CHARACTERIZATION OF AN IMMUNOSTIMULATORY FACTOR ASSOCIATED WITH SPONTANEOUSLY REGRESSING SUBLINE OF MURINE L1210 LYMPHOMA

by

Husam Amin Ghnaim

Submitted in Partial Fulfillment of the Requirements
for the Degree of
Master in Science
in the
Biological Sciences
Program

Advisor _____ May 11, 1992

Dean of the Graduate School _____ June 11, 1992

YOUNGSTOWN STATE UNIVERSITY

June, 1992

4-10-4

THESIS APPROVAL FORM

THESIS TITLE: Characterization of an Immunostimulatory Factor Associated with Spontaneously Regressing Subline of Murine L1210 Lymphoma.

AUTHOR: Husam Amin Ghnaim

DEGREE: Master of Science in Biological Sciences

COMMITTEE MEMBERS	ACCEPT	REJECT
[signature]	✓	
Robert E. Stephens	✓	
[signature]	✓	

DATE: May 11, 1992

1 Copy to Accompany Library Copy

1 Copy to Advisor

1 Copy to Departmental Files

ORAL EXAMINATION APPROVAL FORM

Husam Amin Ghnaim has sucessfully defended a thesis entitled Characterization of an Immunostimulatory Factor Associated with Spontaneously Regressing Subline of Murine L1210 Lymphoma by oral examination on March 12, 1992.
DATE

COMMITTEE MEMBERS	AGREE	DISAGREE
[signature]	✓	
Robert E. Stephenson	✓	
John J. Fenrna	✓	

DATE: May 11, 1992

1 Copy to Accompany Library Copy

1 Copy to Advisor

1 Copy to Departmental Files

ABSTRACT

CHARACTERIZATION OF AN IMMUNOSTIMULATORY FACTOR ASSOCIATED WITH SPONTANEOUSLY REGRESSING SUBLINE OF MURINE L1210 LYMPHOMA

Husam Amin Ghnaim

Master of Science

Youngstown State University, 1992

The cell free ascitic fluid from X-irradiated DBA/2J mice, bearing the spontaneously regressing subline (L1210/MR or MR) of L1210 murine lymphoma, contains a potent immunostimulatory (IST) factor that is capable of stimulating a specific secondary IgG antibody response, when injected into normal mice 3-4 days before priming with an antigen. When the ascitic fluid was fractionated by Ultrogel AcA-22 column chromatography, the IST activity was localized in the high-molecular-weight fraction (>1000 Kd), designated as UG-IST. UG-IST, on the other hand, suppressed the primary IgM antibody response. Further purification of UG-IST by ultracentrifugation in KBr at densities of 1.25, 1.063, and 1.006 g/ml revealed that IST behaved like a high-density lipoprotein (HDL-IST), and was more potent than complete Freund's adjuvant in stimulating the secondary IgG response. Extraction of UG-IST by chloroform-methanol revealed that the IST activity was mostly associated with the aqueous

protein phase. When the extracted lipids were separated by thin-layered chromatography, compounds with Rf values similar to that of cholesterol esters were the only major lipids present. The effect of IST on the production of IgM-bearing cells in the mouse lymphoid organs (the bone marrow, spleen, and the lymph nodes) was also tested. When mice were treated with MR ascitic fluid, UG-IST or HDL-IST 3-4 days prior to organ removal and with or without antigen priming, only the bone marrow showed an increase in the percentage of IgM-bearing cells; all other lymphoid organs tested had no such increase.

We conclude from this study that this unique factor, that is capable of stimulating secondary IgG response, is a high-density lipoprotein containing almost all neutral lipids, and the active moiety may reside in the protein part of this complex. It also increases the percentage of IgM-bearing cells in the bone marrow, even without antigen priming. This IST may also be the same factor which suppresses the primary IgM response, but needs further investigation. We postulate that IST is possibly a specific B-lymphocyte mitogen, which causes the proliferation of certain precursor B-lymphocytes, and it promotes the differentiation and maturation of B cells into plasma cells by enhancing class-switching and the generation of more memory B cells in an animal when primed with a T-dependent antigen.

Abbreviations Used:

AF	ascitic fluid
ARS-BGG	azobenzenearsonate-conjugated bovine gamma-globulin
BGG	bovine gamma-globulin
BSA	bovine serum albumin
CFA	complete Freund's adjuvant
ELISA	enzyme-linked immunosorbent assay
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
HDL	high density lipoprotein
IDL	intermediate density lipoprotein
I.M.	intramuscular
I.P.	intraperitoneal
I.V.	intravenous
IST	immunostimulatory factor
KBr	potassium bromide
LDL	low density lipoprotein
LPS	lipopolysaccharide
MR	the spontaneously regressing subline of the L1210 murine lymphoma
MR-AF	the ascitic fluid of mice bearing L1210/MR tumor
NMS	normal mouse serum
O.D.	optical density
P	the parental subline of the L1210 murine lymphoma
PBA	polyclonal B cell activator
PBS	phosphate-buffered saline

PFC	plaque forming cells
PNPP	para-nitrophenol phosphate
S.C.	subcutaneous
SRBC	sheep red blood cell
TLC	thin-layered chromatography
UG-IST	Ultrogel-purified IST, the preparation obtained from peak number 1 of fractions of Ultrogel column chromatography of MR-AF
VLDL	very low density lipoprotein

ACKNOWLEDGEMENTS

I would like to thank my research advisor, Dr. Peter Koo, for helping me produce a piece of quality research. I feel that Dr. Koo has provided the quality training necessary to allow me to become a productive researcher. I would like also to express my gratitude to the faculty members at Youngstown State University, especially Dr. Robert Leipheimer, my graduate director, and Dr. John Yemma for their guidance and support.

Furthermore, I would like to take this opportunity to thank a few other people who were instrumental in my obtaining a Master's degree. First, I am indebted to my parents for giving of themselves and sacrificing so much in my behalf, while asking so little in return. I appreciate my father's guidance in helping me choose a career in science and for nurturing my aspirations and for helping me attain them. I would like to thank him for his financial support and for allowing my intellectual maturity by encouraging my attendance at the finer institutions of learning.

I would also like to express my appreciation to my friends who saw me through this trying time of life. I would like to give special thanks to Mohammad Abel-Rasoul whose influence on my life is immeasureable. He has stood by me during very hard times, where his kindness and emotional support have enabled me to maintain a positive outlook on life and envision a brighter future.

Finally, I would like to thank the Loeffler Oncology Center (Hartvill, Ohio) for the use of a linear accelerator, and Dr. James Panuska and Kelly Taha for their help and guidance.

TABLE OF CONTENTS

List of Figures:

List of Tables:

CHAPTER I

INTRODUCTION

When progress in clinical immunology revealed an immune impairment or dysfunction associated with allergies, autoimmune diseases, infectious diseases, cancer and AIDS, it became tempting to develop agents that can affect the immune system, primarily for stimulating the immune system to defend the body against infections and to correct immune disorders. When the infections can't be treated, investigators turn their attention to the prevention of many diseases and disorders. To achieve the prevention of diseases the use of vaccines has become very popular, because many vaccines are able to enhance the immune defense system against the causative agents. During vaccination the body is exposed to attenuated or chemically altered infectious agents which do not cause diseases but are capable of stimulating protective immune responses. IgM antibodies are the first class of immunoglobulins produced by B lymphocytes after the first vaccination (the primary immune response) (Gregoriadis et al. 1988). The IgM level reaches its peak level about 5-9 days from the time of the first antigen exposure. In addition to the IgM production, primary immunization will also generate memory B cells. When the body is re-exposed to the same infectious agent, either in its native form or as a vaccine, the secondary immune response is then generated. The main

antibodies in the secondary antibody response are the IgG class. Secondary IgG is produced in much higher concentration than the secondary IgM, and it also reaches its peak level faster (within 3-5 days), and lasts much longer than the primary antibody response (Klein 1978).

Vaccination provides the body with the capability to attain a stronger immune response (that is, the secondary or memory immune response), and to respond quickly whenever the host encounters the same infectious agent again. In the past century, many fascinating developments in the vaccination field have been achieved. The vaccines that are still being used include: 1. whole microorganisms that are either live and attenuated, or dead; 2. purified protein or carbohydrate products of an infectious agent; and 3. chemical agents that resemble the infectious agents but are not directly derived from microorganisms nor are they harmful to the host. Vaccine preparation in the future will include recombinant proteins or synthetic peptides whenever whole microorganisms are found ineffective. This implies the use of agents (adjuvants) to increase the immunogenicity of such molecules for both antibody and T lymphocyte responses, and to render weakly immunogenic vaccines more effective (Gregoriadis et al. 1988).

Adjuvants immunotherapy has stimulated much interest in non-specific active immunotherapy of many dysfunctions. Several immunoadjuvants have been discovered; they include:

1. adjuvants of bacterial origin, 2. several families of polyanionic synthetic polymers, 3. alum or aluminium hydroxide, 4. lipid A or derivatives, etc. (Fenchel et al. 1984). These adjuvants have been shown to be highly active as immunomodulators, where some enhance cell-mediated immunity, others enhance antibody production to test antigens, viruses, and tumor vaccines. Some even alter the lymphoproliferative response of B and T cells to antigens (Fenchel et al. 1984).

Immunoadjuvants have been used successfully in vaccine preparation. For example, aluminium hydroxide is included in the diphtheria/tetanus/pertussis (DTP) trivalent vaccine. In the trivalent vaccine, not only is pertussis an immunoadjuvant itself as well as antigen, but aluminium hydroxide also boosts the immune response to the trivalent vaccine (Chapel et al. 1983).

In spite of their great potentials, immunoadjuvants used in clinical trials in the past have yielded conflicting results regarding their effectiveness as immunotherapeutic agents for immune dysfunctions and neoplasms. The reasons for the failures in many cases are several fold. Many experimental and clinical testing were conducted without a firm biological basis and important guidelines were not fully taken into account. The guidelines include: 1. the role of the host factors on the pharmacokinetics of an agent, thus modifying its potential; 2. the host-parasite or host-tumor

relationship; 3. the influence of associated therapies that could modify the expression of the dysfunction. In addition, until recently little attention was directed toward understanding the pharmacological features and the mode of action of the biological response modifiers to be sure of their efficiency. Meanwhile, many agents came into use before their composition was known, and before the extent of their toxicity, adverse effects, and carcinogenicity was appreciated. The first complication in the use of an adjuvant is the development of a granulomatous reaction, as in the case of using alum, CFA and monophosphoryl lipid A. A second undesiriable effect of adjuvants, such as LPS, is their pyrogenicity that may be potent. Also some adjuvants, such as lipophilic MDP derivatives, can lead to anterior uveitis and blindness, when used in Cynomolgus monkeys (Gregoriadis et al. 1988).

With the great advances that have taken place in immunology during the last decade, at least some of the effects of immunomodifiers are being defined at the cellular and molecular levels (Gregoriadis et al. 1988). Consequently, there has ensued a rather ambiguous interpretation of the value of agents that modify the immune system. Despite the problems that beset its development, immunomodulation remains a fascinating approach to the treatment and prevention of infectious diseases, immunodeficiencies, and neoplasms (Fenchel et al. 1984).

The modification of the biological or immunological response can occur by one or several mechanisms: (a) augmenting anti-antigen immunity by modulating cellular and/or humoral components of the immune system and inducing or restoring effector cells of the immune system, including identifiable subsets of lymphocytes, cells of the monocyte-macrophage lineage, and natural killer cells; or (b) protecting and/or reconstituting stem cell replication in the bone marrow, which also increases the ability of the host to tolerate the damage (Fenchel et al. 1984).

The work being described in this thesis is a continuation of earlier studies on the immunostimulatory factor (IST) by Dr. Peter Koo and his co-workers, who isolated this factor from a subline of the murine L1210 lymphoma designated as L1210/MR or MR. Unlike the parental L1210 lymphoma (P), this MR mutant subline of this tumor is a spontaneously regressing tumor in CD2F1 hybrid mice (Koo et al. 1982). MR and P do not differ significantly in their growth characteristics <u>in vitro</u> or in irradiated mice, but MR cells can protect CD2F1 mice from the lethal effects of P cells, when co-injected (Koo et al. 1982). Certain immunoregulatory factor(s) (IRF) have been found specifically associated with MR tumor only. MR cell extract (MR-CE) and cell free ascitic fluid (MR-AF) contain a potent factor which greatly suppresses both the primary and secondary cytotoxic T cell responses in mice. These factors appear to act directly

on the precursor T cell and not on the sensitized cells (Koo et al. 1981). Both MR and P contain immunoregulatory factor(s) which depress the primary antibody response of mice. But only MR-AF contains a factor which greatly enhances the secondary IgM and IgG antibody responses *in vivo* (Laufer et al. 1981). Fractionation of MR-AF by gel filtration yielded three major U.V.-absorbing peaks (about 80 Kd, 300 Kd, and 1000 Kd), all of which depressed the primary T-killer cells response. Only the 1000-Kd peak material suppressed the primary antibody response and enhanced the secondary antibody response. However, these IRF had little or no effect on the delayed hypersensitivity skin reaction to dinitrochlorobenzene (DNCB) and skin allograft rejection response (Laufer et al. 1981).

The high molecular weight peak was further fractionated by sucrose density gradiant ultracentrifugation into a high-density fraction (HDF) and low-density fraction (LDF). LDF, which is also found in P-AF, suppressed the primary antibody response but still enhanced the secondary antibody response; while HDF, which occurs only in MR-AF, did not affect the primary, but greatly stimulated the secondary antibody response. It was also found that the immunostimulatory factor (IST) is sensitive to protease digestion and heat ($57^{\circ}C$, 30 minutes), whereas the immunosuppressive factor (ISP) is resistant to protease and nuclease, but sensitive to heat and U.V. irradiation (Laufer et al. 1981).

These results suggest that several MR-associated factors act selectively on various immune functions, and that suppression of the secondary cytotoxic T cell mediated immunity, and the primary antibody response does not preclude tumor regression in this system. Also in vivo experimental results suggest that secondary antibodies and macrophages are good candidates as the effectors in MR tumor regression. The secondary antibodies appear to interact with the macrophages in producing the tumor-cytolytic effect in vivo. And it appears that MR-specific immunostimulatory factor and the heightened antibody response may be important for the spontaneous regression of the MR tumor (Koo et al. 1981).

The above-described results agree with those of Carlson and Terres (1976). They found that L1210/MTX - REV subline lymphoma cells were susceptible to cytolysis by 7S antibodies injected into the tumor-bearing mice, whereas the 19S antibodies were totally ineffective. They also indicated that antibody-induced cytotoxicity by normal lymphoid cells (possibly NK cells or lymphocytes with NK-like activity) did not require complement, and the 7S antibodies were responsible for inducing the cytotoxic process. The cell killing might also occur through the phagocytosis of the antibody-coated cells. They found that the host factors involved in the antibody-induced killing were relatively radiation-resistant (670 R)(Carlson et al. 1976). This suggests that macrophages could be involved, since they are

known to be radiation-resistant. Although antibody-induced killing *in vivo* was found to be radioresistant, the protective effect was radiosensitive (Carlson et al. 1976).

In addition Koo (1981) reported that IST factor was most effective when injected into mice 3-4 days before primary immunization. This supports the results of Koo (1980) indicating that this factor may act on non-sensitized immunocytes. A factor with this ability to stimulate an immune response before antigen priming is a non-specific immunostimulatory factor, and it is expected to produce the same effect with any antigen, either pathogenic or non-pathogenic. The enhanced activity on the secondary immune response was achieved without the use of any adjuvants aside from IST administration, followed by primary immunization (Koo et al. 1981).

Many compounds have been found to act as immunomodulatory factors on the immune system in many different ways. Our interest is mainly concerned with substances that are capable of stimulating a host's immune response to a variety of antigens. The first example described in the following is a polyclonal B cell activator (PBA), which is not specific for a single antigen or subset of B lymphocytes that respond to PBA or any other mitogens to produce immunoglobulins. Several criteria which are used to define an agent as a polyclonal B cell activator are listed as follows:

1) Proliferation of lymphoctes induced by the agent must not be due to a previous immunization of the lymphocyte donor by the putative mitogen or by a chemically related structure, which cross-reacts with the agent (Boua et al. 1979).

2) Proliferation of B cells induced by the agent must occur in the absence of T cells. In the case of human lymphocytes, protein A from Staphylococcus aureus (PASA) may serve as a polyclonal B cell mitogen under certain conditions (Forsgren et al. 1976). However, certain so-called "mitogens", such as pokeweed mitogen (PWM), are now known to require T cells (Brochier et al. 1976a and 1976b). Such "mitogens" may stimulate B cell secondarily (for example, by inducing the liberation of T cell-derived lymphokines, such as lymphocyte mitogenic factor which then provides the direct stimulus to B cells), therefore such agents are not considered as true B cell mitogens (Boua et al. 1979).

3) The agent must induce the differentiation and maturation of resting small B lymphocytes into plasma cells (antibody producing cells), which produce antibodies of different specificities that may not crossreact with the stimulant (PBA). Since PBA can trigger B cells non-specifically without help from T cells, such triggered B cells can't mature into memory B cells nor undergo class switching and affinity maturation. Consequently, only IgM antibodies of low affinity are produced by each B cell clone; IgG and other major immunoglobulin classes are generally not produced (Boua

et al. 1979).

Several substances are known to fulfill these criteria when murine lymphocytes are used. Lipopolysaccharide (LPS) from various Gram-negative bacteria (Andersson et al. 1972), enterotoxin B (Janossy et al. 1975), dextran sulfate (Dorries et al. 1974), flagellin and levan (Continho et al. 1973a), and Pneumococcal SIII polysaccharide (Continho et al. 1973b). Lipoprotein(s) have also been shown to affect various immunological responses. The intermediate density lipoprotein (IDL) (isolated at the density between 1.006 g/ml and 1.019 g/ml) injected into the mice resulted in the suppression of the primary humoral response to sheep red blood cells (SRBC) (Barclay et al. 1975), the generation of T lymphocytes, and the immuno-protection against the growth of syngeneic tumor cells. It was later shown that neutral lipid-depleted LDL-In retains full biological activity and receptor specificity (Barclay et al. 1975). These studies suggest that the biologically active component(s) of LDL responsible for immunosuppression resides in one or more of the remaining constituents of the lipoprotein including the lipids and/or apoproteins (Barclay et al. 1975).

From all the studies that have been done with immuno-modulatory factors, we notice variations in the type of such agents, their effects on the immune system, and their mode of action. In comparing the effects of these agents with the effects achieved with the factor in the MR-AF (IST) on the

immune system of mice, we noticed some characteristic differences. As described above IST was most effective when injected intraperitoneally 3-4 days before antigen-priming. The ability of this factor to stimulate both the secondary IgM and IgG antibodies is a unique mechanism of action.

The purpose of the experiments reported in this thesis is to further characterize the immunostimulatory component in the MR-AF (IST) and to determine the site of its action in the lymphoid system by examining its effect on the cells of the lymphoid organs (the spleen, lymph nodes, and the bone marrow). The bone marrow is considered as the Bursa-equivalent organ in adult mammals, which is needed for B cell maturation and differentiation; it is also the site of origin for all hematopoietic cells (Klein 1978). Hence, the bone marrow is of major importance to us in this study, because it could be the site of IST stimulation and the site for the development of precursor B memory cells.

CHAPTER II

MATERIALS AND METHODS

Animals: Six to twelve week old DBA/2J and CD2F1 mice (hybrids of BALB/C X DBA/2J, that accepted skin grafts from DBA/2J mice), bred at Northeastern Ohio Universities College of Medicine, were used throughout these studies. The mice were kept in groups of 3-5 animals per cage (15 X 12 X 25 cm), 12 hours of light/dark, fed freely, and at 20-25°C.

Tumors: The tumor cell line used here is a mutant subline of the murine L1210 lymphoma, designated as L1210/MR. This tumor cell line arose in this laboratory in DBA/2J mice that survived L1210/MTX-Revertant for about one month (Koo and Laufer, 1979). The methotrexate-sensitive L1210/MTX-Revertant cells were originally derived from the methotrexate-resistant mutant (L1210/MTX), which was derived from the parental L1210 cell line designated as L1210/P or P (Koo and Laufer, 1979). The MR line in contrast to L1210/MXT-Revertant or the parental L1210 line, is much less lethal and spontaneously regresses in both DBA/2J and CD2F1 mice. Therefore, MR cells were passaged in irradiated (650 R) DBA/2J mice (Koo and Laufer, 1979).

Ascitic Fluid: Ascitic fluid used in this study was obtained from the X-irradiated (650 R) DBA/2J mice. Mice were injected intraperitoneally (i.p.) with one million cells and the ascitic fluid was collected seven days later from the

peritoneal cavity with phosphate-buffered saline (PBS) without calcium and magnesium under sterile conditions. Part of it was cultured on blood agar under both aerobic and anaerobic conditions to detect for bacterial growth or contaminations. The remaining cell suspension was centrifuged at 223 X g (1000 rpm) for 10 minutes, and the clear ascitic fluid supernatant was obtained and designated as MR-AF. Only uncontaminated MR-AF samples were pooled and used. The cell pellet obtained was washed with PBS, resuspended in dimethyl sulfoxide + 10% fetal calf serum (FCS), and stored at -140° C.

Gel Filtration Column Chromatography: MR-AF (A at 280 nm = 7-15) obtained from the X-irradiated DBA/2J mice was applied to an Ultrogel AcA-22 column (5 X 80 cm). After the column had been equilibrated with PBS and the MR-AF was eluted with PBS. The fractions (5.6 ml each) were collected within 48-hours at 6° C. The optical density for the eluted fractions was determined at 280 nm. The fractions were pooled into three pools with respect to the three major absorbance peaks generated (Figure 1). The pools were concentrated separately by Amicon YM-100 membrane filtration under nitrogen or carbon dioxide positive pressure. The concentrated pools were then stored at -20° C until they were used for i.p. injection into mice to determine their effects on the secondary IgG response and to determine their effects on lymphoid cells in in the immune organs (the spleen, lymph

nodes, and the bone marrow).

KBr Ultracentrifugation: Two-to-three ml of concentrated pool 1 in PBS from the Ultrogel column (Figure 1, first peak) was adjusted to a final density of 1.25 g/ml, or 1.063 g/ml, or 1.006 g/ml with KBr (potassium bromide) in cellulose nitrate tubes (Beckman). Ultracentrifugation of the samples was carried out at 226,240 X g (45,000 rpm) in a TI50 rotor and model LS-75 ultracentrifuge (Beckman) at 12 C for 24 hours (Prezioso et al. 1987). After centrifugation each tube was divided into three fractions which were removed carefully by Pasteur pipets. The top fraction removed equals the volume of the pooled sample that was applied to each tube (2-3 ml) and the remaining volume was divided in half (4-5 ml each fraction). The absorbance at 280 nm was measured for each fraction using the corresponding fraction from PBS tube as control. The individual fractions were then dialysed against PBS at 4^{o} C to remove KBr, and the absorbance for each fraction was measured at 280 nm against PBS after dialysis. The individual fractions after dialysis were tested for their effects on the bone marrow and the secondary antibody response of mice (Prezioso et al. 1987).

Lipid Extraction, Purification, And Thin-Layered Chromatography: Pool 1 from the Ultrogel column (containing IST) was concentrated by Amicon YM-100 membrane filtration,

then it was dried under a stream of nitrogen gas at 40°C. The white powder obtained was extracted with chloroform-methanol (3 : 1, V/V) (Fisher Scientific) at room temperature. The chloroform layer (bottom layer) was collected with a Pasteur pipet, flash evaporated to dryness at 40°C, and then resuspended in chloroform/methanol (9 : 1, V/V). Part of this suspention was applied to a thin-layered chromatographic (TLC) plate (Adsorbosil Plus, Alltech Associates, Inc. Deerfield, IL), which was developed using the solvent system chloroform / methanol / water (65:35:6, V/V/V). Standards included phosphatidylcholine and phosphatidylethanolamine. For the detection of hydrocarbon compounds, iodine vapor was used. For lipid detection, rodamine G-6 in ethanol (Sigma) was used. The detection of phospholipids was attained by using molybdenum blue reagent (Alltech Associates, Inc.). Cholesterol and cholesterol esters were detected by ferric chloride stain, and amino compounds were detected by ninhydrin stain (Bigh et al. 1959; Prezioso, 1988).

After extraction, the chloroform phase (containing lipids) was dried under nitrogen and reconstituted in PBS by vigorous shaking and vortexing. The aqueous phase (containing hydrophilic protein) was dialysed against PBS. Both reconstitutes were then diluted to a concentration equivalent to two O.D. units/ml of the original MR-AF, for i.p. injection into mice.

Cellulose Acetate Membrane Electrophoresis:

Material: Cellulose membrane (sepraphore III), high resolution electrophoresis buffer (Tris-Barbital-Sodium Barbital pH 8.8 and pH 7.0), absorbent pads, Ponceau S solution, Sepra Clear II (0.5% Ponceau S in 7.5% aqueous trichloroacetic acid), 5% acetic acid solution (rinse solution), electrophoresis chamber, power supply, staining trays, and sample applicator. All were obtained from Gelman Instruments, Ann Arbor, Michigan.

Procedure: The samples (3 ul each) were applied by a sample applicator to strips of cellulose membrane. They were then blotted dry and electrophoresed at 350 volts for 45 minutes, and 1-3 milliamperes per strip. Immediately after electrophoresis, the strips were removed, and stained in Ponceau S solution for 10 minutes. The strips were destained in 5% acetic acid solution for approximately 10 minutes to remove excess stain. The strips were fixed on glass slides as follows: The strips were removed from the final rinse tray, and immersed in Sepra Clear II solution for 5 minutes. They were then placed on glass slides, and their ends were folded over the edges of the slides. The slides were then placed in 80-90^{o}C oven for 20 minutes (Gelman 1976).

Antigens And Immunization Protocol:

Antigens: Five percent sheep red blood cells (SRBCs) (Cordis Laboratories, Miami, FL) suspended in saline solution were

used for intravenous (i.v.) immunization. Azobenzenearsonate-conjugated bovine gamma globulin (ARS-BGG) was prepared by coupling 100 mg bovine gamma globulin (Sigma) in 40 ml of 0.1 M $NaHCO_3$ (pH 8.1) with 60 ml of 0.02 M diazotized p-arsanilic acid. The diazotized arsanilic acid was prepared according to Koo and Cebra (1974). The coupling reaction was carried out at 0-5°C by slow addition of diazotized arsanilic acid solution to the bovine gamma-globulin solution. The pH of the solution was maintained between 8.5-9.0 with 5 M NaOH. The reaction mixture was stirred overnight at 0-5°C. The ARS-BGG solution was then exhaustively dialysed against PBS at 5° C (Koo and Cebra, 1974).

Immunization Protocol: Four days before primary immunization 3-5 mice in each group were injected i.p. with a dose of 1 ml of O.D. 2 at 280 nm of the original MR-AF or equivalent amount of test material (pool 1 from ultrogel filtration, or PBS as control). Then at day zero 3-5 animals of each group were killed, and the bone marrow, lymph nodes and the spleen were obtained. Indirect immunofluorescence assays were done with the lymphoid cells to determine the percentage of the IgM-bearing cells in the mice injected with the test materials as compared to the mice injected with PBS. For the other part of the study, at day zero, 3-5 mice in each group were immunized i.v. with a test antigen (5% SRBC). At day seven the mice were killed and their lymphoid organs

obtained to determine the percentage of IgM-bearing cells.

In a separate experiment mouse immune serum was obtained and assayed for IgG level by ELISA. Three to five mice in each group were injected i.p. with the test material four days prior to primary immunization (at day -4), MR-AF was usually centrifuged at 22,340 X g (10,000 rpm) for 15 minutes to remove all traces of precipitate before it was injected into mice. The control group was injected with an equal volume of PBS. At day zero and day nine all groups of mice were injected with 0.5 ml of the 1 mg/ml ARS-BGG solution (0.25 ml I.M. + 0.25 ml S.C.). In another group the mice were primed with ARS-BGG emulsified in complete Frreund's adjuvant (CFA), and nine days later the mice were boosted with 0.5 ml ARS-BGG (1 mg/ml). Finally, at day fourteen the mice were bled through the orbital sinus under ether anesthesia. The blood was allowed to clot for one hour at room temperature, and the clot was broken and allowed to contract at 4° C overnight. The serum was collected and clarified by centrifugation at 2,010 X g (3,000 rpm) for 10 minutes at 4°C. The serum was stored in small aliquots at -20°C before use.

Collection of Lymphoid Cells: Mice were sacrificed by cervical dislocation, and their spleens, lymph nodes and bone marrow were collected into ice-cold RPMI 1640 medium containing 2% FCS and 0.1% NaN_3 . Bone marrow cells were obtained from the femur bone. After the associated tissues

were removed, both ends of the bone were cut and the bone marrow was washed out by the culture media with the aid of a 27G needle. Spleen cells and lymph node cells were dislodged from the lymphoid organs by pressing the organs against a stainless-steel wire screen. Cells were then suspended and washed three times in the RPMI medium. Leukocyte concentration and viability were determined by the trypan blue exclusion. The number of IgM-bearing cells were determined by an indirect immunofluorescent staining method.

Enzyme-Linked Immunosorbent Assay (ELISA):

Materials: All materials were obtained from Sigma. They included: 0.2% bovine gamma globulin (20 mg BGG + 20 mg NaN_3 in 100 ml PBS) prepared fresh, 5% bovine serum albumin 5 g BSA + 20 mg NaN_3 in 100 ml PBS), 1% bovine serum albumin (1 g BSA + 20 mg NaN_3 in 100 ml PBS), mouse antiserum (3-fold dilution in PBS), alkaline phosphatase-conjugated goat anti-mouse IgG, and IgM (1:1000 dilution in 1% BSA + 1 mM $MgCl_2$ + 20 mg NaN_3 in 100 ml Tris-HCl, pH 8.0), 1 mg/ml paranitrophenolphosphate (PNPP) disodium solution (in 1 mM $MgCl_2$+ 1 mM $ZnCl_2$ + 20 mg NaN_3 in 0.1 M glycine buffer, pH 10.4), 96-well flat-bottom tissue culture plates (Fisher Scientific), incubator (37°C + 100% humidity + 5% CO_2in air), ELISA-plate reader (at 405 nm wave length), and PBS was used as washing solution.

Procedure: Equal aliquots (75 ul) of freshly prepared 2% BGG

were added to all the wells in flat-bottomed tissue culture plates and the plates were incubated at room temperature overnight. The BGG solution was decanted by flicking the plates forcefully and the plates were washed twice with PBS. BSA (100 ul of 5% solution) was added to all the wells and the plates were incubated for 1 hour at 37°C to block any non-specific binding sites. The BSA solution was decanted and the plates were washed twice with PBS. Then 45 ul of appropriate dilution of test samples (normal mouse serum, mouse antiserum, 1% BSA, and PBS, were added to the wells (in which 1% BSA and PBS were used as controls). The plates were then incubated at 37°C for 2 hours. The samples were then decanted and the wells washed with PBS three times. Aliquots (75 ul) of alkaline phosphatase-conjugated anti-mouse IgG or IgM antibodies prepared in goats were then added to all wells and incubated for 2 hours at 37°C. The plates were then decanted and washed with PBS three times. PNPP (60 ul) was added to all the wells, and the plates were incubated again for 30 minutes at 37°C. The color change was measured at 405 nm by an ELISA reader.

The results were reported as arithmetic average of the absorbance at 405 nm for each serum dilution used and they were plotted and curve-fitted by the Cricket Graph computer program by Macintosh. Then the slopes of the obtained curves were compared and tested separately for their significance against the slope of the control (PBS) by the unpaired

Student's t-test for the slopes. When $p \leq 0.05$ the difference was considered to be significant.

Indirect Immunofluorescent Assay:

Antibody Absorption: One ml of concentrated stock solution of rabbit anti-mouse IgM was added to a normal mouse spleen cells which had previously been washed in PBS. The mixture was incubated on ice for 30 minutes with occasional agitation, and then centrifuged at 446 X g (2,000 rpm) for 15 minutes. The supernatant, containing the absorbed antibody, was collected and kept frozen at -20°C before use.

Lymphoid Cell Preparation: Bone marrow, spleen, and lymph node single cells were suspended in RPMI 1640 containing 2% FCS and 0.1% NaN_3, centrifuged at 223 X g (1,000 rpm) for 10 minutes, resuspended in the washing solution (PBS + 2% FCS + 0.1% NaN_3), and allowed to settle for 2-5 minutes. The top 1 ml suspension was collected to obtain single - cell suspension, that was adjusted to one million cells per ml. Cell viability was determined by trypan blue exclusion.

Cell Staining: Tubes containing one million dissociated lymphoid cells were centrifuged at 223 X g (1,000 rpm) for 5 minutes to remove the supernatant. To the cell pellets, 100-ul aliquots of the absorbed normal rabbit serum, or absorbed normal horse serum (1:10 dilution in PBS) were added, and the

mixtures incubated on ice for 20 minutes. They were then washed three times with the washing solution by centrifugation at 223 X g for 5 minutes each. This step was to block any non-specific antibody binding to the pellets. To the experimental tubes, a previously determined dilution of absorbed rabbit anti-mouse IgM (100 ul) was added. And to another set of control tubes, the same dilution of absorbed normal rabbit serum (100 ul) was added to control for the non-specific binding. All tubes were placed on ice for 30 minutes, and then washed three times by centrifugation at 223 X g for 5 minutes. To all resulting pellets FITC-conjugated goat anti-rabbit IgG (100 ul) was added, and the mixtures were incubated on ice for 30 minutes. The cells were again washed four times with the washing solution by centrifugation as above. The cells were then resuspended in 500 ul 0.5% para-formaldehyde (which fixes and stabilizes the stain in the dark for one week). Wet mounts of cells were prepared on microscope slides and were sealed at the edges by nail polish. The wet mounts were read under a fluorescent microscope (Todd et al. 1981; Fearon et al. 1983).

Results were reported as the arithmetic average of the percentage of fluorescent cells. Differences between the experimental groups and the control were determined separatly by the unpaired Student's t-test. When P was $\leq$ 0.05 the differences were considered to be significant.

CHAPTER III

RESULTS

In order to purify and characterize the immunostimulatory component in the ascitic fluid of mice bearing the spontaneously regressing subline of the the murine lymphoma L1210 (MR), several purification techniques were employed, and the partially purified IST was characterized with respect to its immunological and physiochemical properties. The results are presented below.

Ultrogel Filtration Column Chromatography:

MR-AF from several groups of irradiated and MR tumor bearing mice was collected and applied to an Ultrogel column at 6°C. The optical densities (at A = 280 nm) of the collected fractions were plotted against the fraction number. As shown in Figure 1, three major peaks were identified: the first peak at fraction number 175-190 (also designated as UG-IST) , the second peak at fraction number 191-340, and the third peak at fraction number 340-370. The fractions within each peak were pooled. The resulting pools were separately concentrated by Amicon YM-100 membrane under N_2 or CO_2 positive pressure.

To determine which pool contained the immunostimulatory effect on the secondary IgG response, different groups of mice (3-5 mice per group) were injected with the different

pools i.p.; another group was injected with MR-AF i.p. as a positive control to make sure that the MR-AF used was itself active. The third group was injected with PBS as a negative control. All these injections were done four days before the primary immunization with ARS-BGG. On days 0 and 9 all mice were immunized with ARS-BGG, and at day 14 they were bled and their sera were assayed by ELISA for anti-BGG IgG antibodies. The results are shown in Figure 2, where MR-AF and UG-IST (pool 1) are shown to significantly stimulate the secondary anti-BGG IgG antibody level (9-27 - fold increase) in comparison with that of the PBS control. Pools 2 and 3 elicited only about a 3-fold increase (not significant).

To determine the effect of UG-IST on the primary IgM antibody response, different groups of mice were injected i.p. with either UG-IST or PBS (as a control) four days before the primary ARS-BGG immunization. Seven days after the immunization the mice were bled, and their sera were assayed by ELISA for the level of IgM anti-BGG antibodies. The results, as shown in Figure 3, indicate that UG-IST slightly suppresses the primary IgM production by about 2-fold (significant).

Ultracentrifugation Results:

UG-IST was applied to KBr at different densities (1.25, 1.063, and 1.006 g/ml), and three fractions were collected from each density tube as described in the methods. The

optical densities of the dialyzed fractions in PBS were measured at 280 nm, and the results were shown in Table 1.

To determine which density fraction contained the stimulatory effect, different groups of mice were injected with different fraction samples four days before primary ARS-BGG immunization. The mice were immunized in the same manner as for testing the effect of MR-AF described above. Then the mouse serum was assayed by ELISA to determine the effect of the different fractions on the secondary anti-BGG IgG antibody response.

Figure 4 shows the effect of the fractions from ultracentrifugation at KBr density of 1.25 g/ml on the secondary antibody response of mice. The results indicate that F 1 (the top fraction of the tube) was the most active fraction. This fraction normally contains the high density lipoproteins (HDL), low density lipoproteins (LDL), very low density lipoproteins (VLDL), and chylomicrons (CM) (Prezioso, 1988). For the fractions obtained at KBr density of 1.063 g/ml, the immunostimulating activity was distributed among the different fractions as shown in Figure 5, although F3 contained more activity than the other two fractions. At KBr of 1.063, the top fraction normally contains LDL, VLDL, and CM; while the lower fractions contain HDL, and protein (Prezioso, 1988). For the fractions obtained at density of 1.006 g/ml, most of the immunostimulatory effect was detected in F 3 (the bottom fraction), as shown in Figure 6.

At a density of 1.006 g/ml the fraction at the top of the tube normally contains VLDL and CM, while the bottom fraction contains HDL and proteins; LDL is distributed among all the fractions (Prezioso, 1988).

The immunostimulatory effect of UG-IST was compared to that produced by complete Freund's adjuvant (CFA) on the secondary IgG response to ARS-BGG in mice. The results are shown in Figure 7, which demonstrates that there is a three-fold increase in the effect of UG-IST, and about a two-fold increase in the effect of F 1 in comparison with CFA.

Lipid Extraction And Thin-Layered Chromatography:

To determine the lipid content in the UG-IST (the immunostimulatory fraction from the Ultrogel column) and F 1 fraction (from ultracentrifugation in KBr at density of 1.25 g/ml), were extracted and the lipid phases were analyzed by thin-layered chromatography, along with lipid standards, by using different solvent systems for total lipid and neutral lipids. The results are shown in Figures 8 and 9. Figure 8 is the chromatogram for separating total lipids, and it shows that the vast majority of the lipids present have mobilities (R_f values) like cholesterol esters, together with two minor phospholipids (sphingomyelin (SP), and phosphatidylcholine (PC)). Figure 9 shows the type of neutral lipids present in the extract and one minor spot at the origin representing phospholipids. This chromatogram

shows that there are at least four neutral lipid spots with high Rf values like cholesterol esters.

Also, the two phases from the chloroform:methanol extraction (after dialysis and reconstitution in PBS) were tested for their effects on the secondary response to ARS-BGG. The results in Figure 10 demonstrate that most of the immunostimulatory activity was in the aqueous phase (protein phase), which shows a 12-fold increase in the amount of IgG produced in comparison to the PBS control. This was slightly less than that produced by an equivalent amount of MR-AF injected (about a 20-fold increase over the PBS control), but it was 6 times more potent than the lipid phase material.

Cellulose Acetate Electrophoresis:

To determine the electrophoretic mobility of the protein content of UG-IST, concentrated UG-IST or the aqueous phase from UG-IST extraction (the protein phase) was applied onto Sepraphore III cellulose acetate membrane strips and electrophoresis was run in tris-barbital-sodium barbital buffer at pH 8.8 or pH 7.0 for 45 minutes. Normal mouse and human serum were used as controls. The results (not shown) indicated that only one major protein in UG-IST was detected by Ponceau S stain for proteins, and that protein did not leave the origin of application during electrophoresis under both pH conditions, while under the same conditions both normal human and mouse serum were separated normally into

alpha-, beta-, and gamma-globulins.

The Effect of the Immunostimulatory Factor on IgM-Bearing Cells of mouse Lymphoid Organs:

To determine the effects of the immunostimulatory factor on the development or distribution of lymphoid cells in mouse lymphoid organs (the bone marrow, the spleen, and lymph nodes), different groups of mice were tested as described in the methods.

Lymphoid cell dissociates were stained by the indirect immunofluorescent assay described in the methods to determine the percentage of cells bearing IgM surface markers. As shown in Table 3 when MR-AF, or UG-IST, or F 1 (from the ultracentrifugation in KBr at density of 1.25 g/ml) was injected i.p. into mice , 2-to-3-fold increase in the IgM-bearing cells in the bone marrow were noted in comparison to the mice that were injected i.p. with PBS. Pools 2 and 3 from the ultrogel column on the other hand had no significant effect. The non-specific staining was always below 5%. But when the percentages of IgM-bearing cells in the lymph nodes and spleen were estimated under identical conditions (shown in Tables 4 and 5), there were no differences between the experimental groups and the control group.

The percentage of IgM-bearing cells in lymphoid organs was also estimated after MR-AF, UG-IST or PBS was injected i.p. and followed by primary SRBCs intravenous immunization.

Bone marrow, spleen and lymph node cells were collected seven days after the immunization and stained by the indirect immunofluorescent assay. The results are shown in Tables 6, 7 and 8. Only the bone marrow showed a 3-fold increase in the percentage of IgM-bearing cells in the mice that were injected with MR-AF or UG-IST when compared with mice that were injected with PBS, while lymph nodes and spleen did not show any significant increase.

Figure (1)

The fractionation of MR-AF on Ultrogel AcA-22 Column. MR-AF (A at 280 nm = 10-15, 30 ml) was equilibrated and eluted with PBS. Absorbance of each collected fraction (5.6 ml) was determined at 280 nm, and the fraction numbers were plotted against their absorbance.

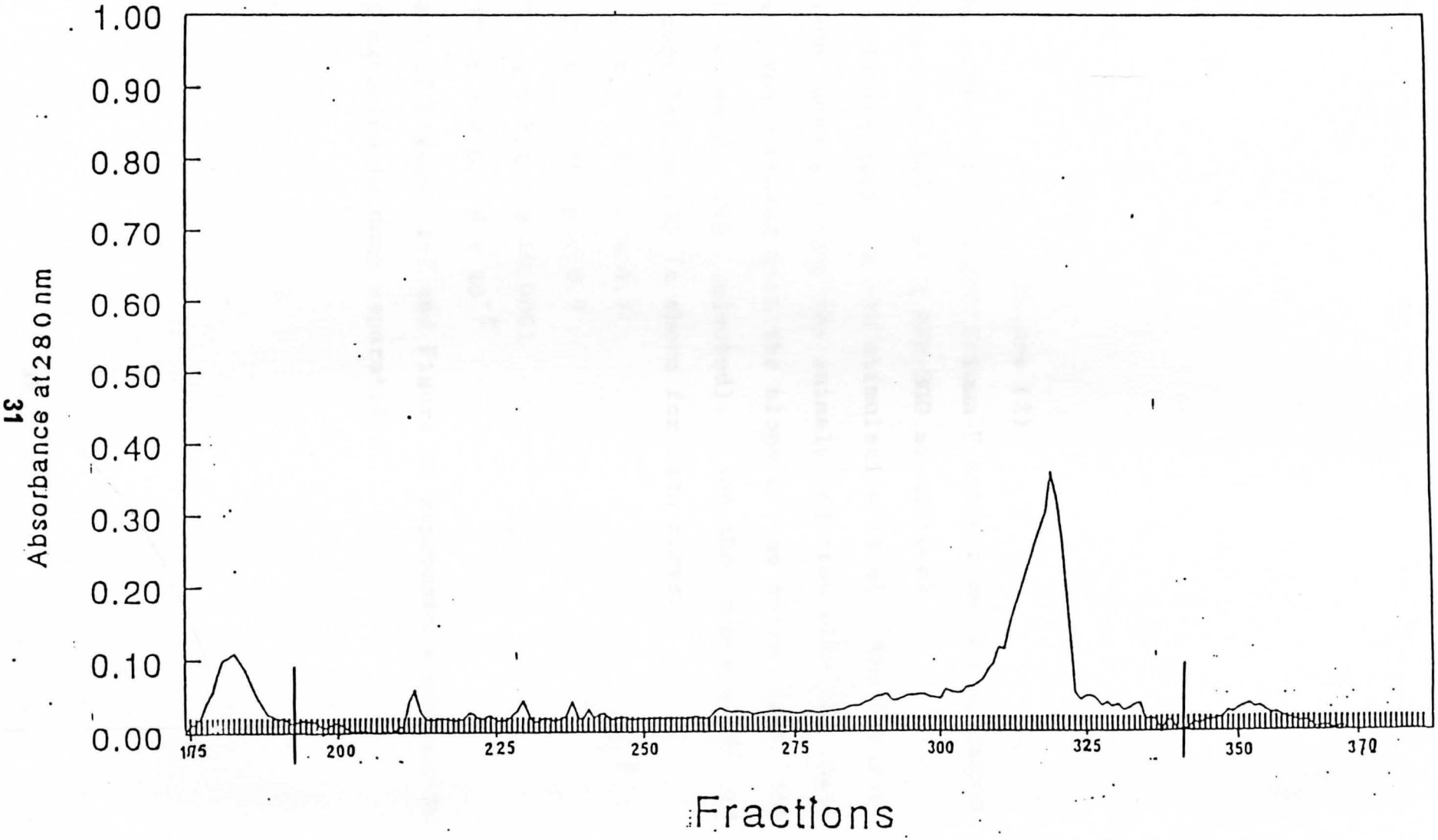
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
Absorbance at 280 nm
175
200
225
250
275
300
325
350
370
Fractions

Figure (2)

The effect of Ultrogel Column Fractions on the secondary IgG response to BGG (using ARS-BGG as antigen).

For determining the the stimulation level, the slope of the curve obtained from the animals injected with the test material was compared with the slope of the curve from the control animals (PBS injected). And the coefficient of linear correlation (R) is shown for each curve.

- Pool 2: $t = 0.37$, $P = 0.7$
- Pool 3: $t = 0.39$, $p = 0.7$
- MR-AF : $t = 2.1$, $p = 0.0001$
- UD-IST: $t = 4.03$, $P < 10^{-6}$

(Each of Figures 2-7 and Figure 10 represent a summary of several experiments done separately).

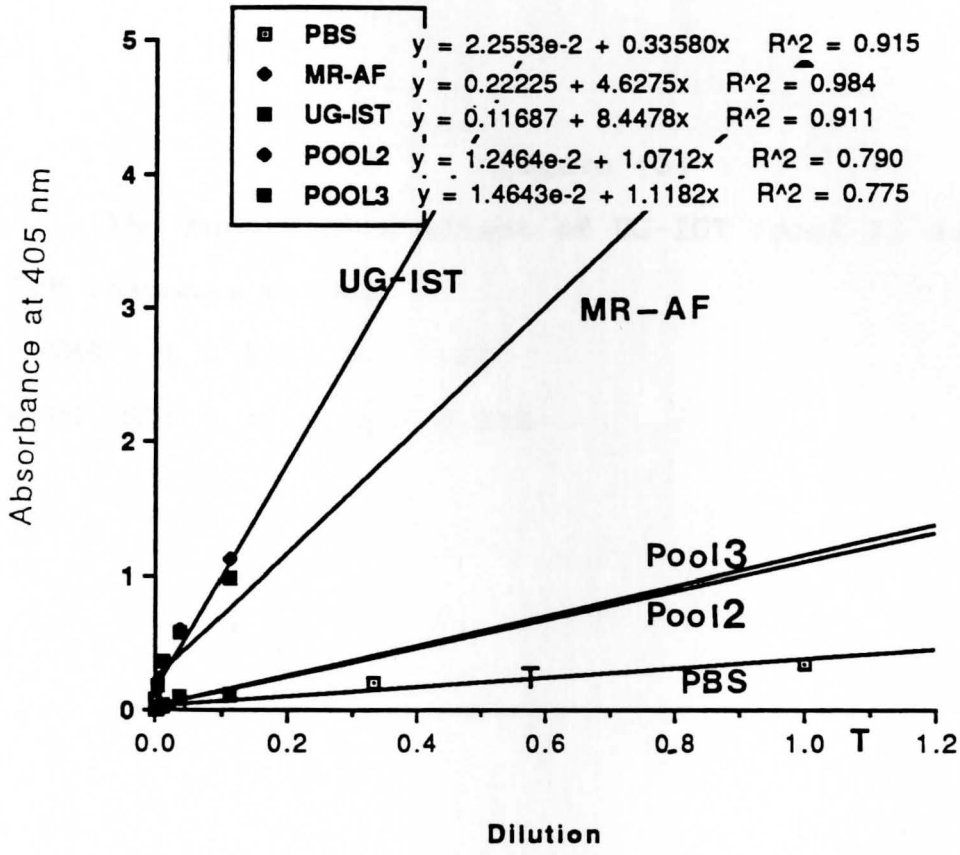
PBS y = 2.2553e-2 + 0.33580x R^2 = 0.915
MR-AF y = 0.22225 + 4.6275x R^2 = 0.984
UG-IST y = 0.11687 + 8.4478x R^2 = 0.911
POOL2 y = 1.2464e-2 + 1.0712x R^2 = 0.790
POOL3 y = 1.4643e-2 + 1.1182x R^2 = 0.775
UG-IST
MR-AF
Pool3
Pool2
PBS
Absorbance at 405 nm
Dilution
0
1
2
3
4
5
0.0
0.2
0.4
0.6
0.8
1.0
1.2

Figure (3)

The suppressive effect of UG-IST (pool 1) on the primary IgM response to SRBC.

- NMS : t = 2.3, p = 0.02
- UG-IST: t =2.6, p = 0.012

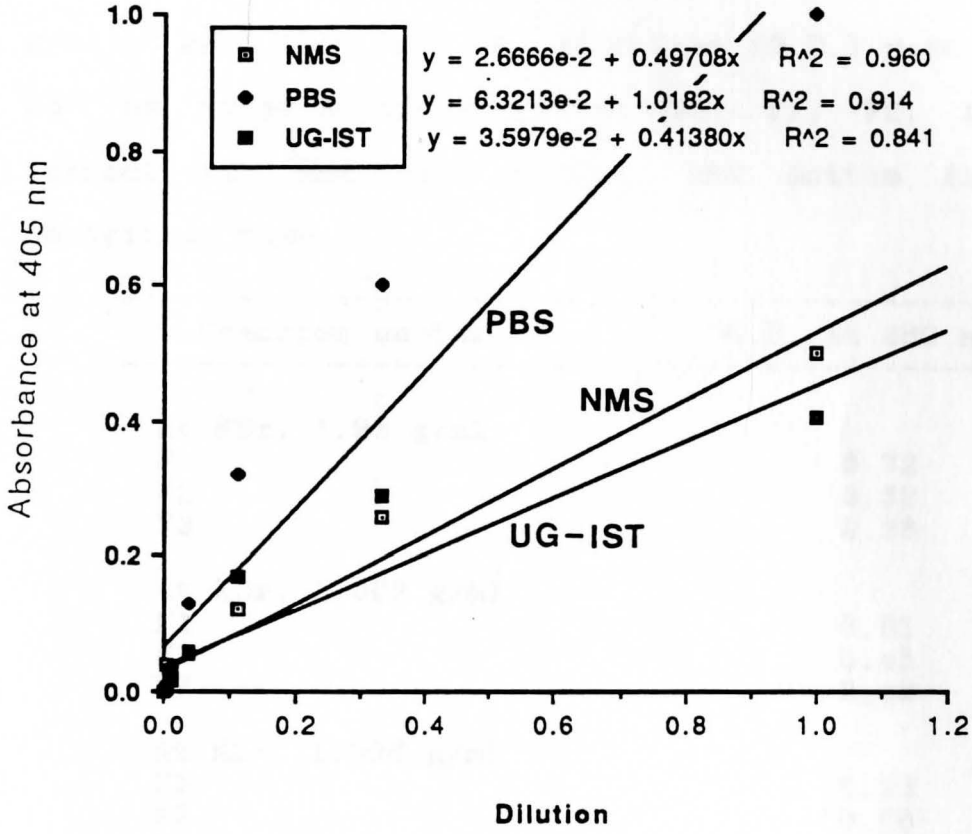
NMS
PBS
UG-IST
y = 2.6666e-2 + 0.49708x R^2 = 0.960
y = 6.3213e-2 + 1.0182x R^2 = 0.914
y = 3.5979e-2 + 0.41380x R^2 = 0.841
PBS
NMS
UG-IST
Absorbance at 405 nm
Dilution
0.0
0.2
0.4
0.6
0.8
1.0
1.2

TABLE 1

Ultracentrifugation fractions (obtained after centrifugation of UG-IST in KBr at densities of 1.25, 1.063, or 1.006 g/ml), and their optical densities (O.D.) were measured at 280 nm after dialysis against PBS. F1, F2, and F3, are respectively the top, middle, and bottom fractions in a centrifuge tube.

Fraction number	O.D. at 280 nm
At KBr, 1.25 g/ml	
F1	0.72
F2	0.32
F3	0.38
At KBr, 1.063 g/ml	
F1	0.51
F2	0.45
F3	0.42
At KBr, 1.006 g/ml	
F1	0.13
F2	0.26
F3	0.61

Figure (4)

The effect of ultracentrifugation at KBr density of 1.25 g/ml on the distribution of UG-IST in fractions F1, F2, and F3, and their effect on the secondary IgG response to BGG (using ARS-BGG as antigen).

- F1: $t = 8.2$, $p < 10^{-6}$
- F2: $t = 3.8$, $p = 0.0004$
- F3: $t = 3.9$, $p = 0.002$

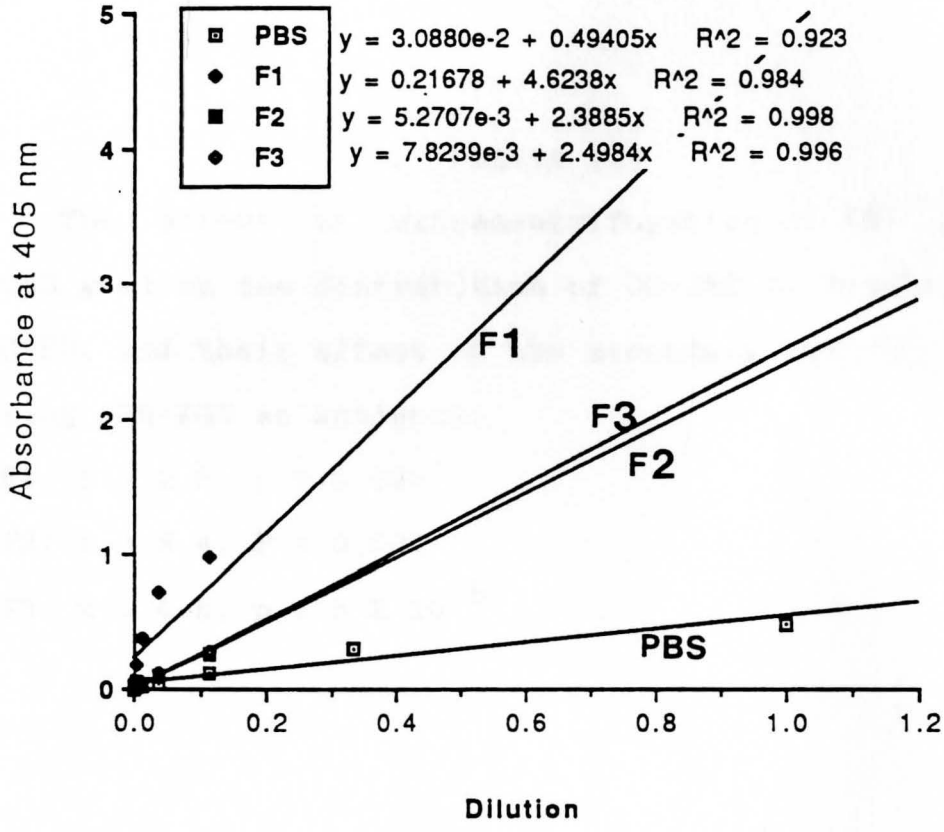
PBS
F1
F2
F3
y = 3.0880e-2 + 0.49405x R^2 = 0.923
y = 0.21678 + 4.6238x R^2 = 0.984
y = 5.2707e-3 + 2.3885x R^2 = 0.998
y = 7.8239e-3 + 2.4984x R^2 = 0.996
F1
F3
F2
PBS
Absorbance at 405 nm
Dilution
0
1
2
3
4
5
0.0
0.2
0.4
0.6
0.8
1.0
1.2

Figure (5)

The effect of ultracentrifugation at KBr density of 1.063 g/ml on the distribution of UG-IST in fractions F1, F2, and F3, and their effect on the secondary IgG response to BGG (using ARS-BGG as antigen).

- F1: $t = 2.9$, $p = 0.005$
- F2: $t = 3.4$, $P = 0.001$
- F3: $t = 4.5$, $p = 5 \times 10^{-5}$

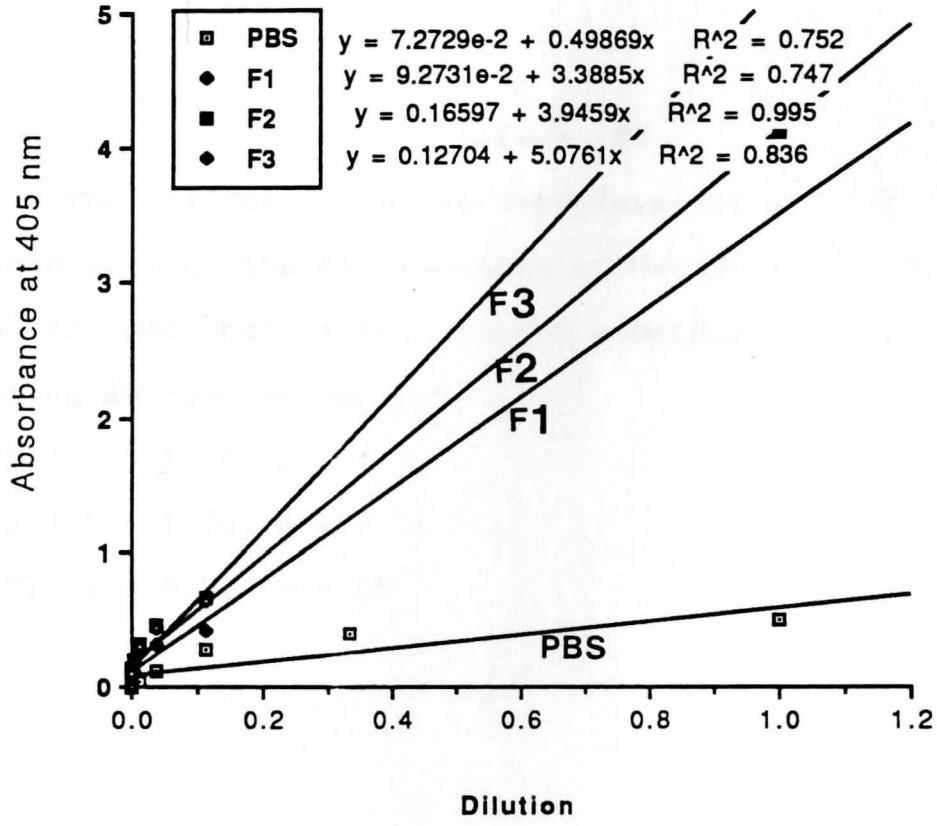
PBS
F1
F2
F3
y = 7.2729e-2 + 0.49869x R^2 = 0.752
y = 9.2731e-2 + 3.3885x R^2 = 0.747
y = 0.16597 + 3.9459x R^2 = 0.995
y = 0.12704 + 5.0761x R^2 = 0.836
F3
F2
F1
PBS
Absorbance at 405 nm
Dilution
0
1
2
3
4
5
0.0
0.2
0.4
0.6
0.8
1.0
1.2

Figure (6)

The effect of ultracentrifugation at KBr density of 1.006 g/ml on the distribution of UG-IST in fraction F1, F2, and F3, and their effect on the secondary IgG response to BGG (using ARS-BGG as antigen).

- F1: $t = 1.45$, $p = 0.15$
- F2: $t = 1.55$, $p = 0.12$
- F3: $t = 5.7$, $p < 10^{-6}$

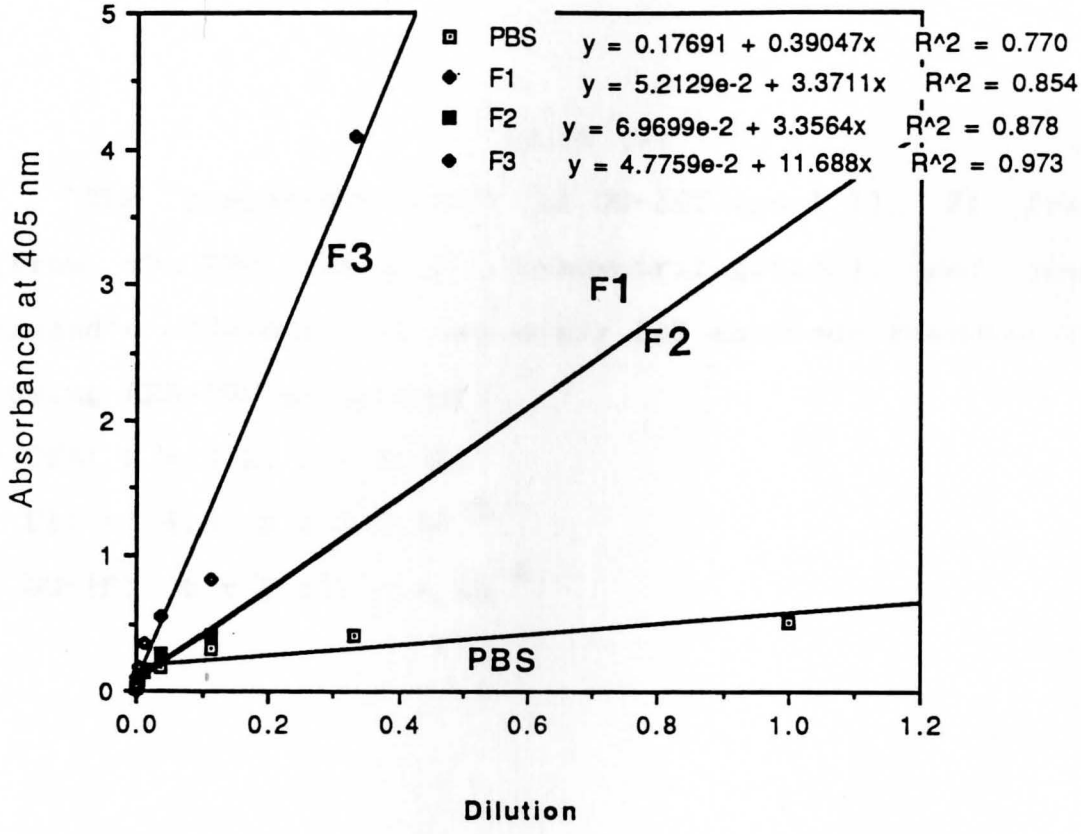
PBS y = 0.17691 + 0.39047x R^2 = 0.770
F1 y = 5.2129e-2 + 3.3711x R^2 = 0.854
F2 y = 6.9699e-2 + 3.3564x R^2 = 0.878
F3 y = 4.7759e-2 + 11.688x R^2 = 0.973
F3
F1
F2
PBS
Absorbance at 405 nm
Dilution
0
1
2
3
4
5
0.0
0.2
0.4
0.6
0.8
1.0
1.2

Figure (7)

The comparison effect of UG-IST (pool 1), F1 fraction (from the KBr 1.25 g/ml ultracentrifugation), and complete Freund's adjuvant , on secondary IgG antibody response to BGG (using ARS-BGG as antigen).

- CFA: $t = 2.2$, $p = 0.03$
- F1: $t = 4.6$, $p = 2 \times 10^{-5}$
- UG-IST: $t = 9.05$, $p < 10^{-6}$

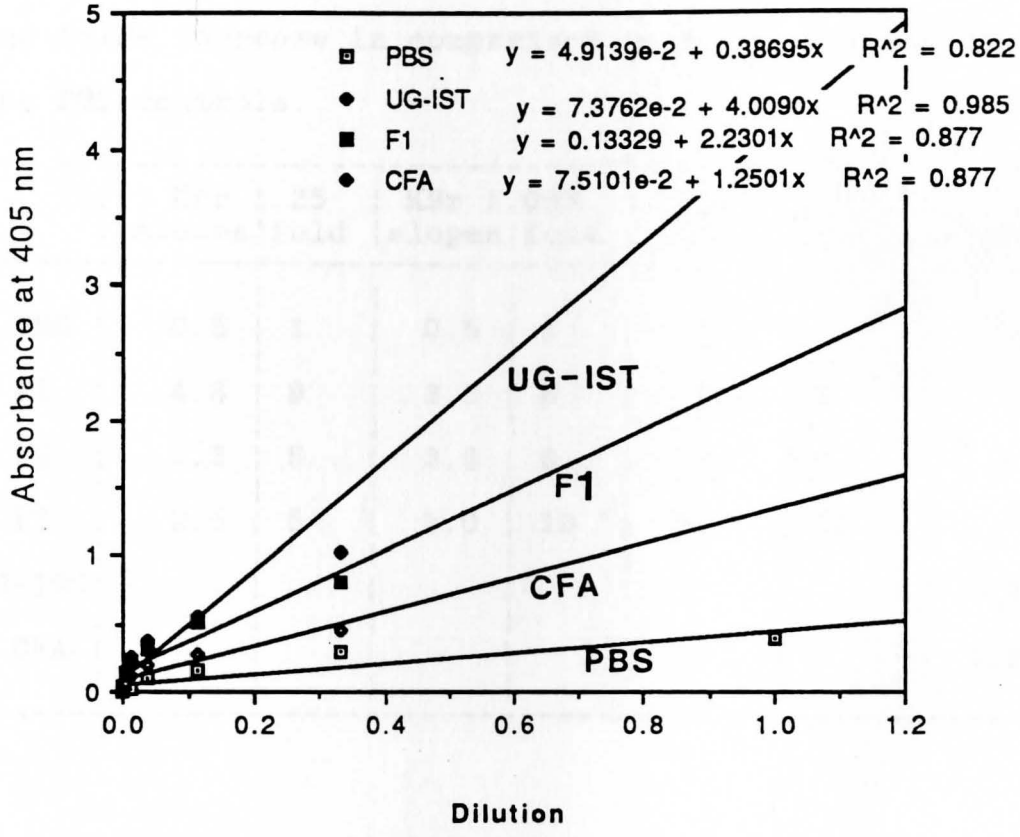
PBS y = 4.9139e-2 + 0.38695x R^2 = 0.822
UG-IST y = 7.3762e-2 + 4.0090x R^2 = 0.985
F1 y = 0.13329 + 2.2301x R^2 = 0.877
CFA y = 7.5101e-2 + 1.2501x R^2 = 0.877
UG-IST
F1
CFA
PBS
Absorbance at 405 nm
Dilution
0
1
2
3
4
5
0.0
0.2
0.4
0.6
0.8
1.0
1.2

TABLE 2

A summary of the data presented in Figures 4 to 7, and Figure 10, with respect to the slopes of the best fit plots, and folds increase in comparison with the slopes obtained for the PBS controls.

	KBr 1.25		KBr 1.063		KBr 1.006		CFA	
	slopes	fold	slopes	fold	slopes	fold	slopes	fold
PBS	0.5	1	0.5	1	0.4	1	0.4	1
F1	4.6	9	3.3	6	3.3	8		
F2	2.3	5	3.9	8	3.3	8		
F3	2.5	5	5.0	10	11.7	30		
UG-IST							4.0	10
CFA							1.2	3

Figure (8)

Thin-layered chromatograms for (A) lipid standards (spot # 1), and UG-IST (spot # 2 and 3); (B) F1 from ultracentrifugation in KBr at density of 1.25 g/ml (spot # 1), phospholipid standards (spot # 2). The TLC plates were run in $CHCl_3$: MeOH : H_2O (65:35:6, V/V/V), at room temperature. Then the plates were developed under iodine vapor.

CE = cholesterol ester, PG = phosphatidylglycerol, PE = phosphatidylethanolamine, PI = phosphatidylinositol, PC = phosphatidylcholine, and SP = sphingomyelin.

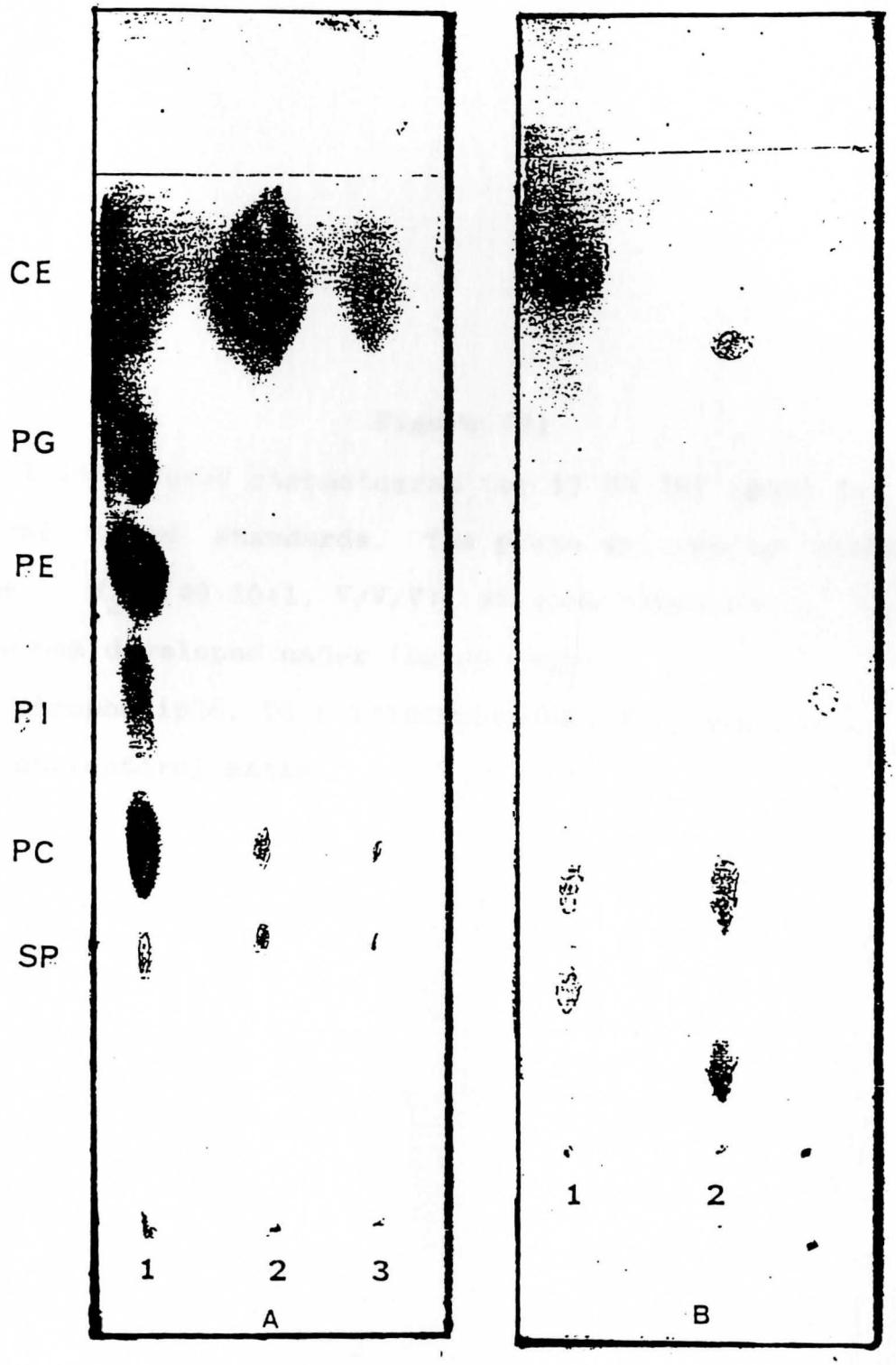
CE
PG
PE
PI
PC
SP
1
2
3
A
1
2
B

Figure (9)

Thin-layered chromatogram for 1) UG-IST (pool 1), and 2) neutral lipid standards. The plate was run in n-hexane : ether : H_2O (40:10:1, V/V/V), at room temperature. Then the plate was developed under iodine vapor.

PL = phospholipid, TG = triglycerides, DG = diglycerides, and CE = cholesterol ester.

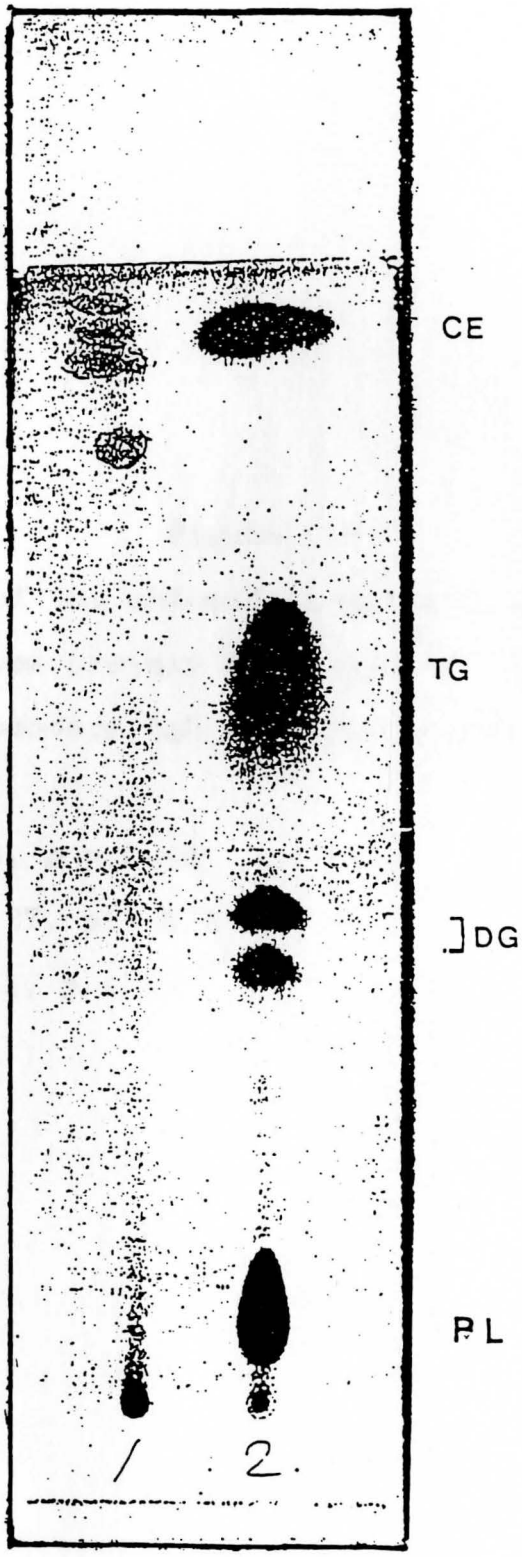
CE
TG
]DG
PL
1
2

Figure (10)

The effect of the chloroform-methanol extraction phases (i.e., the aqueous protein phase and the chloroform or lipid phase) on the secondary IgG response to BGG (using ARS-BGG as antigen).

- Lipid: $t = 0.74$, $p = 0.46$
- Protein: $t = 4.27$, $p = 8 \times 10^{-5}$
- UG-IST: $t = 8.11$, $p < 10^{-6}$

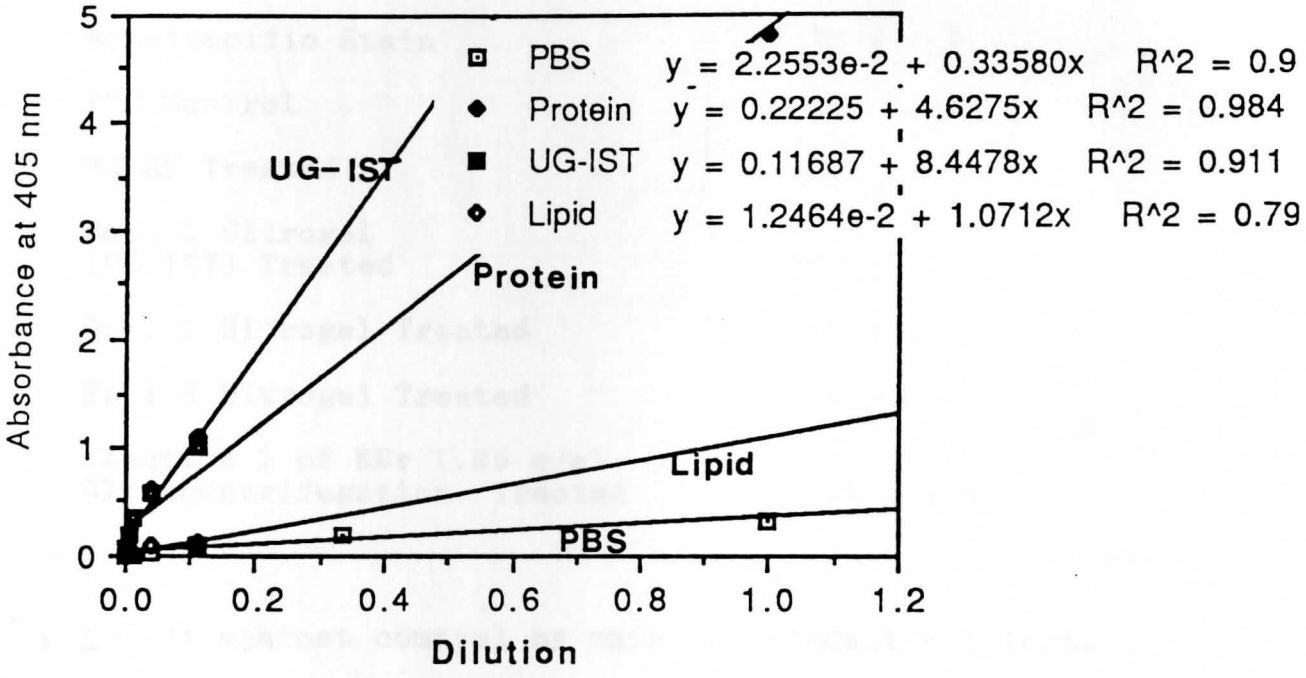
PBS y = 2.2553e-2 + 0.33580x R^2 = 0.9
Protein y = 0.22225 + 4.6275x R^2 = 0.984
UG-IST y = 0.11687 + 8.4478x R^2 = 0.911
Lipid y = 1.2464e-2 + 1.0712x R^2 = 0.79
UG-IST
Protein
Lipid
PBS
Absorbance at 405 nm
Dilution
0
1
2
3
4
5
0.0
0.2
0.4
0.6
0.8
1.0
1.2

TABLE 3

The effects of MR-AF and its purification products on the bone marrow IgM-bearing cells in unimmunized mice, four days after i.p. injection of the test sample.

Mouse Group	Percent Fluorescent Bone Marrow Cells ± sd
Non-specific Stain	3.9% ± 0.8
PBS Control	13.2% ± 2
MR-AF Treated	28.8% ± 2 *
Pool 1 Ultrogel (UG-IST) Treated	34.2% ± 3 *
Pool 2 Ultrogel Treated	13.4% ± 2
Pool 3 Ultrogel Treated	12.9% ± 2
Fraction 1 of KBr 1.25 g/ml Ultracentrifugation Treated	28.3% ± 2 *

* $p \leq 0.01$ against control by unpaired Student's t-test.

(Each of Tables 3-8 is a summary of several experiments done separately).

TABLE 4

The effects of MR-AF and Pool 2 from the Ultrogel column on IgM-bearing cells in the lymph nodes of unimmunized mice, four days after i.p. injection of the test sample.

Mouse Group	Percent Fluorescent Lymph Node Cells ± sd
PBS Control	42.6% ± 4
MR-AF Treated	43.8% ± 4 *
Pool 1 (UG-IST) Treated	41.3% ± 3 *

* $p > 0.05$ against control by unpaired t-test.

TABLE 5

The effects of MR-AF and Pool 1 from the Ultrogel column on IgM-bearing cells in the spleen of unimmunized mice, four days after i.p. injection of the test samples.

Mouse Group	Percent Fluorescent Spleen Cells ± sd
PBS Control	32.1% ± 4
MR-AF Treated	33.3% ± 3 *
Pool 1 (UG-IST) Treated	34.6% ± 3 *

* $p > 0.05$ against control by unparied t-test.

TABLE 6

The effects of MR-AF and Pool 1 from the Ultrogel column on IgM-bearing cells in the mouse bone marrow after primary SRBCs immunization. Eleven days after the treatment of the test samples and 7 days after the primary immunization the lymphoid cells were obtained and stained by the indirect immunofluorescent assay.

Mouse Group	Percent Fluorescent Bone Marrow Cells ± sd
PBS Control	14.2% ± 2
MR-AF Treated	33% ± 2
Pool 1 (UG-IST) Treated	34.1% ± 3

$p \leq 0.01$ against control by unpaired Student's t-test.

TABLE 7

The effects of MR-AF on the IgM-bearing cells in the lymph nodes. The cells were stained for IgM surface markers 11 days after MR-AF treatment and 7 days after the primary SRBCs immunization.

Mouse Grroup	Percent Fluorescent Lymph Node Cells ± sd
PBS Control	41.8% ± 3
MR-AF Treated	39.9% ± 3

$p > 0.05$ against control by unpaired t-test.

TABLE 8

The effects of MR-AF on IgM-bearing spleen cells. The cells were stained for IgM surface markers 11 days after treatment with MR-AF and 7 days after primary SRBCs immunization.

Mouse Group	Percent Fluorescent Spleen Cells ± sd
PBS Control	33.9% ± 4
MR-AF Treated	28.9% ± 3

$p > 0.05$ against control by unpaired t-test.

CHAPTER IV

DISCUSSION

During the past few years much of the work in the area of immunomodulation and the agents involved was focused on the development of agents which would enhance or modify the immune response against foreign antigens, autologus antigens and tumors. These agents vary in their chemical properties; they include proteins, protein complexes, lipids, lipopolysaccharides (LPS), microbial products, synthetic chemicals (e.g., vitamin A analogues), etc. (Fenchel et al. 1984). Also the mode of action varies from one agent to another. Some act as lymphocyte mitogens (like B cell mitogens); others act by stimulating the immunogobulin production by stimulating B cell in different manners at various or specific stages of the immune response. For example, CFA, which contains Mycobacterium, is capable of stimulating the immune response best when injected with the antigen in the primary immunization (Gregoriadis et al. 1988). Some of these agents act directly on effector cells, such as LPS which acts on B cells directly to increase antibody production (Moller et al. 1975). Some act indirectly by enhancing <u>in vivo</u> the production of biomediators that in turn act on effector cells; for example, pokeweed mitogen requires T helper lymphokines to stimulate B cells (Brochier et al. 1976a). Other agents act by stimulating,

protecting and/or reconstituting stem cells in the bone marrow, thus increasing the ability of the host to resist the invasion of foreign agents or tumor, and to tolerate the damage (Fenchel et al. 1984). One of the most important kinds of immunomodifiers is adjuvants, which act by increasing the immunogenicity of poorly immunogenic or non-immunogenic antigens in both humoral and cell-mediated responses.

The present study verifies and substantiates the earlier reports on the immunostimulatory factor that is found in the cell-free ascitic fluid of mice bearing spontaneously regressing L1210/MR cells (Koo, 1981). In addition, we further purified the immunostimulatory factor and demonstrated its effect on the murine lymphoid cells of various major lymphoid organs, which are involved in the development, growth, and differentiation of lymphocytes. Several previous studies on this ascitic fluid by Koo et al. (1979-1982) were done to determine whether and how the secondary humoral response may play any significant role in the spontaneous regression of the MR tumor. It was shown that a potent immunostimulatory factor may be specifically associated with the MR regressor and it can selectively enhance the secondary humoral immunity in mice (Laufer et al. 1981). Koo and his coworkers also demonstrated that when MR-AF is injected into normal mice before immunization with SRBCs, there was an increase in the number of IgG and IgM

plaque - forming cells (PFC) in the mice spleens after the secondary SRBCs immunization. Under normal circumstances, the level of the secondary IgM response is about the same or lower than the level achieved in the primary response. The MR-AF-stimulated secondary IgM antibody levels in all instances exceeded the primary IgM response and were approximately equal to or even surpassed the normal unstimulated secondary IgG level. At the same time the number of IgM PFC in the primary response was decreased in the mouse spleens. This unusual, and in fact unique, phenomenon can be explained at least in part by the three following possibilities : 1. MR-AF and its purified products contained two separate factors; an immunostimulatory factor for promoting the development of IgM-bearing precursor memory B cells in the bone marrow and an immunosuppressive factor which acts on primary B cells. 2. The immunostimulatory factor for the secondary antibody response is the same factor that suppresses the primary antibody response. But it is stimulatory to secondary antibody response, by promoting the production of IgM-bearing memory cells and by promoting the switch of IgM cells to IgG cells after primary immunization. 3. The immunostimulatory and the immunosuppressive activities are on the same factor, but this factor affects primary B cells and secondary B cells differently.

The above proposed postulates are based on the following key experimental observations. When MR-AF was applied to an

Ultrogel column and the eluted materials were tested for their effects on the secondary IgG antibody response of mice, IgG antibody level was found to increase by more than 20-fold when the high-molecular-weight pool (UG-IST) was tested and compared to the amount of IgG produced in PBS control or when compared to the amount produced by mice injected with other Ultrogel column pools. This stimulation was achieved even by BGG, which is a relatively poor immunogen. But when UG-IST was tested to determine the level of IgM produced in a primary response to SRBC (a strong immunogen in mice), it was found that the level of primary IgM (the major immunoglobulin produced in the primary immune response) was suppressed in comparison to that produced by the control mice. These findings are consistent with the postulates described above that IST is involved in the enhancement of the class-switching and the generation of memory B cells. These postulates also suggest that the secondary antibody production may be under the control of a separate B cell development somewhere in the differentiation pathway. A mechanism that would account for a decline in the primary response and the enhancement of the secondary response is as follows: UG-IST (the stimulatory factor) may block the differentiation of B cell at certain stage of the primary response, or may promote rapid switches of the primary B cells to memory B cells, thus affecting the expression of primary antibody-producing cells. The primary B cells or a

certain subset of B precursors are converted to memory B precursors by UG-IST, and the memory cells may then be selectively stimulated by antigen to undergo further B cell differentiation. This hypothesis is supported by the findings of Bixler et al. (1980) who implied that progenitors to secondary B cells belong to a cell subpopulation separable from precursors to primary antibody-forming cell clones.

When UG-IST was subjected to KBr ultracentrifugation (at 1.25 g/ml), the immunostimulatory activity was recovered in the top fraction (F1), which normally includes all the lipoproteins (HDL, LDL, VLDL, and chylomicrons). When the density of KBr in ultracentrifugation was lowered to 1.063 g/ml, most of the activity was recovered from the middle and bottom fractions (F2 and F3), which normally contains HDL and proteins; the top fraction (F1), which is supposed to contain LDL, VLDL, and chlyomicrons, showed less activity. Therefore, we conclude that the IST activity appears to be associated with HDL. The activity was also recovered from the bottom fraction (F3) in KBr at a density of 1.006 g/ml; this suggests that the activity is not associated with VLDL, and is not a lipid being carried by all lipoproteins. Also as shown in Figures 4, 5, 6, and 10, and Table 2, all the other fractions during the purification procedures show some immunostimulatory effect, which is most probably due to the contamination of these fractions with IST from the most

active fraction during the separation processes. Since IST is very potent, even a very small amount of contamination in a fraction can be detected.

One of the most interesting results that was obtained was the ability of UG-IST to stimulate the secondary anti-BGG IgG response by 2-3-fold more than that achieved by CFA. (CFA is known for its immunoenhancing effect when it is injected into animals together with a variety of antigens). Several lines of evidence suggest that the stimulation induced by IST is not due to endotoxin contamination : 1. Endotoxin is heat - stable and the immunostimulatory factor is heat-sensitive (56^{o} C for 45 minutes) (Laufer et al. 1981). 2. MR-AF was tested to be completely devoid of any bacterial contamination. 3. Endotoxin does not act immunologicaly like IST, which is effective only when injected into animals prior to antigen priming. 4. Our results also demonstrate that the immunostimulatory activity is localized in the high-molecular-weight fraction , 1000 kd, when MR-AF was fractionated by Ultrogel AcA-22 column. These high M.W. substances are not lipid micells because lipids can be effectively absorbed by the gel matrix of the column. 5. Extraction of IST by chloroform/methanol retains more IST activity in the aqueous phase than in the lipid phase. This suggests that the active moiety does not reside in the lipid part of the molecule. 6. The immunostimulatory effect was

localized in the high-density fraction when MR-AF was fractionated by a linear sucrose density gradiant (0-60%) during ultracentrifugation (Koo, 1981); endotoxin, on the other hand, is expected to float in this aqueous solution.

When UG-IST was extracted for lipids and the extracted material was analyzed by TLC, the lipids in UG-IST had R_f values similar to the cholesterol esters, although insignificant amounts of phosphatidylcholine and sphingomylin were also present. In order to demonstrate which part of the UG-IST (lipid or protein) contained the stimulating factor, both protein and lipid extracts were injected separately into two different groups of mice to determine their effects on the secondary IgG response to BGG. When the antisera were obtained and assayed by ELISA, the immunostimulatory activity was found to be associated with the aqueous phase (protein). This finding corroborates the findings of Koo (1981), who reported that the immunostimulatory activity of MR-AF (IST) was abolished by trypsin digestion and heat (56° C for 45 minutes). This suggests that the immunostimulatory agent is possibly a lipophilic protein complexed primarily by cholesterol-like compounds, forming large molecular weight complexes with the characteristics of HDLs.

The MR-AF and its purification products (UG-IST and F1 at KBr 1.25 g/ml) were also tested to evaluate their effects on the cells of the main lymphoid organs of mice. Koo (1981) showed that the stimulatory factor increased the number of

IgG and IgM plaque - forming cells in mice after secondary SRBC immunization. We, therefore, tested the level of lymphocytes in the various lymphoid organs before immunization and after treatment with MR-AF and the purification products. MR-AF, UG-IST, and F1 (all of which were able to increase the level of anti-BGG IgG in the secondary antibody response) were able to increase the number of IgM-bearing cells in the bone marrow by 2-3-fold when injected four days before obtaining the bone marrow, even without prior exposure to any antigen. Also, when the animals were injected with MR-AF or UG-IST and followed by primary immunization with 5% SRBCs, there was a two-fold increase in the number of IgM-bearing cells in the bone marrow 7 days after the immunization. But when the spleen and lymph nodes were similarly tested there were no increases in the level of IgM-bearing cells with or without immunization.

This characteristic action of IST in the bone marrow suggests that IST may be a specific B lymphocyte mitogen, which causes proliferation of precursor memory B lymphocytes prior to stimulation by antigens or other mitogens. Also, this stimulatory factor may promote the differentiation and maturation of memory B cells into plasma cells (antibody producing cells) after antigen priming, since it was able to induce the production of large amounts of secondary anti-BGG IgG antibodies when compared to either the control animals

or the CFA-treated animals. Aside from the possibility that IST may act like a B cell mitogen by inducing the proliferation of B stem cells in the absence of T cell help, IST may act directly on T cells which when stimulated may produce another factor that in turn acts on the stem cell in the bone marrow to stimulate the production of the IgM-bearing cells. If this is the scenario, the IST that is found in the MR-AF is not a true B cell mitogen.

The evidence for the occurrence of an immunostimulatory substance in tumor-bearing animals has been accumulated (Bodger et al. 1979). The stimulatory factor (M.W. = 30,000-100,000) that occurs in human cancer ascitic fluid enhances the primary antibody response to SRBC <u>in vitro</u> and is heat-labile (80^{o}C for 10 minutes) (Bodger et al. 1979). Also, macrophages have been implicated to produce a heat-labile enhancing factor and a heat-stable inhibitor (Connolly et al. 1979). The supernatants obtained from the tumor-bearing host macrophages, when compared with their normal counterparts, possess a greater ability to enhance mixed lymphocyte reaction reactivity <u>in vitro</u> (Connolly et al. 1979). These kinds of studies on tumor models may provide us with the opportunities to identify and study the mechanisms of regulation and potentiation of anamnestic humoral responces by biomediators, for improving the host's response to neoplasms and vaccines.

REFERENCES

Andersson, J., Sjoberg, O. and Moller, G. Introduction of immunoglobulin and antibody syntesis *in vitro* by lipopolysaccharides. Eur. J. Immunology 2: 349-355 (1972).

Barclay, M., and Skipiski, V.P. Lipoprotein in relation to cancer. Proc. Biochem. Pharmacol. 10 : 76-111 (1975).

Bilgh, E. G., and Dyer, W. J. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37 : 911-917 (1959).

Bixler, G. S., and J. Booss. Establishment of immunologic memory concurrent with suppression of the primary immune response during acute cytomegalovirus infection of mice. L. Immunology 125 :893- 904 (1980).

Bodger, A. M., and S. R. Cooperband. An immunostimulatory substance in the ascitic fluid of patients with cancer metastatic to the peritoneum. Cellular Immunology 45 : 15-25 (1979).

Boua, C., S. Broder, A. Dimitriu, and T. A. Waldmann. Polyclonal activation of human B lymphocytes by Nocardia Water Soluble Mitogen (NWSM). Immunological Rev. 45 : 69-92 (1979).

Brochier, J., Abou-Hamed, Y. A., and Revillard J. B. Study of human T and B lymphocytes with hetrologous antisera. I. Preparation, specificity and properities of antisera. Immunology 31 : 749-762 (1976a).

Carlson, G. A., and G. Terres. Antibody-induced killing *in vivo* of L1210 / MTX-R cells quantitated in passively immunized mice with I 131 iododeoxyuridine-labled cells and whole-body measurement of retained radioactivity. J. Immunology 117 : 822-834 (1976).

Chapel, H., and Haneney, M. Essentials of Clinical Immunology, second edition (1983).

Connolly, K. M., and K. D. Elgert. Regulation of T cell mixed lymphocyte reactivity : demonstration of enhancing and inhibitory activity in tumor-bearing host macrophage supernatants. Cell Immunology 45 : 94- 106 (1979).

Continho, A., and Moller, G. Mitogenic properities of the thymus-independent antigens. Nature New Biol. 245 : 12-27 (1973a).

Continho, A., and Moller, G. mitogenic properities of the thymus-independent antigen pneumococcal polysaccharide S III. Eur. J. Immunology 3 : 608-621 (1973b).

Dorries, R., Schimple, A., and Wecker, E. Action of dextran sulfate as a direct B cell mitogen. Eur. J. Immunology 4 : 230-237 (1974).

Fearon, D. T., and Collins, L. A. Increased expression of C3b receptor on PMN leukocytes induced by chemotactic factor and by purification procedures. J. Immunology 130 : 370-381 (1983).

Fenchel, R. L., and Chirigose, M. A. Immune modulation agents and their mechanisms. Immunology Series, Vol. 25 (1984).

Forsgren, A. Svedjelund, A., and Wigzell, H. Lymphocyte stimulation by protein A of staphylococcus aureus. Eur J. Immunology 6 :207-214 (1976).

Gelman Instrument Company. Serum protein electophoresis system (1976).

Gregoriadis, G., Allison, A. C., and Poste, G. Immunological adjuvants and vaccines. Series A: Life Sciences, Vol. 179 : 1-10 (1988).

Janossy, G., and Greaves, M. Functional analysis of murine and human B lymphocytes subsets. Transpl. Rev. 24 : 177-195 (1975).

Klein, J. Immunology - the Science of Self-Nonself Dicrimination (1978).

Koo, P. H., and Cebra, J. J. Affinity labiling of distinctive lysyl residue within the second hypervariable region of gamma 2 chain of guinea pig anti-p-azabenzenearsonate antibody. Biochemistry 13 : 184-195 (1974).

Koo, P. H., and Laufer, C. S. Immunoregulatory factor associated with a spontaneously regressing subline of the lymphoma L1210. Federation Proceedings 38 : 4027-4036 (1979).

Koo, P. H. Immunostimulatory factor specifically associated with the spontaneously regressing subline of murine lymphoma L1210. Fourth International Congress of Immunology. Abstract. Paris, France. J. L. Preud'homme and V. A. L. Hawhen. (Eds), p 10 (July 1980).

Koo, P. H. Immunostimulatory factor specifically associated with a spontaneously regressing murine lymphoma L1210. J. Immunology 127 :373-382 (1981).

Koo, P. H. Growth and antigenic properities of a spontaneously regressing subline of murine leukemia L1210. Eur. J. Cancer and Clinical Oncology. 18 : 2623-2628 (1982).

Laufer, C. S., Fazely, F., and Koo, P.H. Selective regulation of immune response by factors associated with a spontaneously regressing murine leukimia L1210. Federation Proceedings 40 : 1038- 1047 (1981).

Linton, P. et al. B cell development. Molecular and Cellular Biology 1 : 75-89 (1988).

Moller, G., Andersson, J., and Sjoberg, O. Mitogens as probes for immunocyte activation and cellular cooperation. Transplant. Rev. 11 : 131-177 (1972).

Prezioso, J. A., and Koo, P. H. Natural cytotoxins in human plasma : Isolation and characterization of phospholipids associated with cytotoxic lipoproteins. Molecular Toxicology 1 191-208 (1987).

Prezioso, J. A. Isolation and characterization of cytotoxic peroxidized lipoproteins in human plasma. A dissertation submitted to the Kent State University Graduate College. Kent, Ohio (1988).

Roff, H., Deverux, D., and Maloney, G. Human monoclonal antibody with protective activity against E. Coli and Neisseria meningitids group B infection. J. Infectous Diseases 157 : 118-125 (1988).

Todd, R. F., Nadler, L. M., and Schlossman S. F. Antigens on human monocytes identified by monoclonal antibodies. J. Immunology 126 : 1435-1446 (1981).