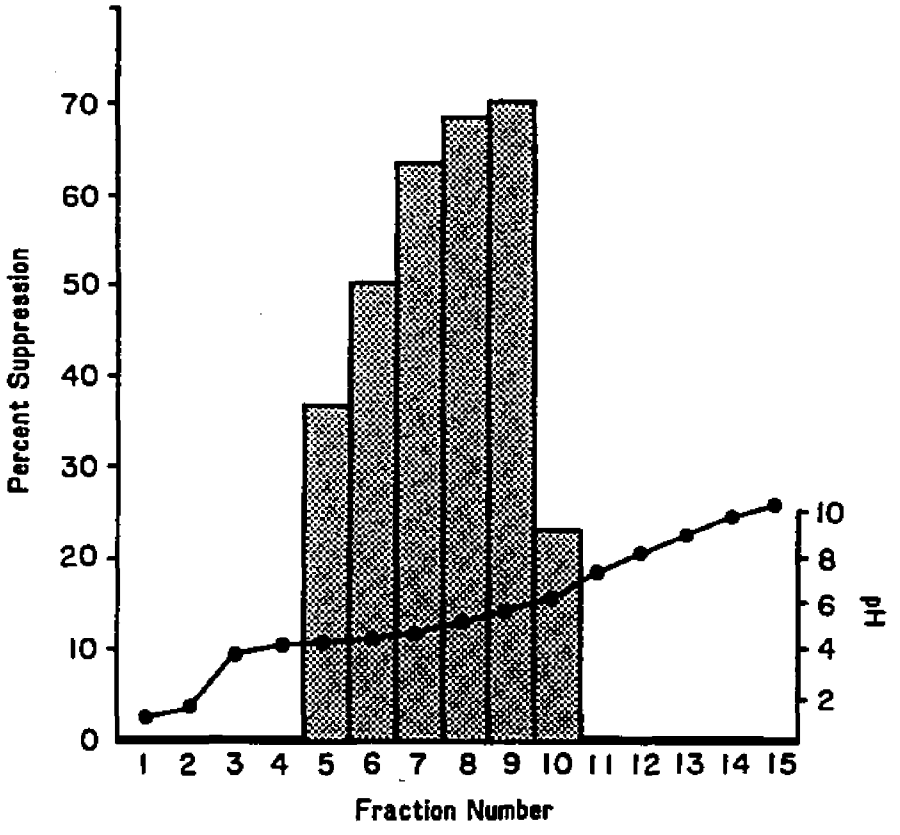

Percent Suppression
0
10
20
30
40
50
60
70
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Fraction Number
pH
2
4
6
8
10

TABLE 7

Summary of Suppressor Isoelectric Points

Ampholines[a]	Range[b]	pI of Suppression[c]
Ampholyte	3-10	4.26, 4.55
Ampholyte	3-10	4.75, 5.16
Ampholyte	3-10	4.27
Pharmalyte	3-10	4.4, 4.6, 4.88
		5.25, 5.63, 6.3
Pharmalyte	4-7	5.41
Pharmalyte	5-8	5.0

Mean pI ± S. D. = 5.0 ± 0.6

[a]Brand name of polyamino-polycarboxylic acid.

[b]pH range.

[c]Isoelectric point of significant suppressive fractions.

not shown) it was learned that the blastogenic effect of con A was not significantly altered by the presence of nondialyzable polybuffer constituents. That the suppressor was bound or inhibited by the polybuffer was not investigated.

Anesthesia and Its Relationship to Suppressive Activity. Patients about to undergo tonsillectomy were anesthetized with combinations of the general anesthetics ethrane, nitrous oxide, and sodium pentothal. To test the possibility that these drugs were related to the observed suppression, the mean per cent suppression for supernatant fluids from 14 patients were classified according to the anesthesia used and analyzed by one way ANOVA (Table 8).

There was no significant difference between groups and it was concluded the anesthesia had no effect on the degree of suppression.

Mitogenic Activity in Tonsil Supernatant Preparations. Concentrated crude supernatant fluids from tonsillar lymphocytes grown in the absence of serum or any stimulatory agent were tested for their ability to stimulate blastogenesis in allogeneic PBL.

As shown in Table 9, 13 of the 30 supernatants tested significantly stimulated the incorporation of ^{3}H-thymidine corresponding to mitogenic indices from 2 to 7.2. Fourteen supernatants had MI's greater than one but less than two, while four supernatants had no significant effect. The mean mitogenic index was 2.4.

As also indicated by the data (Table 9), there was no apparent relationship between total protein in the crude preparation, primary culture blastogenesis and the degree of stimulation.

Augmentation in Tonsil Supernatant Fluids. In the bioassays for suppression, it was noted that several of the supernatant preparations

TABLE 8

Anesthesia

Group[a]	Percent Suppression[b,c]
I Ethrane, N_2O, Pentothal	31
	11
II Flourane, N_2O	11
	64
	40
	7
	13
	16
	83
	30
III Ethrane, N_2O	11
	44
	45
	36

[a] Anesthesia types grouped according to combination used.

[b] Percent suppression calculated as described in text.

[c] F from one-way analysis of variance was 0.0239.

[d] F = 11. No significant difference.

TABLE 9

Comparison of Mitogenic Activity with Total Protein and Non-Induced Blastogenesis of Primary Culture

Supernatant Number	Volume[a]	Primary cpm[b]	Protein Concentration[c]	AB+[d]	MF cpm[e]	MI[f]
1	200	2,445 ± 390	348	1,206 ± 340	3,583 ± 623	2.9
2	300	5,120 ± 759	550	1,850 ± 769	7,098 ± 119	3.8
3	300	3,029 ± 668	464	1,654 ± 675	8,296 ± 463	5.0
4	300	9,288 ± 1,415	600	1,097 ± 564	7,919 ± 977	7.2
5	300	5,262 ± 535	330	783 ± 139	3,618 ± 1,682	4.6
6	300	21,596 ± 1,997	130	783 ± 139	2,373 ± 351	3.0
7	150	9,064 ± 642	115	783 ± 139	1,764 ± 872	2.3
8	550	9,383 ± 483	100	783 ± 139	1,944 ± 521	2.5
9	70	4,516 ± 411	50	783 ± 139	976 ± 151	1.2
10	250	1,937 ± 368	90	1,912 ± 207	9,725 ± 3,633	5.0
11	84	2,717 ± 297	140	1,912 ± 207	2,551 ± 1,272	1.3
12	136	10,597 ± 1,100	590	1,912 ± 207	3,467 ± 1,625	1.8
13	1000	12,430 ± 1,433	580	799 ± 277	712 ± 272	NS

TABLE 9 (Cont)

Comparison of Mitogenic Activity with Total Protein and Non-Induced Blastogenesis of Primary Culture

Supernatant Number	Volume[a]	Primary cpm[b]	Protein Concentration[c]	AB+[d]	MF cpm[e]	MI[f]
14	600	7,193 ± 871	340	799 ± 277	1,125 ± 238	1.4
15	200	18,790 ± 816	160	799 ± 277	1,359 ± 376	1.7
16	116	7,694 ± 992	150	942 ± 360	4,229 ± 3,128	4.5
17	100	3,380 ± 323	30	942 ± 360	741 ± 316	NS
18	600	7,373 ± 1,188	330	942 ± 360	1,103 ± 266	1.2
19	830	15,016 ± 1,568	1120	942 ± 360	927 ± 445	NS
20	247	12,878 ± 1,337	320	942 ± 360	1,265 ± 667	1.3
21	232	11,875 ± 660	390	942 ± 360	1,270 ± 330	1.3
22	167	29,285 ± 1,951	150	789 ± 344	670 ± 225	NS
23	550	14,985 ± 1,755	265	789 ± 344	1,386 ± 597	1.8
24	550	7,967 ± 843	300	789 ± 344	1,070 ± 502	1.4
25	350	11,629 ± 2,277	420	789 ± 344	835 ± 505	NS
26	200	9,993 ± 499	380	567 ± 211	954 ± 754	1.7

TABLE 9 (Cont)

Comparison of Mitogenic Activity with Total Protein and Non-Induced Blastogenesis of Primary Culture

Supernatant Number	Volume[a]	Primary cpm[b]	Protein Concentration[c]	AB^{+}[d]	MF cpm[e]	MI[f]
27	200	9,522 ± 466	400	567 ± 211	1,178 ± 174	2.0
28	200	8,260 ± 638	382	567 ± 211	2,194 ± 947	3.9
29	200	6,787 ± 595	460	567 ± 211	1,274 ± 663	2.2

[a]Volume of original supernatant (ml).

[b]cpm ± S. D. (n = 6) of non-induced blastogenesis in the primary culture.

[c]Protein concentration (µg/ml) in crude supernatant as determined by Lowry (97).

[d]Cpm ± S. D. (n = 6) of AB^{+} control in bioassay.

[e]Cpm ± S. D. (n = 6) of mitogenic supernatant in bioassay.

[f]Mitogenic index calculated as described in text.

had an enhancing effect on con A-induced blastogenesis. Since augmentation required the presence of con A, the degree of enhancement (per cent augmentation) was calculated by comparing the tested supernatant cpm with the con A control (10 µg/ml) cpm. Significance was determined by taking the difference between means of test supernatant and control and establishing 95% confidence limits about the difference. The error used in calculating the limits was from one way ANOVA.

Of the many supernatant fluids tested in the suppressor/augmentor assay (Table 10), 14 caused significant incorporation of ^{3}H-thymidine greater than that of con A control values. The amount of augmentation varied greatly ranging from 12% to 211%.

When the per cent augmentation was compared with total protein and primary culture cpm, there was no observable relationship (Table 11).

Mitogenic Factor and Augmenting Dose Response Curves. In some supernatants it was possible to observe both mitogenic and augmenting activities. Four of these supernatants were examined for dose responsiveness based on total protein in the crude preparation. Serial dilutions of the supernatants were made and subjected to the MF and suppressor/augmentor assays. Representative dose curves of a doubly active supernatant are shown in Figures 9 and 10. The mitogenic activity (Fig. 9) behaved in a dose dependent fashion and seemed to approach a plateau of saturation at higher concentrations.

The slope of the dose response curve for augmentation (Fig. 10) was quite different from the dose curve obtained from MF. There was no apparent increase in cpm as a function of increasing dosages. However, linear regression analysis revealed that the MF and augmenta-

TABLE 10

Effect of Tonsil Cell Culture Fluids on Transformation (Augmentation) of Normal PBL Stimulated with Con A[a]

Experiment Number	Sample Number[b]	cpm[d]	Augmentation[e]	Δ Mean[f]	Significant[g]
1	Con A[c]	34,577 ± 8,875			
	1	107,547 ± 5,995	211%	+72,970 ± 13,135	+
	2	50,489 ± 12,085	46%	+15,912 ± 13,135	+
2	Con A	79,793 ± 14,494			
	3	112,023 ± 3,852	40%	+32,230 ± 16,696	+
3	Con A	99,711 ± 6,674			
	4	116,084 ± 5,541	16%	+16,373 ± 14,583	+
4	Con A	88,953 ± 1,631			
	5	111,043 ± 6,845	25%	+22,090 ± 6,407	+
5	Con A	69,331 ± 4,390			
	6	114,165 ± 23,763	65%	+44,834 ± 25,398	+
	7	108,589 20,744	57%	+39,259 ± 25,398	+

TABLE 10 (Cont)

Effect of Tonsil Cell Culture Fluids on Transformation (Augmentation) of Normal PBL Stimulated with Con A[a]

Experiment Number	Sample Number[b]	cpm[d]	Augmentation[e]	Δ Mean[f]	Significant[g]
	8	123,197 ± 14,310	78%	+53,866 ± 25,398	+
	9	115,865 ± 14,184	67%	+46,534 ± 25,398	+
6	Con A[c]	72,716 ± 8,683			
	10	90,453 ± 10,794	24%	+17,737 ± 14,167	+
7	Con A	62,972 ± 8,403			
	11	81,782 ± 7,183	30%	+18,810 ± 10,484	+
8	Con A	64,986 ± 4,142			
	12	83,359 ± 3,529	28%	+18,372 ± 8,530	+
9	Con A	71,770 ± 1,013			
	13	99,804 ± 3,451	39%	+28,033 ± 8,890	+
10	Con A	63,022 ± 3,151			
	14	70,492 ± 1,687	12%	+ 7,469 ± 6,689	+

TABLE 10 (Cont)

Effect of Tonsil Cell Culture Fluids on Transformation (Augmentation) of Normal PBL Stimulated with Con A[a]

Experiment Number	Sample Number[b]	cpm[d]	Augmentation[e]	Δ Mean[f]	Significant[g]
11	Con A[c]	73,984 ± 3,537			
	15	80,663 ± 10,336	9%	+ 6,679 ± 9,945	+
12	Con A	56,714 ± 2,673			
	16	62,609 ± 7,701	10%	+ 5,859 ± 10,272	-
	17	65,966 ± 9,588	16%	+ 9,252 ± 10,272	-
13	Con A	79,793 ± 14,494			
	18	88,234 ± 3,519	11%	+ 8,441 ± 16,696	-

[a]Tonsil cells were cultured in the absence of stimulation. The supernatants were assayed by their effect *e* on PBL cultured in the presence of Con A.

[b]Numerals refer to individual patients' tonsils.

[c]Con A refers to radioactivity incorporated by PBL stimulated with Con A in the absence of tonsil cell culture fluid.

TABLE 10 (Cont)

Effect of Tonsil Cell Culture Fluids on Transformation (Augmentation)

of Normal PBL Stimulated with Con A

---

[d] Mean cpm ± S. D. (n = 6).

[e] Percent augmentation of radioactivity incorporated by PBL stimulated with Con A in the presence of tonsil cell culture fluid compared to that incorporated in the absence of tonsil cell culture fluids as described in text.

[f] The difference between means of test supernatant and control ± 95% confidence limits about the difference.

[g] The error used to calculate limits was from analysis of variance.

(+) indicates significant augmentation.

TABLE 11

Comparison of Augmentation with Total Protein and Non-Induced Blastogenesis of Primary Culture

Supernatant Number	Primary Culture Parameters: Protein Concentration (µg/ml)[a]	Volume (ml)[b]	Blastogenesis (cpm)[c]	Bioassay of Primary Culture: Percent Augmentation
1	348	200	2,445 ± 390	30
2	550	300	5,120 ± 759	29
3	464	300	3,029 ± 668	33
4	600	300	9,288 ± 1,415	12
5	140	84	2,717 ± 297	40
6	590	136	10,597 ± 1,100	11
7	160	200	18,790 ± 816	18
8	150	116	7,694 ± 992	2
9	30	100	3,380 ± 323	7
10	1120	830	15,016 ± 1,568	7
11	320	247	12,878 ± 1,387	13

TABLE 11 (Cont)

Comparison of Augmentation with Total Protein and Non-Induced Blastogenesis of Primary Culture

Supernatant Number	Primary Culture Parameters: Protein Concentration (µg/ml)[a]	Volume (ml)[b]	Blastogenesis (cpm)[c]	Bioassay of Primary Culture: Percent Augmentation
12	390	232	11,875 ± 660	16
13	382	200	8,260 ± 638	7
14	460	200	6,787 ± 595	7

[a]Protein concentration (µg/ml) in primary culture supernatant preparation as determined by Lowry technique (97).

[b]Volume of original supernatant fluid (ml).

[c]Cpm ± S. N. (n = 6) of non-induced blastogenesis of primary culture.

[d]Percent suppression in bioassay of primary supernatant culture fluids.

Figure 9. Serial dilutions of concentrated MF active supernatant fluids. Activity expressed in cpm as a function of total protein.

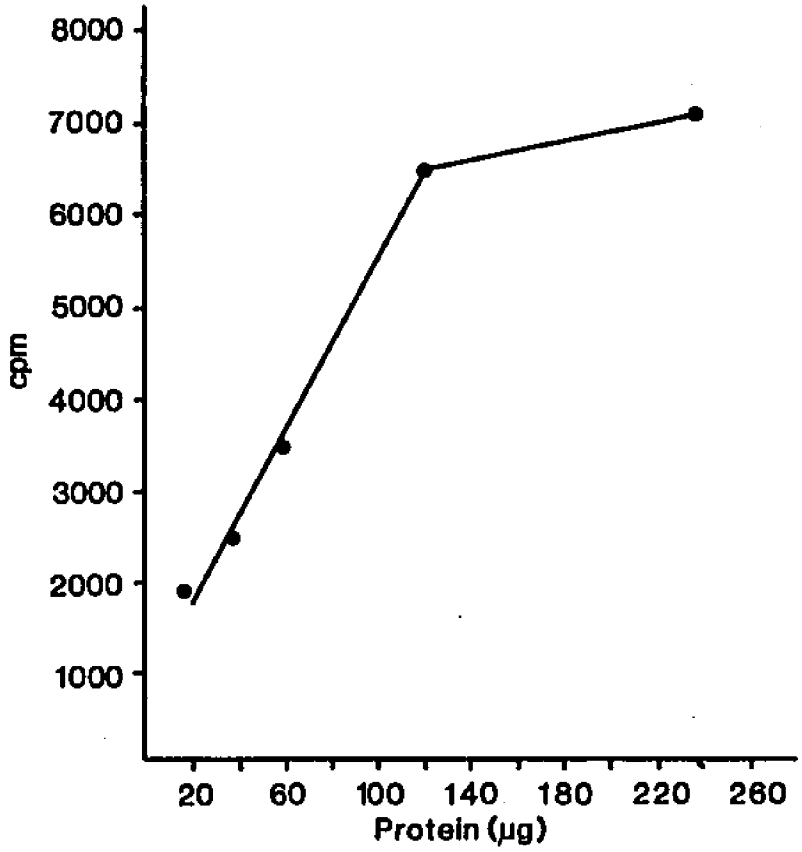
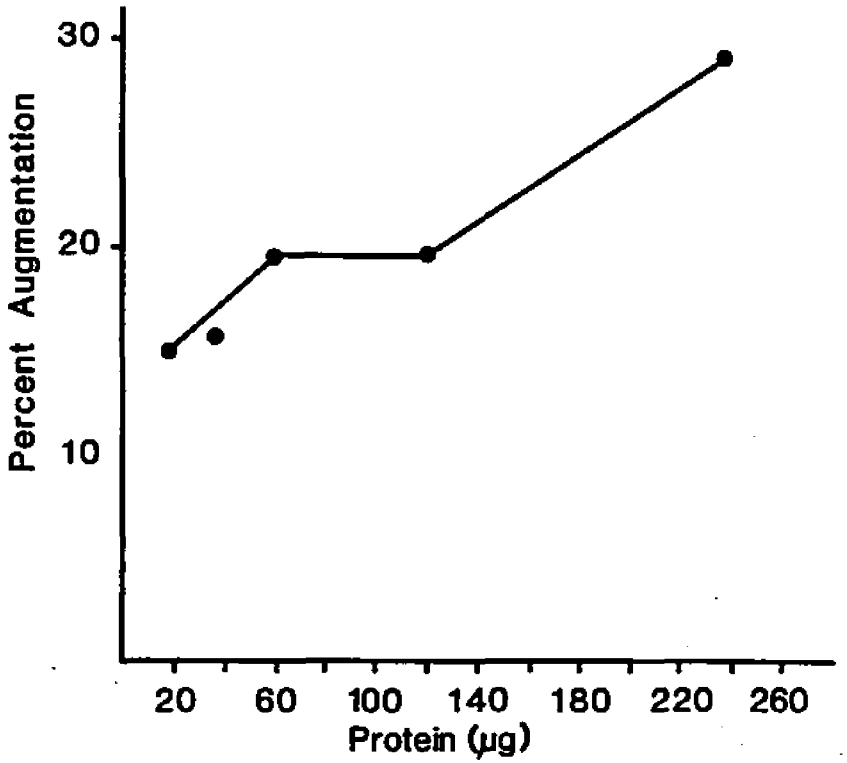

8000
7000
6000
5000
4000
3000
2000
1000
cpm
20
60
100
140
180
220
260
Protein (µg)

Figure 10. Serial dilutions of concentrated augmenting active supernatant fluids. Activity expressed as percent augmentation as a function of total protein.


30
20
10
Percent Augmentation
20
60
100
140
180
220
260
Protein (µg)

tion dose curves were more complex than straight lines (data not shown) and peak points of both curves were significant based on Dunnett's test.

Molecular Sieve Chromatography of Supernatant Fluids with Suppressor and Augmentor Activities. In experiments to determine the suppressor, it was possible to detect both suppressive and augmenting activities in a single supernatant preparation.

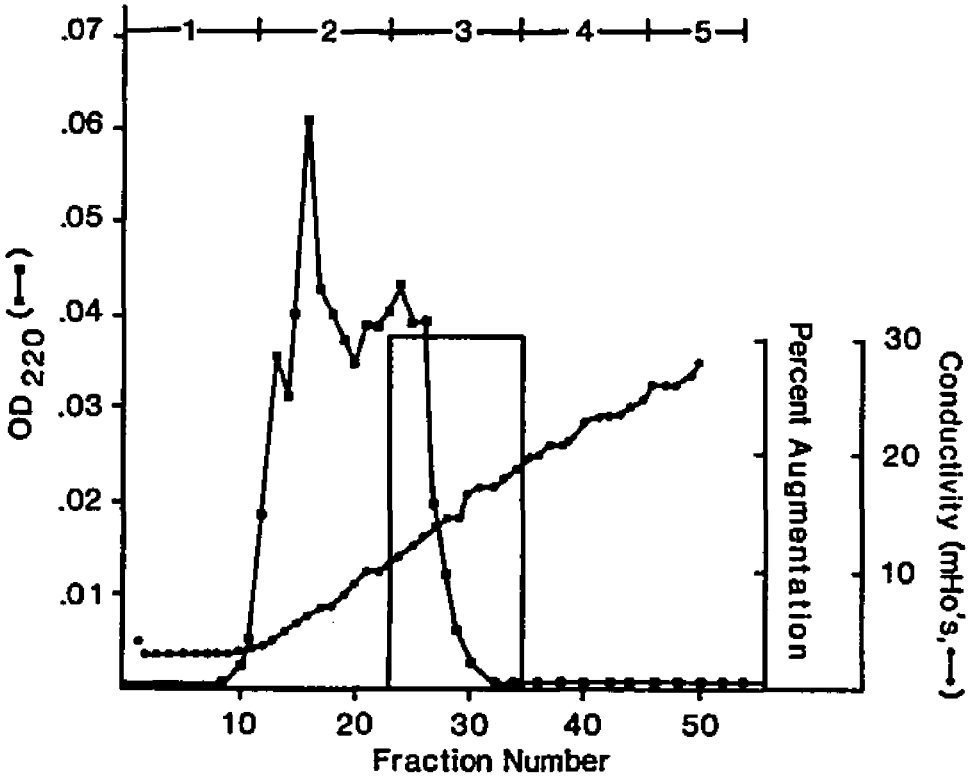
Fractionation on Sephadex G-100 of suppressor-active supernatant fluids (Fig. 5) repeatedly demonstrated one pool to contain the augmenting effect. The active fractions corresponded to the elution volume of carbonic anhydrase and, therefore, its apparent molecular weight was about 30,000.

Anion Exchange Chromatography of Augmenting Supernatant Fluids. Anion exchange chromatography of both suppressive and augmenting supernatant preparations was performed using a sodium chloride gradient of 0 to 1 M. Fractions of 1.0 ml were collected. The protein (OD_{220}) content and conductivity of each fraction was determined, and then they were pooled as shown in Figure 11. The pools were prepared for the suppressor/augmentor assay in the usual manner and subsequently tested for augmenting activity.

In most experiments, augmentation was expressed in the 0.15 to 0.25 M salt fractions regardless of the activity (i.e. suppression or augmentation observed in the assay of the crude preparation.) Augmenting activity corresponded to the minor optical density peak of the biphasic curve.

On one occasion, augmenting activity was noted in the 0.38 M salt fractions. There was, however, no detectable absorbance at

Figure 11. Anion exchange chromatography of augmenting active supernatant fluid. OD_{220} (▪-- · ▪), conductivity (mHo's; ●——●), percent augmentation (boxed area).


1
2
3
4
5
.07
.06
.05
.04
.03
.02
.01
OD 220 (—)
Percent Augmentation
30
20
10
Conductivity (mHo's, —)
10
20
30
40
50
Fraction Number

OD_{220} of these fractions.

Determination of Augmentor Producing Cell Population. To determine the cellular origin of augmenting activity, tonsillar cells were separated on the basis of their adherence properties.

Although culture controls (Table 12) demonstrated significant augmentation with respect to con A control values, Dunnett's test (data not shown) indicated there was no significant difference in the amount of augmentation observed in the culture controls and the adherent or non-adherent qualities of the cell population.

TABLE 12

Adherent Cell Study For Augmenting

Experiment	Culture	CPM[a]
1	Con A	72,716 ± 8,683
	Control[b]	75,296 ± 8,146
	Adherent[c]	81,927 ± 6,216
	Non-Adherent	76,533 ± 5,654
2	Con A	72,716 ± 8,683
	Control	92,767 ± 10,797
	Adherent	80,704 ± 7,166
	Non-Adherent	93,675 ± 10,586
3	Con A	37,829 ± 2,634
	Control	45,639 ± 3,966
	Adherent	37,735 ± 2,726
	Non-Adherent	46,146 ± 4,499
4	Con A	74,804 ± 8,350
	Control	80,776 ± 6,663
	Adherent	79,454 ± 12,863
	Non-Adherent	83,758 ± 9,115
5	Con A	56,714 ± 2,674
	Control	70,613 ± 5,875
	Adherent	74,607 ± 11,265
	Non-Adherent	67,194 ± 9,619

Adherent Cell Study for Augmenting

Experiment	Culture	CPM[a]
6	Con A	59,485 ± 5,439
	Control[b]	47,905 ± 7,760
	Adherent[c]	47,080 ± 8,817
	Non-Adherent	57,184 ± 6,010

[a]Cpm ± S. D. (n = 6)

[b]Control cultures assayed as usual

[c]Adherent cell population allowed to adhere to tissue flask for 2 hr, then the non-adherent cells was decanted. The adhered cells were rehydrated to the same volume. All cultures were incubated for 24 hr.

CHAPTER 4

DISCUSSION

Transformation and proliferation of small lymphocytes *in vitro* follows activation by antigens or by various mitogens. Among the consequences of activation is that both T and B lymphocytes secrete certain products called lymphokines (102), and a rather large number of bioactivities have been attributed to these soluble mediators of immune response. *In vitro*, lymphokines cause phenomena which mimic type IV tissue reactions, and more recent perceptions are that some of these functions immunoregulatory. It is widely presumed that lymphokines also are produced *in vivo* by lymphocytes activated by natural antigens.

Tonsils are lymphatic organs which comprise similar percentages of T and B lymphocytes (103, 104); and very low percentages of monocytes (104). Their cells produce the lymphokines lymphotoxin (105), colony stimulating factor (106), T cell growth factor (54), and at least one suppressor factor (65) subsequent to *in vitro* activation. Since the proliferative response of tonsil cells in some humans is apparently evident by frequent episodes of tissue swelling, it is likely that tonsil cells would be engaged in immunoregulatory activities. During the subsiding phase, it seems possible that suppression of cellular proliferation might be induced by a soluble mediator.

A major problem inherent in the isolation and purification of most lymphokines has been the lack of substantial quantities of starting material for biochemical analysis. For this reason, it was thought that tonsils would provide a large and readily available source of lymphokine producing cells.

The cell culture methodology for both the primary tonsillar

lymphocyte cultures and the bioassays had been well established in the literature and was adapted to these experiments with modifications. Unique to this study was the absence of serum during the primary culture, the use of Ficoll-Paque to isolate the mononuclear fraction of the tonsillar tissue, and the absence of *in vitro* stimulation of the primary culture.

Normally, lymphocytes grown in primary culture for any length of time required at least 10% serum. However, it was found that the lack of serum in our cultures for 24 hr did not cause significant loss of viability. Furthermore, growth of these cells in media free from exogenous serum contaminants facilitated purification of endogenous protein.

Bouyant density flotation on Ficoll-Paque had been used routinely to isolate mononuclear cells from whole blood but rarely from tonsillar tissue. The use of Ficoll-Paque was chosen for these studies for two reasons. First, separation by Ficoll-Paque eliminated unwanted tonsillar cell types whose products may have interferred with the bioassay. Secondly, it was generally accepted that all three mononuclear cell types i.e. T cells, B cells, and monocytes were required for the production of most lymphokines (107) and Ficoll-Paque effectively separated these cells (95% mononuclear cells) from other tonsil cells and debris, and improved the yield of viable mononuclear cells.

Preliminary experiments in which con A was incubated with the tonsillar lymphocytes and then subsequently removed by adsorption to Sephadex, revealed that supernatant fluids of cultures incubated with con A caused less ^{3}H-thymidine incorporation in the bioassay than did control cultures. This result was reasoned to be indicative of a

suppressive substance present in the supernatant preparation.

Tonsil lymphocytes were shown to be synthesizing DNA in the absence of *in vitro* stimulation. The extent of incorporation of ^{3}H-thymidine by these cells varied from a few thousand to greater than ten thousand cpm at the time of measurement. These data confirmed those of Drucker et al. (108) who termed the phenomenon "preactivation". Similar observations were made by Williams and Korsmeyer (109) in studies with human cord blood lymphocytes.

In these experiments, tonsil lymphocytes activated *in vivo* presumably as a consequence of naturally occurring antigen insult secreted suppressive material which significantly inhibited the expected response of PBL to con A. This was in agreement with the work of Wolf et al. (65) who suggested that tonsil T lymphocytes elaborated a substance which suppressed the response of PBL to phytohemagglutinin and certain antigens.

The rather wide range of suppression percentages reported here probably were at least partially attributable to the genetic variability between the tonsillar cells and the donor target cells for the bioassay. To circumvent this problem, many different supernatant fluids were tested in a single assay as logistically as possible. Ideally, lymphocytes from a single individual would have caused less heterogeneity with respect to suppression but this could not have been accomplished readily.

After the initial discovery of the suppressive activity, studies were undertaken to determine the relationship, between the degree of non-induced proliferation of the primary cultures, total protein in the crude preparations and the percent suppression

in the bioassay.

Intuitively, one might predict that an increase in total protein would be accompanied by an increase in percent suppression as well as an increase in non-induced blastogenesis of the primary culture. Similarly, one would expect a concomitant increase in percent suppression and non-induced blastogenesis. However, data accumulated from several experiments indicated there was no correlative increase or decrease in suppression as a function of any of the measured parameters.

The active substance apparently was protein since trypsin treatment of the crude preparation destroyed the suppressive effect in nearly all supernatant fluids tested. However, one supernatant preparation with a relatively high OD_{260} value, was still suppressive after enzyme treatment. This might have been due to the reported suppression of mitogen stimulation by nucleic acids (66).

The apparent molecular weight of the suppressor factor, as determined by molecular sieve chromatography, was >100,000. Attempts to further define the molecular weight by sieving through Sephadex G-200 and Bio Gel P-150 resulted in loss of biological activity. Tibbets et al. (91) described a suppressor which inhibited PHA stimulation of lymphoid cells. The factor was produced by mitogen-stimulated PBL and had an apparent molecular weight of >100,000 d. The suppressor identified by Wolf et al. (65) was also in excess of 100,000 d.

The suppressor material adhered strongly to the anion exchanger DEAE, eluting in the 0.4 M-0.6 M salt fraction. This step in purification gave only a slight increase in specific activity. Polyacryl-

amide gel electrophoresis showed more homogeneity of the anion exchange fraction with respect to the crude, concentrated preparation in that the active fraction contained one lightly staining band, while 7-8 bands were detected in the crude preparation. The gels, although loaded with concentrated partially purified suppressor, were not inbued to the extent that homogeneity was confirmed.

The rather diffuse range of isoelectric points reported here for the suppressor activity may belie charge heterogeneity. This heterogeneity was evidently not due to sugar residues since PAS staining (sensitive to 5 μg) detected no glycoproteins for the concentrated, crude preparation nor the active anion exchange fraction. Loss of amide groups, cyclization of pyrollidone groups or non-specific aggregation of the suppressor with *de novo* protein in the primary culture may account for the variation in charge.

The fact that the suppressor material was nondialyzable suggested that the activity was not attributable to thymidine secreted by monocytes during generation of the supernatants (110), or to prostaglandins E, or E_2. The suppressor from the preactivated tonsil cells was not the normal immunosuppressive protein described by others (74, 75) and it was not some other serum-derived factor because neither plasma nor serum was present (72, 73, 76); nor was suppression due to complement components since heating at 56° C for 30 min, which inactivates complement, had no effect on suppression. It was not likely that the suppressor was a bacterial product such as endotoxin (111) or a fungal product elaborated during culture because prior to culture the tonsils or tonsil cells were kept in media which was heavily fortified with antibacterial and antifungal chemicals. The culture medium was

fortified also, and the cultures were asceptic at harvest. The suppressor apparently was not immunoglobulin G, A, M, or E or α2-macroglobulin. It did not seem likely that α_1-acid glycoprotein, which inhibited the response of lymphocytes to con A (77), could have been transferred on tonsil tissue in a quantity sufficient to be inhibitory in our experiments.

The binding site of con A has been shown to be specific for α-D-manopyranose, α-D-glucopyranose or sterically similar residues (29) and, consequently, is specifically inhibited by methyl-α-D manopyranose. The active fraction obtained from anion exchange chromatography, however, did not comprise any stainable glycoproteins and it was concluded that the suppression did not occur by nonspecific binding of con A to sugar moieties.

The viability of target cells consistently was 90% or greater. Thus the suppressive agent did not act by causing extensive death of target lymphocytes. The suppressor was not lymphotoxin since this lymphokine did not act on human lymphocytes.

It was suggested in the literature that certain general anesthetics (112, 113, 114) suppress the immune response by acting on lymphocytes. Rats injected with 1.5% halothane developed a transient, reduced capacity to induce lysis of antibody-coated target cells compared with those from untreated animals. The result reported here indicated statistically that suppression occurred irregardless of the type of anesthesia used.

In most supernatant culture fluids, suppression was the predominant biological activity observed. Occasionally, however, cultures

comprised material which enhanced the expected activation of PBL by con A. Augmentation was also present in suppressor supernatant preparations. Evidently, the suppressor masked the augmenting activity until separation of the two molecules was effected on G-100 or anion exchange chromatography columns.

It was possible that the enhancing effect was due to T cell growth factor since this lymphokine has been shown to augment the effect of mitogens (62). Warren et al. (66) previously reported that tonsillar T lymphocytes were capable of producing a T cell growth factor like substance which promoted the growth of lymphocytes in long term culture.

T cell growth factor has been shown to elute from anion exchange columns in 0.07 M NaCl and to have an apparent molecular weight of 15,000. In these experiments, however, the augmentor was eluted from the ion exchanger in 0.15-0.25 M NaCl and was shown to be 30,000 in molecular weight. These discrepancies may possibly be accounted for by the aggregation of endogenous protein which has been shown to occur in cell cultures grown without serum (115).

Since T cell growth factor is produced by the non-adherent T lymphocyte, studies were undertaken to separate the tonsillar lymphocytes into adherent and non-adherent populations. No definitive conclusion could be drawn from these experiments presumably due to the inability to adequately separate the two populations in the absence of serum.

In nearly all unstimulated tonsil lymphocyte supernatant fluids tested, there was at least minimal mitogenic activity present. Mitogenic factor apparently was different from the augmentor since (a) the mitogenic activity did not require lectin co-stimulation for the

expression of biological activity, (b) MF activity was demonstrable in the presence of the suppressor, while the augmentor was not, and (c) the dose response curves for the two activities, which were derived from the same supernatant fluids, were different shapes.

Some lymphokines are presumed to have an immunoregulatory role. The suppressor, augmentor, and mitogenic factor described here may function to regulate cellular proliferation. It is not yet certain that they act in a biologically relevant manner. They do inhibit or augment the proliferative response to a plant lectin or, as is the case with mitogenic factor, act independently of mitogen-stimulated blastogenesis; but these are experimental phenomena engineered *in vitro*. The mechanism of suppression, augmentation, and mitogenic activity is not understood. However, the action of these molecules in the presence of one another *in vitro* may provide clues as to their *in vivo* functions.

In summary, our initial objectives were achieved in so much as we demonstrated the existence of three soluble factors produced by tonsil mononuclear cells which had different affectations on PBL proliferation. Furthermore, isolation and partial characterization of the suppressor factor was facilitated since there were no serum factors contaminating the primary cultures. Alternatively, we did not confirm immunoregulatory properties i.e. the relationship, between the three factors *in vitro* or *in vivo*.

These experiments have raised several questions which, if answered, would add to our understanding of how the immune system functions. For example, how can MF and the suppressor be present in the same supernatant; is it produced by the same or different cell types? Secondly,

does the suppressor inhibit the augmentor stoichiometrically? This could be determined only by first separating the two entities then adding them back to each other in titrating doses. Thirdly, how does the suppressor function mechanistically, i.e. does it directly affect con A target cells or does it turn on suppressor cells which then inhibit the proliferation of con A responsive cells?

In light of these questions, the immediate direction of this research seems evident. Initially, the anion exchange fraction containing suppressor activity should be submitted to PAGE and stained with silver stain. This would help determine purity since the silver stain is more sensitive than the Coomassie stain. Next, the same anion exchange fraction should be examined for trypsin sensitivity to eliminate the possibility of suppression by nucleic acids. Assuming these experiments yield the expected results, the anion exchange fraction could then be applied to generating rabbit anti-suppressor antibodies. Suppressor specific antisera would greatly facilitate further purification of the factor as well as help answer the immunoregulatory questions presented above.

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Hodge, T. W. and F. P. Inman, "Biochemical Characterization of a Soluble Suppressor Factor Produced by Tonsil Lymphocytes". Fed. Proc. 41, 574. 1982, Abs. 1777.

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