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A Study Of Molecular Events In Lipopolysaccharide - Stimulated Murine B Lymphocytes (Two-Dimensional Gels, Lipid A, Receptors).

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A STUDY OF MOLECULAR EVENTS IN LIPOPOLYSACCHARIDE-STIMULATED MURINE B LYMPHOCYTES

by

KATHERINE ANNE GOLLAHON

A DISSERTATION

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ABSTRACT OF DISSERTATION
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This study investigated two independent aspects of lipopolysaccharide (LPS) induced murine B cell mitogenic responses. First, two-dimensional gels of lysates from LPS-stimulated B lymphocytes were compared with two-dimensional gels of lysates from control nonstimulated B lymphocytes. Densitometric readings of the protein spots on these gels were determined. No difference was found qualitatively or quantitatively between the proteins of cell lysates from stimulated and non-stimulated cell cultures after 24 hours of incubation. Although these results were unexpected, it was felt to be a valid estimation of protein synthetic events in the cell. It is possible that other types of molecular events, changes in membrane potential, calcium influx or changes in cyclic nucleotides, may be more important in the first 24 hours or LPS stimulation. In addition, it was found that the general protein content of the cell does not vary significantly (less than 6%) between T lymphocytes and macrophages, and B lymphocytes which suggests that the basic protein content of these cell types was identical.

Second, lipid A binding proteins were identified from spleen cell lysates. Lipid A is the portion of LPS which is responsible for most of the biological activity of that molecule. The proteins which were

identified were 62,000, 60,000 and 28,000 daltons. These proteins could be identified in cell lysates from both LPS responsive and hyporesponsive mouse strains. The 62,000, 60,000 and 28,000 dalton proteins bind lipid A with a high affinity, such that the lipid A-protein complex was maintained even in a sodium dodecyl sulfate-polyacrylamide (SDS-polyacrylamide) gel. A fourth protein which had a molecular weight of 45,000 daltons was also found in the sucrose gradient fraction which contained increased amounts of ^{125}I lipid A, the radioactive tracer in this system. This molecule failed to bind ^{125}I lipid A in an SDS-polyacrylamide gel which indicated that it either binds ^{125}I lipid A but is dissociated by SDS or that a second nondetectable protein binds the ^{125}I lipid A. The fact that the 60,000 and 62,000 dalton protein could be co-isolated with the 45,000 dalton protein raises the possibility that these two molecules may be aggregated by LPS on cell surface.

Abstract Approved by: Committee Chairman Jerry R. McGhee

Program Director Richard J Conn

Date 12/2/85 Dean of Graduate School Alan A Boswell

DEDICATION

This work is dedicated to my grandfather, Les Faller.

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LIST OF ABBREVIATIONS

BHF	B cell helper factor
Bu-LPS	Butanol water extracted lipopolysaccharide
cAMP	cyclic adenylate monophosphate
cGMP	cyclic guadinylate monophosphate
Con A	concanavalin A
DFP	diisopropylfluorophosphate
EGTA	Ethyleneglycol-bis-N,N'-tetracetic acid
FCS	fetal calf serum
Ig	immunoglobulin
KDO	ketodeoxyoctonate
LPS	lipopolysaccharide
M	molar
mM	millimolar
mRNA	messenger RNA
NEPHGE	nonequilibrium pH gel electrophoresis
NP-40	Nonidet P-40
NWSM	Nocardia water soluble mitogen
PBS	phosphate buffered saline
PHA	phytohemagglutinin
Ph-LPS	phenol water extracted lipopolysaccharide
poly A:U	polyriboadenylic-polyribouridylic acid
poly I:C	polyriboinosinic-polyribocytidylic acid
PPD	purified protein derivative of tuberculin
PWM	pokeweed mitogen
SDS	sodium dodecyl sulfate
sIgD	surface immunoglobulin D

sIgM	surface immunoglobulin M
TEMED	N,N,N',N' -tetramethylethylenediamine
TI-1	T cell independent antigen type 1
TI-2	T cell independent antigen type 2

INTRODUCTION

The studies described in this work concern the molecular biology of lipopolysaccharide activation of murine B lymphocytes. In order to fully understand the approach taken, one must be familiar with B lymphocytes, B lymphocyte mitogens, the animal models which made these studies possible, and receptor models involved in cell regulation. Each of these topics will be discussed below.

B Lymphocytes. The embryonic origin of the lymphocyte is a mesenchymal stem cell (1). This cell develops into a pluripotent stem cell (2,3) which can give rise to macrophages, neutrophils, eosinophils, megakaryocytes and erythrocytes, as well as lymphocytes. All of these cells are found circulating in the bloodstream and, with the exception of the erythrocyte, play a role in host defense. A B lymphocyte is a mononuclear cell which contains a large nucleus, a small amount of cytoplasm and immunoglobulin on the cell surface. B cells, together with macrophages and other antigen-presenting cells, provide a major line of defense against invading organisms, both viral and bacterial, and foreign cells.

Lymphocytes can be divided into two major groups, T lymphocytes and B lymphocytes (4). T lymphocytes are cells which have been processed or modified in the thymus and are the major cell type involved in cellular immunity, i.e., cytotoxicity, delayed-type hypersensitivity, and graft versus host reactions. In addition, T cell can modulate B lymphocyte activity via factors and direct cell to cell contact. Thus, cellular immunity and immune regulation are functions of T lymphocytes.

The B lymphocyte is the cell which will differentiate into a plasma cell, the antibody-secreting cell. B lymphocytes or B lymphocyte-like cells are present in all vertebrates (5,6). It has been shown that vertebrates, as low on the evolutionary scale as cyclostomates, produce antibodies. Although B lymphocytes originate from the same stem cell as T lymphocytes, the maturation of B lymphocytes does not take place in the thymus. In chickens, B lymphocytes originate in the bursa of Fabricius (7,8), while in humans and mice the fetal liver is the embryonic source and the bone marrow is the adult source of B lymphocytes (9). The stem cell goes through a series of developmental stages to become a B lymphocyte and then a plasma cell. Antibody secreted by plasma cells can directly inactivate an invading organism, combine with antigen, serve as an activator of complement, or assist in cellular immune responses such as antibody-dependent cellular cytotoxicity. Since only plasma cells secrete antibody, it is important to understand how the pluripotent stem cell develops into a plasma cell. These differentiation steps occur in the organs of the lymphoreticular system which will be discussed next.

After the initial identification of T and B lymphocytes, the organs of the lymphoreticular system, e.g., lymph nodes, spleen and Peyer's patches, were reexamined for the presence or absence of these cells. T and B lymphocytes and antigen-presenting cells (macrophages and dendritic cells) exhibit specific spacial arrangements which are similar among the major organs of the immune system. Lymph nodes are lymphoid tissues located along the lymphatic vessels of the body. They are widely distributed and serve as sites for antigen processing and presentation. The outer cortex of lymph nodes mainly consists of B lymphocytes (10).

Within this cortex are lymphoid follicles. The primary follicles have no germinal centers, while the secondary follicles have germinal centers of B lymphocytes surrounded by T lymphocytes. Within the germinal centers are macrophages and dendritic cells which take up and process antigen for presentation to B and T lymphocytes. In general, the cortex is T independent in that an athymic mouse still has a fully developed outer cortex region. The medulla is that region of the lymph node through which the lymphoid cells leave the tissue. This region is also T cell independent. There is a region between the medulla and outer cortex called the paracortical region where the majority of the T cells reside. The paracortical region is absent in lymph nodes of athymic mice and thus, is T cell dependent.

The spleen is a major lymphoreticular organ with several functions. A primary immune function includes antigen processing and presentation to B and T cells. The spleen consists of two main areas (11,12), the red pulp and the white pulp. The red pulp has non-immune functions. The white pulp is a sphere of lymphoid cells which are arranged into germinal centers. On one side of the spherical area, B cells are the predominant cells along with some macrophages and dendritic cells, while the other side consists of T cells. Thus B cells, T cells and macrophages are arranged in close proximity in lymphoid tissues, to allow antigens to be processed and presented and for cell interactions which result in cellular and humoral immune responses.

The Peyer's patches (13) are discreet nodes which line the small intestine, and comprise the gut-associated lymphoreticular system (14). The Peyer's patches are covered by unique epithelial cells called M cells for their membranous and microfolding appearance. These cells

are in constant contact with the lumen of the intestine and continually sample environmental antigens and transport them into the Peyer's patch. Again, there are distinct T and B cell regions in the Peyer's patch which constitute germinal centers much like those of the lymph node and spleen. The proximity between T lymphocytes, B lymphocytes and antigen-presenting cells in the germinal centers is necessary to stimulate the B cells to differentiate into plasma cells which will produce specific antibody (16,17). The interactions which take place among T cells, B cells, and macrophages and antigen can be mimicked by lipopolysaccharide which provides an artificial means of examining the developmental process of B cells into mature plasma cells.

The state of maturation of a B lymphocyte can be defined in two different ways. In one system, the maturational state is described by various cell surface and cytoplasmic markers, while in the other system, functional activity is used to determine the maturational state of the cell. In the simplest scheme, five distinct stages of B cell maturation can be identified based on the site of immunoglobulin expression, i.e., stem cell, pre-B cell, immature B cell, mature B cell and plasma cell. The stem cell, the pluripotent cell previously discussed, has no immunoglobulin expressed either in the cytoplasm or on the cell surface. The pre-B cell expresses cytoplasmic mu chain and has been described by Owen et al. (9) as making up 30% of all small lymphocytes in the bone marrow. The immature B cell expresses IgM on its cell surface (18-20); then as this cell matures, a second immunoglobulin isotype, IgD, appears on the cell surface (21). The relative amount of surface IgD (sIgD) to surface IgM (sIgM) increases as the cell matures. The mature B cell is capable of responding to antigen (or mitogen). Finally, the plasma

cell, a larger cell with an extensive endoplasmic reticulum, has a single class of immunoglobulin in its cytoplasm and this immunoglobulin is secreted.

In addition to immunoglobulin, other surface antigens are expressed on B cells as a function of their maturational state (22). One of these antigens, Lyb 5, has been especially useful in defining stages of B cell maturation. Cells which do not express Lyb 5 on their cell surface are thought to be less mature than cells which express Lyb 5. B cells which lack Lyb 5 do not respond to the class 1 T independent antigens (see Animal Model section) or to lipopolysaccharide (23). Thus, a functional subset of B lymphocytes is distinguished by a specific cell surface marker.

It is not possible to describe all B cell surface determinants which have been extensively reviewed by others (22,24-26); however, a brief summary is presented in Figure 1 and Table 1. Figure 1 is a compilation of cell surface antigens which are expressed at different maturational states. The cell which responds to lipopolysaccharide is emphasized since this is the cell which is of interest in the present work. Among the expressed antigens are Ia, a class II histocompatibility antigen, and surface IgD. Both sIgD and Ia have been implicated as LPS receptors (see B Lymphocyte Mitogens section). IgD is an immunoglobulin and is normally expressed on mature B cell membranes. Ia is a molecule which is involved in immune regulation. Table 1 lists the major cell surface determinants of B lymphocytes, their respective molecular weights and chromosomal location.

In summary, B lymphocytes are mononuclear cells which originate in the bone marrow. These lymphoid cells differentiate into plasma cells

Figure 1. Ontogeny of murine B lymphocyte cell surface antigens. This figure is a schematic representation of the appearance of cell surface markers on murine B lymphocytes. Blank rectangles show that the antigen is absent from that cell. Rectangles which are single cross hatched show that the marker is present on the cell. Double cross hatch of a rectangle shows that the antigen is present on an LPS responsive cell.

Figure 1

Murine B Lymphocyte Maturation

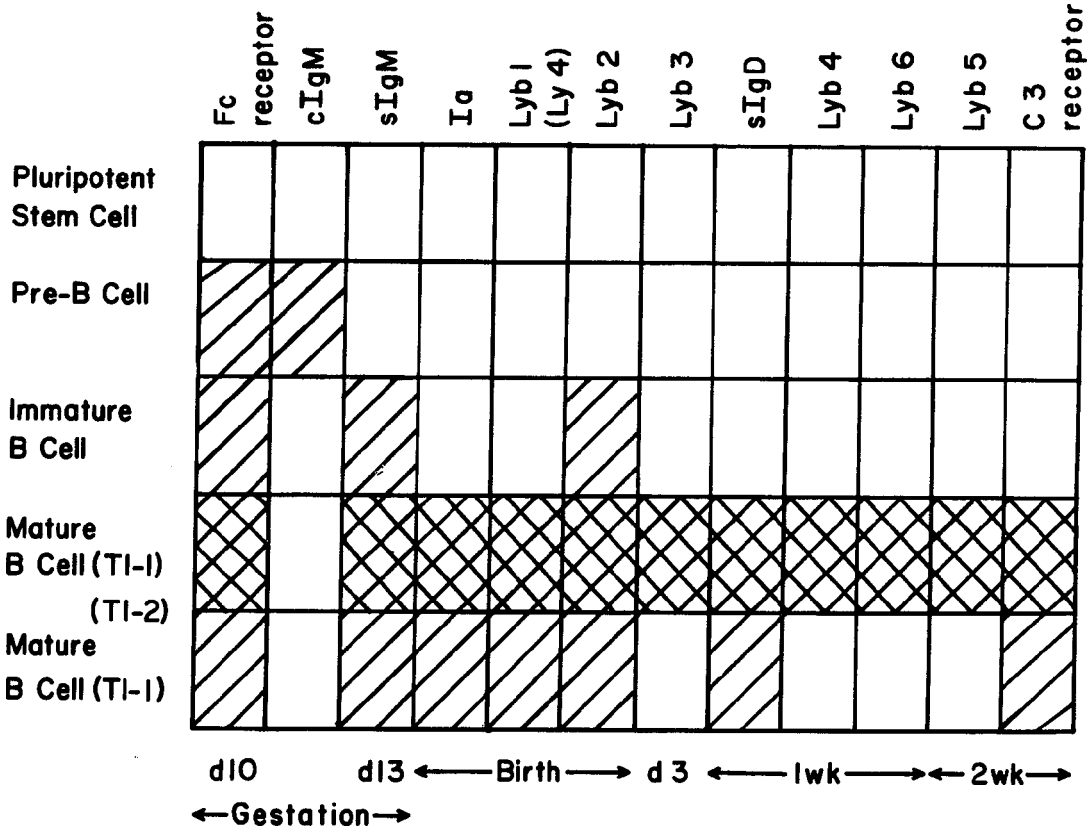


TABLE 1

Characteristics of Murine B Cell Surface Antigens

Antigen	Molecular Weight	Chromosome Location
sIgM	180,000	12
sIgD	185,000	12
Ia	60-58,000	17
Lyb 2	45-40,000	4
Lyb 4	45,000	4
Lyb 6	45,000	4
Lyb 3	68,000	unknown
Lyb 5	unknown	unknown

and display a series of cell surface antigens during maturation. These antigens have been used to define B lymphocyte subsets. Each subset responds to particular mitogens. The structure, function, and source of many B cell mitogens are discussed in the next section.

B Lymphocyte Mitogens. Mitogens are substances from plants, bacteria, or synthetic sources which have been used to nonspecifically stimulate cells to proliferate and/or differentiate in much the same way that antigen stimulation or T cell help would trigger B lymphocytes. These substances have been used to examine various molecular events which occur during the development of a B cell. A series of B cell mitogens have been identified, including pokeweed mitogen (PWM), purified protein derivative of tuberculin (PPD), dextran sulfate, Nocardia water-soluble mitogen (NWSM), polynucleotides, anti-immunoglobulin, and lipopolysaccharide (LPS). Most plant extracted mitogens were found to be specific for T lymphocytes. One exception was pokeweed mitogen, which was

extracted from the roots of Phytolacca americana (28). It was demonstrated to be a single glycoprotein with a molecular weight of 32,000 and is thought to have 33 intrachain disulfide bonds, making it a very rigid molecule (29). This mitogen stimulated both T and B cells in mice.

Two synthetic B cell mitogens are the polynucleotides and dextran sulfate. Two polynucleotides, polyriboadenylic-polyribouridylic acid (poly A:U) and polyriboinosinic-polyribocytidylic acid (poly I:C) have been used to manipulate the immune system. A recent review by Johnson (30) describes many of the activities of these molecules. There seems to be a consensus that poly A:U regulates B cells via T cell factors while poly I:C appears to act directly on B lymphocytes. Poly I:C has been shown to act on the same B cell population as LPS (31). Dextran sulfate, another synthetic molecule, was shown to stimulate ^{3}H-thymidine uptake in athymic mice (32). This molecule acts on a less mature set of B lymphocytes than poly I:C.

The effects of anti-immunoglobulin on B lymphocytes are somewhat complicated. The effects of anti-μ have been shown to be either suppressive (33) or stimulatory (34). In general, anti-μ has been shown to be suppressive for neonatal B lymphocytes or lymphocytes with a low sIgM to sIgD ratio (33). In addition, treatment of B lymphocytes from adult or neonatal mice with anti-μ could block the proliferative effects of LPS. This was thought to be due to the endocytosis of sIgM which either triggered some suppressive event or to removal of sIgM which was necessary for interaction with LPS. In other studies by Sieckmann (34), anti-μ has been shown to induce mitogenesis without stimulating polyclonal B cell activation. This effect was both dose-and time-dependent.

Anti-μ-triggered mitogenesis was absent in CBA/N mice which suggests that triggering involves an Lyb 3^+, Lyb 5^+ cell (see Animal Models section). Thus, the effects of anti-μ depend upon the maturational state of the B cell.

Surface IgD appears later in B cell differentiation than sIgM (19). The ratio of sIgD to sIgM increases as the cell matures and this ratio has been used to determine the maturational state of the B lymphocyte. In vitro and in vivo effects of anti-IgD have been shown to be the same (35,36). When antigen was administered with anti-IgD, there was a specific enhancement of antigen-specific antibody with no evidence for polyclonal B cell activation. In vitro experiments have shown that anti-IgD, like anti-μ, will stimulate a proliferative response, but not a polyclonal antibody response. It can be concluded that anti-immunoglobulins are good mitogens but not polyclonal B cell activators.

Bacteria have served as the primary source of B cell mitogens. Purified protein derivative of tuberculin was shown by Sultzer and Nilsson (37) to be a potent B cell mitogen which, unlike PWM, has little or no effect on thymocytes. Another B cell mitogen is the water soluble extract from Norcardia opoca (38). Little information is available on the structure of this mitogen. A third B cell mitogen of bacterial origin is lipopolysaccharide, a major component of the outer wall of Gram-negative bacteria. Structurally, LPS can be divided into three components, the O-specific side chain, the core polysaccharide and lipid A (39). The O-specific side chain contains the same repeating sequence which is a subunit of either a linear trisaccharide or a branched tetrasaccharide or pentasaccharide. The chains vary in length and have as many as 25 repeating units. It is the O side chain structure which

confers most of antigenic properties to this molecule. The core is a set of eight sugars which serve as attachment for the O-side chain. The first three core sugars are common sugars, the next two are heptoses and the final three are ketodeoxyoctonate (KDO). The KDO sugars are attached to β 1-6 diglucosamines to which are linked five fatty acids and two phosphates. This latter complex is termed lipid A. It is the lipid A which is responsible for the majority of the biological effects of LPS (39). Lipid A is the endotoxic moiety and can cause death of the animal, abortion, as well as macrophage activation and mitogenesis and polyclonal activation of the B lymphocytes.

An early question in the analysis of B cell mitogens concerned which B lymphocytes were activated by a given mitogen. To address this problem, Bona et al. (40) used a suicide experiment to show that distinct populations of B lymphocytes are triggered by Norcardia, LPS and dextran sulfate. Spleen cell cultures were incubated with one of three mitogens with or without 5-bromo-deoxyuridine. The cultures were exposed to fluorescent light, washed and again incubated with one of the mitogens, and ^{3}H-thymidine uptake was measured. These investigators showed that successive treatment of cells with the same mitogen, i.e., LPS followed by LPS, did not result in additional stimulation; however, the use of different mitogens stimulated different subpopulations of B lymphocytes. Subsequent studies have shown that dextran sulfate stimulates the least mature set of B cells, LPS the next most mature set and PPD and Nocardia a more mature set (41). In addition, recent work by Bergstedt-Linqvist et al. (42) has shown that dextran sulfate and LPS can work synergistically. Further evidence for mitogens acting on separate B cell subpopulations comes from the fact that various mitogens trigger a

predominance of one immunoglobulin isotype over another (43 rev.); lipopolysaccharide triggers more IgG_{2b} and IgG_3 while PWM triggers more IgG_1 and IgG_3. Finally, the fact that LPS is a poor mitogen for the CBA/N mouse B lymphocyte (23) (CBA/N mice lack a B cell subset; see Animal Models section) also supports mitogen-specific stimulation of B cell subsets.

Very little is known about the mechanisms involved in mitogenesis and the mechanisms of B cell mitogens are no exception. A review by Decker and Marchalonis (44) has emphasized some of the known mitogen-induced biochemical events which occur in lymphocytes, T lymphocytes in particular, which include cation fluxes, cyclic nucleotide changes, and phosphorylation of nuclear proteins. Each of these events will be discussed as they relate to LPS activation of B lymphocytes along with other reactions.

Cation changes in LPS stimulated B cells have been studied by several laboratories. Owens and Kaplan (45) used the potassium congener ^{86}Rb to measure potassium uptake by the spleens of athymic mice in the presence and absence of LPS. They observed a doubling of ^{86}Rb uptake over a 2-hour period, after 8-16 hours of culture with LPS. Uptake of ^{86}Rb was not studied at earlier times following LPS treatment.

There are two reports which examined calcium ion fluxes. Miller and Moticka (46) used EGTA, a calcium chelator, to inhibit the activity of calcium. They demonstrated that EGTA could inhibit LPS-stimulated mitogenesis at 0, 6, 24, and 48 hours. They also showed that calcium chloride (1 mM) suppressed LPS-triggered mitogenesis, if added at 0 or 6 hours, and enhanced LPS-triggered mitogenesis if added after 24 hours. Diamanstein and Ulmer (47) have shown that there is a calcium ion

dependent and independent period during LPS stimulated mitogenesis. They suggest that calcium may be required for a step immediately preceding DNA synthesis but not for any early events.

As a consequence of these studies, Rosenstreich and Blumenthal (48) examined seven different ionophores in attempts to stimulate a mitogenic response. Only one ionophore, excitability inducing material, could cause an increase in ^{3}H-thymidine uptake, while gramicidin A, a functionally similar ionophore, failed to stimulate a mitogenic response. Morrison and Rudbach (49) have studied calcium ion fluxes in platelets. They suggest that LPS intercalates into the membrane of the platelet, which destabilizes the membrane and causes a calcium ion influx. These events seem to be sufficient to activate platelet aggregation. The fact that LPS binds equally well to the lymphocytes of the LPS hyporesponsive mouse strain, C3H/HeJ (see Animal Models section) as to the lymphocytes of the syngeneic, LPS responsive C3H/HeN mouse strain (50), would suggest that binding of LPS and whatever ion fluxes occur as a result of the membrane destabilization are not enough to induce B cell mitogenesis. Thus, calcium ions cannot trigger mitogenesis, but may be necessary for mitogenesis to occur.

The role of cyclic nucleotides in LPS-stimulated mitogenesis remains unclear. While cyclic AMP (cAMP) is known to initiate a series of events leading to activation in most cells, early studies by Bourne et al. (51) suggested that cAMP is a down regulator for immune responses in general and for inflammatory responses in particular. Although one can make a case for either cAMP or cyclic GMP (cGMP) as a regulatory molecule in B lymphocytes, Watson (52) has shown that cGMP changes are most important. First, it was shown that LPS stimulates a transient increase in

cGMP (and no effect in cAMP), and that external cGMP could trigger B cell mitogenesis. LPS triggers an increase in cGMP levels, while dextran sulfate, poly I:C and PPD do not (53). Exogenous cGMP stimulated nude spleen cells but not thymocyte cultures, while exogenous cAMP actually depressed the LPS response. From these studies, one can conclude that LPS triggers an increase in cGMP which is integral for B lymphocyte mitogenesis.

Cyclic AMP is known to trigger a cascade of events in which enzymes are phosphorylated. Thus, an increase in cGMP could also stimulate events which lead to phosphorylation of proteins. Phosphorylation of nuclear chromosomal proteins in LPS stimulated cells has been studied by Stott and Williamson (54, 55). In extensive studies, they showed an increase in the phosphorylation of several nuclear proteins ranging in size from 10,000 to 75,000 in molecular weight. Two hours after stimulation with LPS, the tightly bound nuclear proteins were the most highly phosphorylated. At four hours, the nonhistone chromosomal proteins and nucleoplasmic proteins began to undergo phosphorylation. In addition, a number of cytoplasmic proteins were phosphorylated, which could be seen as early as two hours. Since phosphorylation is a mechanism used to activate enzymes, it is not surprising that phosphorylation occurs in cell proliferation and differentiation.

Calcium ion influx, phosphorylation of nuclear proteins and increases in cGMP are probably events secondary to a yet unknown LPS-cell interaction. It is clear that LPS acts initially at the membrane level, but there is also evidence that LPS may act internally as well. An electron-micrograph study by Bona et al. (56) demonstrated that both thymocytes and spleen cells capped and shed LPS, but spleen cells also internalized

the LPS into the cytoplasm and nucleus, and LPS was localized over the chromatin. In two separate studies using radiolabeled LPS and differential centrifugation techniques (57, 58), it was shown that the majority of the retrievable radioactivity was found in the nuclei of spleen cells. Tavakoli and Moon (59) have shown that lipid A will bind chromatin proteins but not naked DNA which suggests that there may be a nuclear lipid A binding protein. Freedman et al. (60) showed that basic plasma membrane proteins (30,000 to 60,000 MW) disappear with LPS stimulation. Whether or not these proteins are internalized or shed is not known. Finally, Watanabe and Ohara (61, 62) fused the karyoplasts of cells from a mouse strain which was hyporesponsive to LPS with the cytoplasts from cells of mice which respond to LPS in a normal manner. In this study, they showed that the nucleus of the hyporesponsive mouse strain was capable of responding to LPS when the membrane of the responsive cell surrounded it. In a recent study (63), cell fusion techniques were used to "microinject" the cytoplasm of a B lymphocyte from the C3H/HeN mouse strain (responsive to LPS) into the B lymphocytes of the C3H/HeJ mouse strain (hyporesponsive to LPS). They showed that a 100,000 molecular weight protein would allow C3H/HeJ B lymphocytes to respond to LPS. They concluded that a cytoplasmic protein was necessary for mitogenesis.

The nature of the binding protein or receptor has been the focus of a number of studies. Coutinho and colleagues (64) prepared an antiserum which would bind to LPS responsive lymphocytes but not to LPS hyporesponsive lymphocytes. They suggested that the antiserum recognized the LPS receptor. This finding has been disputed by Watson et al. (65) and Gregory et al. (50) who showed that lymphocytes from

responsive and hyporesponsive strains of mice bind lipid A at comparable levels. Two separate studies have used either LPS or lipoprotein columns to attempt to isolate the LPS binding protein. In the first work, Yokoyama and coworkers (66) reported that seven different proteins could be eluted from the LPS column. These proteins were 73,000, 62,000, 42,000, 32,000, 24,000, 23,000 and 12,000 in molecular weight. They presented evidence that H-2 and IgD were the critical binding proteins. In the second work, Bessler et al. (67) found five binding proteins, three of which (the 73,000 62,000 and 32,000 molecular weight proteins) corresponded with those reported in the previous paper. Although no evidence was presented for the involvement of Ia or sIgD, a recent abstract by Wolfert et al. (68) suggests that Ia and sIgD are crosslinked by LPS during mitogenesis. Dithiobisphenylazide, a photoreactive crosslinking agent, was used to show that several molecules are crosslinked in the presence of LPS. Ia and a fragment of IgD were immunoprecipitated from the crosslinked molecules. Additional support for the role of Ia has come from Niederhuber and colleagues (69), who have shown that anti-Ia antibody can inhibit LPS stimulated mitogenesis.

Finally, a study which examined enzymatic activity during mitogenesis was done by Ku and co-workers (70). In this study, PPD and LPS stimulated mitogenesis were examined. They used diisopropylfluorophosphate (DFP) to inhibit serine esterase activity and showed mitogenesis was inhibited with DFP during the first 18 hours of incubation. They further demonstrated that the inhibition correlated with the cleavage of a protein (107,000 to 55,000 molecular weight). This protein was B cell specific, but not LPS specific since PPD could also activate cleavage of this protein.

In summary, LPS is a glycolipid which stimulates a specific subset of B lymphocytes. This reaction is calcium dependent and triggers an increase in cGMP, phosphorylation of proteins and cleavage of a protein by serine protease. The actual receptor remains to be defined, but it appears to be membrane bound, at least initially, and may be cytoplasmic soon after LPS activation. In addition, there may be a separate cytoplasmic component which is necessary for mitogenesis to occur.

Animal Models. Several mouse strains have been used to facilitate the study of LPS activation of B lymphocytes (Table 2). Mice which cannot respond to LPS or lipid A have been particularly useful. C3H/HeJ mice are LPS resistant and have been used to investigate the molecular mechanisms involved in LPS activation. The LPS resistance in C3H/HeJ mice is manifested in several ways, which include: 1) decreased

TABLE 2

Characteristics of Mouse Strains

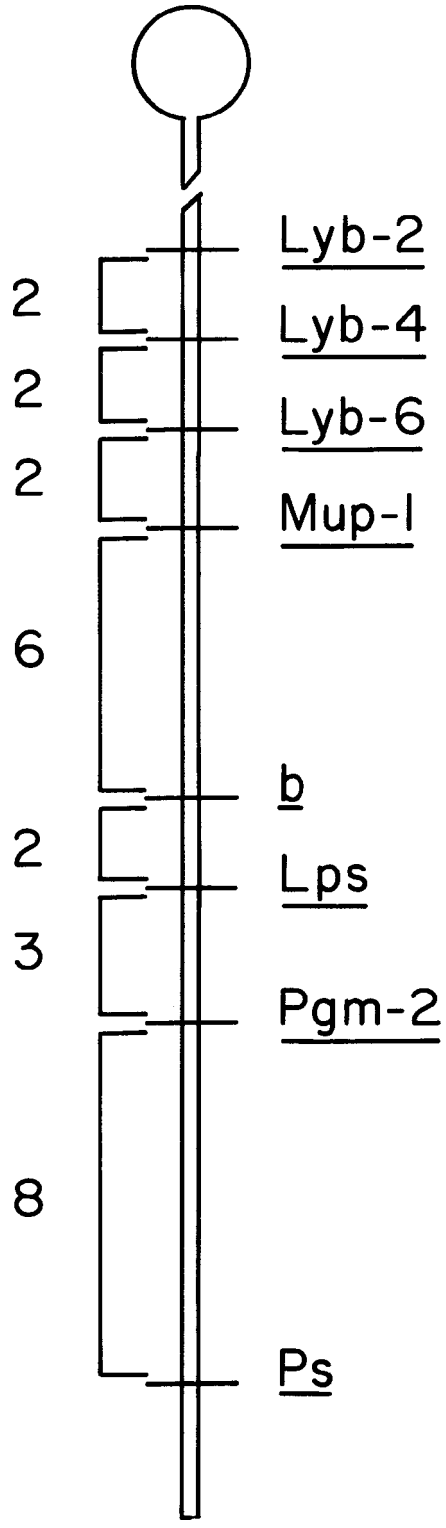
Strain of Mouse	Responses to LPS	Known Defects
CBA/N	No	x-linked
C3H/HeJ	No	Lps gene 4th chromosome
C3H/HeN	Yes	None

susceptibility to endotoxin death, 2) failure of LPS to stimulate polyclonal B cell activation, and 3) failure of LPS to stimulate B cell mitogenic activity. The use of C3H/HeJ and LPS sensitive, syngeneic C3H/HeN mouse strains have made it possible to define the location of the gene responsible for LPS unresponsiveness. In these studies,

recombinant inbred mice were used to locate the $\underline{Lps}^{d}$ (d=defective) gene on the fourth chromosome (71, 72). The $\underline{Lps}^{d}$ gene is closely linked to genes coding for the major urinary protein (MUP-1) and the B cell differentiation antigens Lyb 2, Lyb 4, and Lyb 6 (Figure 2).

As previously stated, the genetic defect can be demonstrated by decreased susceptibility of the host to endotoxin death. While C3H/HeN mice are highly susceptible to LPS (LD_{50} of approximately 100 μg) and C3H/HeJ mice are less susceptible to LPS (LD_{50}=15-20 mg), F1 hybrids [from crosses between C3H/HeN ($\underline{Lps}^{n}/\underline{Lps}^{n}$; n=normal) and C3H/HeJ ($\underline{Lps}^{d}/\underline{Lps}^{d}$) mice] show an intermediate responsiveness to the endotoxic effects of LPS. Alternately, the LPS defect can be demonstrated in the polyclonal expansion of B cells of the two mouse strains. When spleen cells for C3H/HeN, C3H/HeJ, and F1 hybrid mice are stimulated with LPS, the F1 animals exhibit intermediate polyclonal responses when compared to the high level seen with C3H/HeN mice, and the low number in C3H/HeJ animals. It has been shown, using F1 mice, that this intermediate reponse is a result of allelic exclusion (73) rather than codominant gene expression. In allelic exclusion, there is an all or none expression of the $\underline{Lps}$ phenotype. Each individual B cell in the F1 mouse either expresses the $\underline{Lps}^{d}$ gene and is unresponsive to LPS or expresses the $\underline{Lps}^{n}$ gene and is responsive to LPS. The C3H/HeJ mouse, which is unresponsive to phenol-water extracted LPS (Ph-LPS), is fully responsive to butanol-water extracted LPS (74), since the latter preparation also contains the B cell mitogen, lipid A-associated protein. As stated in the previous section, the failure of the C3H/HeJ mouse to respond to Ph-LPS is not due to an inability of their B cells to bind LPS, since several authors have shown that the C3H/HeJ mouse B cell can bind

Figure 2. Fourth chromosome of the mouse. This figure respresents the eighth linkage group on the fourth chromosome of the mouse. Lyb 2, Lyb 4 and Lyb 6 are genes for B lymphocyte surface antigens. Mup 1 is the gene for major urinary protein 1. The b gene is for brown coat color. Lps is the gene which controls B lymphocyte response to LPS. Pgm 2 is the gene which codes for phosphoglucomutase-2 and Ps is the gene which codes for polysyndactyly.



VIII

Ph-LPS (50, 75). Thus, the failure seems to lie in an inability of LPS to trigger certain biochemical events involved in B cell activation.

The second mouse strain which has been used to study the effects of LPS on B lymphocytes is the CBA/N mouse strain. This strain was first identified by Amsbough et al. (76), who showed that the CBA/N mouse carried an x-linked defect which prevented them from responding to pneumococcal polysaccharide. The development of antisera which recognized specific B cell surface antigens showed that CBA/N mice lacked specific B lymphocyte surface antigens, Lyb 3 and Lyb 5 (77, 78 rev.), and thus, lack a B lymphocyte subset. This mouse strain has been used to define two types of T independent (TI) antigens. A TI antigen is capable of inducing an antibody response without T cell help. The properties of TI antigens have been summarized in a recent review by Mosier and Subbarao (77). These antigens are high molecular weight substances with repeating units, slowly metabolized, frequently mitogenic and polyclonal B cell activators, and often have a carbohydrate or bacterial cell wall component. T independent antigens which fail to stimulate an antibody response in CBA/N mice have been called TI type 2 (TI-2) antigens, while TI antigens which elicit an antibody response in this strain have been called TI type 1 (TI-1) antigens. Because the defect is x-linked, if one crosses CBA/N female mice with normal male mice then the defect would be expressed in the F1 male progeny and the F1 female mice would be normal. Huber and Melchers (23) used F1 hybrid mice to examine LPS responsiveness in the CBA/N mouse strain. Their studies showed that B cells from male (CBA/N x BALB/c) F1 mice failed to respond to LPS, lipoprotein and Norcardia water soluble mitogen. These results suggest that a Lyb 3^+, Lyb 5^+ cell is responsive to LPS

while an Lyb 3^-, Lyb 5^- B cell is not responsive to LPS.

These two mouse strains have been important tools in the study of the precise B lymphocyte subset, Lyb 3^+, Lyb 5^+ cells, which is responsive to LPS. The C3H/HeJ and syngeneic C3H/HeN pairing has allowed a genetic defect (LPS resistance) to be identified (chromosome 4) and has been instrumental in many of the studies described in this section and the previous section. There is no doubt that these mouse strains will continue to be used in the investigation of LPS-activated mitogenesis.

Receptors. A receptor, as it is defined in the classical sense, is a molecule which has a binding site with high affinity for a specific molecule. Interaction between the receptor and ligand stimulates a specific set of events for that given cell. There are numerous reviews on receptors and their mechanisms of action (79, 80-82). The best understood receptor-ligand interactions are those between hormones and their receptors. On a B lymphocyte, the best characterized receptors are surface immunoglobulin, Fc receptors, and the receptors for complement. Before discussing the receptors on the B lymphocyte, two classic hormone receptors will be examined, the receptor for insulin and the receptor for progesterone.

The insulin receptor is a peptide hormone receptor, which is membrane bound and has two major components, alpha and beta (83, 84). Two beta subunits occur, beta and beta-one. In a recently proposed model for the insulin receptor (83), the representation resembles immunoglobulin and contains two alpha chains (the heavy chain equivalent) and the two beta chains (the light chain equivalent). Unlike the immunoglobulin molecule, two different beta chain molecules can be associated with the alpha chains which accounts for the three different molecular weights

that have been reported for the intact molecule, 350,000, 320,000, and 290,000. When insulin interacts with this receptor, a number of events are induced which include an increase in cAMP and a calcium ion influx (80, 85). The receptor is then internalized and has been shown to migrate to several subcellular locations which include the Golgi region (85) and the nucleus (86). Originally, this internalization was thought to be artifactual, but is now thought to play an integral role in the regulatory mechanism of the insulin receptor.

Binding of insulin to the insulin receptor results in negative cooperativity. This means that even though the insulin receptor has more than one binding site for insulin, once a single molecule is bound, it is difficult to bind additional molecules. It has been suggested that internalization of the receptor prevents binding of additional insulin molecules, but a precise explanation awaits further investigation. Another peculiarity of the insulin-insulin receptor interaction was demonstrated by Clark and Harrison (87) who have shown that a covalent linkage is established between insulin and the insulin receptor.

Progesterone is a steroid hormone which activates the transcription of mRNA for avidin in the chicken oviduct (88, 89). Unlike insulin receptors, the receptors for steroid hormones are cytoplasmic rather than membrane bound. Steroid receptors are DNA binding proteins or histone binding proteins and thus, directly activate transcription. The progesterone receptor has two components with molecular weights of 71,000 daltons (A) and 114,000 daltons (B). These components are synthesized separately and combine in the cytoplasm. When progesterone interacts with the receptor, a number of changes occur which include transport to the nucleus and binding of the receptor to DNA. It is proposed that

one component of the receptor, A, stabilizes the DNA and that the other, B, binds specific receptor sites and enhances specific transcription.

These two hormones regulate the cells by totally different mechanisms. Insulin, a peptide hormone, works via a membrane receptor to activate a number of biochemical changes and include calcium ion influx and phosphorlyation of proteins, which lead to stimulation of the cell. Progesterone, a steroid hormone, interacts with a cytoplasmic receptor and directly activates mRNA transcription. Do B lymphocyte receptors mimic either of these regulatory schemes? B lymphocyte surface IgM parallels the insulin model. The Fc receptor has been termed "liponomic regulation" (90) and represents a unique regulatory mechanism, while the complement receptor events remain undefined. These three receptors will be discussed in detail below.

The interactions of surface immunoglobulin with antigen can cause either activation or suppression of the B lymphocyte (see B Lymphocyte Mitogens section), as has been demonstrated with anti-immunoglobulin. The biochemical events which follow surface immunoglobulin-antigen interaction are not as well defined as the hormone system. It has been shown by Nishizawa et al. (91) that anti-immunoglobulin will activate phosphorylation of nonhistone nuclear proteins. The phosphorylation could be stimulated with the cytosol from anti-Ig stimulated cells. In a subsequent study (92), this group described a cytoplasmic protein with a molecular weight of 150,000 which was cleaved by a serine protease to a 45,000 dalton protein. It was suggested that the presence of this cleaved protein was necessary for phosphorylation to occur. This system is not unlike the PPD and LPS-triggered serine protease system of Ku et al. (70) which was previously discussed.

The molecular events which occur when the Fc portion of the antibody molecule interacts with the Fc receptor have received some attention during the last few years. These receptors regulate the composition of membrane lipid, and thus the term liponomic regulation has been used to describe this system. The Fc receptor for IgE, although not found on B lymphocytes, has been shown to activate two separate methylases (93) which change a phosphatidyl ethanolamine to phosphatidyl choline. This change is thought to alter the fluidity of the plasma membrane. The Fc receptor for IgG on a B lymphocyte has been shown to have phospholipase A2 activity (94). The phospholipase cleaves fatty acids which vary in length from 16 to 20 carbons. This not only alters membrane fluidity but also serves as a source for arachidonic acid, which in turn is a precursor for a number of biologically active substances which include prostaglandins and leukotrienes. These results suggest that altered membrane fluidity can play a role in B cell regulation.

The receptors for the components of complement have recently been reviewed (95). B lymphocytes have receptors for C3b, C3d and β_1H. The C3b receptor is a glycoprotein with a molecular weight of 205,000 daltons (96), while C3d has a molecular weight of 50,000 daltons. The C3bi and 1βH receptors have yet to be characterized. The B lymphocyte events which are triggered by C3b-C3b receptor interactions are the release of β_1H and the release of macrophage chemotactic factor. The functions of the other three ligand-receptor interactions have yet to be defined.

If one were to use the hormone receptor model systems, which model does LPS most closely follow? My colleagues and I have previously proposed that there is a cytoplasmic receptor for LPS which is similar to the steroid hormone receptor (97). We proposed this based upon the

cytoplasmic and nuclear association of LPS in triggered B cells. However, one can make an equally strong case for an insulin type receptor. Table 2 summarizes the events involved in peptide-, steroid-, and LPS-receptor interactions. All three receptors have a number of events in common. In the work presented here two aspects of LPS stimulation have been examined. First, two lipid A binding proteins were identified and partially characterized. Second, computer assisted two-dimensional gel analysis was used to examine the proteins of B lymphocytes from the LPS responsive and hyporesponsive mouse strains.

TABLE 3

Comparison of Steroid Hormone-, Peptide Hormone- and LPS-Receptors

	Receptor Type		
	Steroid Hormone	Peptide[1] Hormone	LPS
RECEPTOR SITE			
membrane	no	yes	yes
cytoplasmic	yes	endocytosed	possible
nuclear	yes	possible	possible
CELLULAR EVENT			
Ca^{+2} influx	no	yes	yes
cyclic nucleotide change	no	yes	yes
protein phosphorylation	no	yes	yes
chromatin binding	yes	no	yes

[1] Insulin receptor was used as a model.

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Characterization of Lipid A Binding Proteins: An LPS Receptor Found?

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ABSTRACT

This study has examined molecular aspects of the interaction between lipid A and B lymphocytes. From lysates of spleen cells incubated with ^{125}I-lipid A, we have identified two lipid A binding protein with a molecular weights of 60,000 and 62,000 daltons. These molecules bind ^{125}I lipid A in the presence of detergent, 2% Nonidet-P40 or 2% sodium dodecyl sulfate. A small molecule, 28,000 daltons, co-isolated with the 60,000 and 62,000 dalton proteins. The 28,000 dalton molecule also bound lipid A in the presence of detergent. In addition, a fourth molecule with a molecular weight of 43-45,000 daltons was identified in a sucrose gradient fraction which contained elevated levels of ^{125}I-lipid A but no direct interaction between this molecule and ^{125}I-lipid A was established.

INTRODUCTION

It was shown over ten years ago that lipopolysaccharide (LPS) from the outer membrane of gram-negative bacteria is mitogenic for murine spleen cells (1). Subsequent studies showed that LPS stimulated B lymphocytes to divide (2) and that the effect was confined to the lipid A portion of the LPS molecule (3). A number of approaches have been taken to determine the molecular events involved in LPS-triggered B cell activation. It has been shown that LPS-stimulated mitogenesis 1) is calcium ion dependent (4), 2) induces an increase in cGMP levels (5,6), 3) causes phosphorylation of nonhistone chromosomal proteins (7), and 4) activates a serine protease (8). In addition, it has been shown that LPS can localize in the nucleus of lymphocyte (9).

Another major approach to analysis of LPS effects on B lymphocytes has been to examine the effects of anti-IgM and anti-IgD which can mimic LPS activity and anti-Ia which can block the effects of LPS. Kearney et al. (10-11) have shown that treatment of mature lymphocytes with high doses of anti-IgM can inhibit LPS-triggered mitogenesis. Others (12) have shown that lower doses of anti-IgM are able to induced mature B cells to proliferate, but did not stimulate polyclonal B cell activation to immunoglobulin (Ig) synthesis. Anti-IgD has also been shown to stimulate B cells to divide (13). Niederhuber et al. (14) used an antiserum to Ia to block the proliferative response to LPS.

A number of groups have attempted to identify the actual LPS receptor. Coutinho et al. (15) produced an antiserum which recognized the B lymphocytes from LPS responsive C3H/Tif mice but not B lymphocytes from an LPS nonresponsive mouse strain which suggests that there is a

surface antigen difference between LPS responsive and nonresponsive strains. This antibody was subsequently used to identify a second LPS nonresponsive mouse strain (16). However, Gregory et al. (17) have shown that lymphocytes from C3H/HeJ mice, which are hyporesponsive to LPS, and C3H/HeN, a mouse strain which responds normally to LPS, bind lipid A equally well, which suggests that the defect in the hyporesponsive mouse strain is not due to a surface LPS-binding protein. This finding suggests that binding of LPS is not sufficient to trigger mitogenesis. Yokoyama et al. (18) used LPS attached to a Sepharose LPS column to identify seven cellular proteins which bind LPS. Indirect evidence was provided which showed that three of these proteins were H-2K, H-2D and Ia antigens. In this regard, Wolfert et al. (19) used a crosslinking agent to determine which cell surface molecules bound LPS. Several molecules were isolated; the major protein had a molecular weight of 95,000-100,000 and could be immunoprecipitated with anti-Ia. Finally, Springer et al. (20) isolated a lipoglycoprotein (M.W. = 256,000) from human erythrocytes, which they called the lipopolysaccharide receptor complex. No B cell equivalent for this complex has been identified to date.

In this study, we have combined the techniques of sucrose density gradient centrifugation, polyacrylamide gel electrophoresis, and autoradiography to identify two proteins from B cell lysates which bind lipid A and a third which co-isolates with ^{125}I lipid A. The nature of each molecule and possible interactions between these molecules are discussed.

MATERIALS AND METHODS

Preparation of Spleen Cells and B Lymphocytes. Spleens were removed aseptically from 7-12 week old C3H/HeN (obtained from the Core Facility for Immunocompromised Mice, Comprehensive Cancer Center at UAB) and C3H/HeJ (Jackson Laboratories, Bar Harbor, ME) mice. Single cell suspensions were obtained by processing spleens through a 60 gauge stainless steel mesh. The cell suspension was washed once with RPMI 1640 medium (GIBCO, Grand Island, NY). Single cell suspensions were treated with Tris-ammonium chloride solution (21) for 5 min at 37°C to lyse red blood cells, washed once in RPMI 1640 and adjusted to 14×10^4 cells/ml in phosphate-buffered saline (PBS; 0.14 M NaCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl), containing 5% fetal calf serum (FCS) (HyClone, Logan, UT).

The method used for purification of B lymphocytes was an adaptation of the procedure described by Wysocki and Sato (22). Goat anti-mouse immunoglobulin (5 ml; 25 μg/ml) in 0.05 M Tris (pH 9.5) was added to Petri dishes (100 x 15 mm; Falcon 1029). The plates were gently swirled to distribute the reagent over the entire surface of the plate, incubated for 40 min at room temperature, and then washed four times with PBS, and once with PBS containing 1% FCS. Three ml of the spleen cell suspension was added to each Petri plate, the plates were rotated to distribute the cells and incubated for 70 min at 4°C. After 35 min of the 70 min incubation, the plates were gently rotated again to insure even coating of the cells onto the plate. Each plate was washed five times with PBS and 1% FCS, followed by vigorous pipetting to remove the cells which were attached to the Petri plate.

A mitogenesis assay was used to determine the purity of the B lymphocyte population. Purified B lymphocytes or whole spleen cells were adjusted to 5 x 10^6 cells/ml in RPMI 1640 supplemented with L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (GIBCO). Aliquots of the appropriate cell preparation (5 x 10^5 cells/0.1 ml) were dispensed into 96-well flat bottom microtiter plates (Linbro Chemical Co., McClean, VA) and were stimulated with phytohemagglutinin (PHA; 2 μg/ml; Burroughs-Wellcome, Triangle Park, NC), concanavalin A (Con A; 0.5 μg/ml; Miles Laboratories, Elkhart, IN), or butanol-water extracted lipopolysaccharide (Bu-LPS; 25 μg/ml) (23). The plates were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. Cultures were pulsed with ^{3}H-thymidine (0.5 μCi/well; New England Nuclear [NEN], Boston, MA) for the last 6 hr of a 48 hr incubation and cells were harvested with a MASH II (Microbiological Associates, Walkersville, MD) onto glass filter paper. Paper discs were dried, placed into mini vials (Wheaten, Millville, NJ) and resuspended in 3 ml of Scint-0 (Packard Instrument Co., Downers Grove, IL). The amount of radioactivity was determined in a Beckman LS 8000 scintillation counter. If purified B lymphocyte cultures had an experimental/control index (cpm of stimulated cells/cpm of cells in culture medium only) of one (or less) in the presence of PHA or Con A, they were considered to be free of T cell contamination. A good experimental/control index for purified B lymphocytes cultured with Bu-LPS or Ph LPS was 10 or greater.

Labelling of Lipid A. One mg of commercially obtained lipid A (List Laboratories, Campbell, CA) or lipid A derived from Escherichia coli K 235 LPS by the method described by Galanos et al. (24) were

radioiodinated using a modification of the method described by Zimmerman and Kern (25). One mg of lipid A was mixed with 0.25 ml of chloroform, 0.25 ml of glacial acetic acid containing one μmol/ml of iodine monochloride and one mCi of $Na^{125}I$ (NEN). This mixture was incubated for 5 min at room temperature in a hood, and then one ml of 0.15M NaCl-0.01 M phosphate buffer, pH 7.2, containing 30 μmol KI/ml was added. The mixture was spun in a microfuge for 5 min and then the aqueous layer was removed. The wash step was repeated 5 times. The chloroform was then evaporated by gently passing a stream of nitrogen gas over it. Ten μl of triethylamine was added to the pellet, followed by addition of 1 ml of the phosphate-buffered saline. The specific activity for an average labelling was about 20 μCi/mg of lipid A.

Sucrose Gradients. The 5-30% sucrose gradients were poured in a centrifuge tube (1/2 inch and 2 inch Ultra-Clear, Beckman, Palo Alto, CA). B lymphocytes or spleen cells which had been cultured with ^{125}I lipid A (5 μg/ml) and Ph-LPS (25 μg/ml) were harvested after 24 hr of incubation from 24-well culture plates (5×10^6 cells/ml, 1 ml/well). These cells were washed in PBS and then lysed with 2% Nonidet P-40 (NP-40). The lysates were adjusted to 50,000 cpm/0.1 ml and 0.1 ml was carefully layered onto each gradient. The gradient was spun at 35,000 x g for 18 hr in a Beckman model LS-50 (SW 50.1 rotor) centrifuge. Fractions were collected from each gradient tube (0.17 ml/fraction) and counted in a Beckman gamma counter. The fractions which contained ^{125}I activity were retained for electrophoresis on sodium dodecyl sulfate (SDS)-polyacrylamide gels.

Polyacrylamide Gels. Twenty-five μl of the fraction from a sucrose gradient was mixed with 25 μl of dissociation buffer [10% (w/v) glycerol,

5% (v/v) 2-mercaptoethanol, 2% SDS and 0.0626 M Tris-HCl, pH 6.8] and 40 µl of the mixture was added to a well of the SDS-polyacrylamide gel. A Laemmli gel system (26) was used to visualize the radioactive sucrose fractions. The resolving gel (10% acrylamide/bisacrylamide, 2% SDS and 0.375 M Tris-HCl, pH 8.8) was poured (0.7 mm thick) and aged overnight. A 3% acrylamide stacking gel containing 0.1% SDS and 0.12 M Tris HCl (pH 6.8), was layered onto the resolving gel. The gel was run in a Tris-glycine buffer (0.02 M Tris, 0.19 M glycine and 0.1% SDS, pH greater than 8) at 4°C at 20 mA for approximately 4 hr or until the bromphenol blue dye reached the bottom edge of the gel. The gel was removed, silver stained (27), dried and incubated with photographic film at -70°C for 20 days.

For two-dimensional gel analysis, the first dimension was a nonequilibrium pH gel electrophoresis (NEPHGE) system (28). B lymphocytes or splenocytes which had been incubated with ^{125}I-lipid A for 24 hr were harvested, centrifuged over Ficoll-hypaque (29) to remove dead cells, and washed 3 times in PBS. Ten million cells were added to a tube and centrifuged at 2,000 x g. The supernatant was discarded and 100 µl of lysis buffer [9.5 M urea; 2% (w/v) NP-40; 2% Ampholines, LKB 3.5-10; and 5% 2-mercaptoethanol] was added to the cell pellet. This preparation was frozen at -70°C until used in the first dimension of the two-dimensional gel. The NEPHGE gel consisted of 9.2 M urea, 2% NP-40, 4% acrylamide/bisacrylamide, 2% Ampholines, 20 µl of 0.87 mM ammonium persulfate and 12 mM N,N,N',N'-tetramethylethylenediamine and was poured into 130 mm x 2.5 mm tubes to a height of 12 cm and then overlaid with a solution of 8 M urea and 1% Ampholines. After polymerization (1 hr), the overlay buffer was replaced with the lysis buffer and allowed

to equilibrate for 1 hr. The tubes were placed in an electrophoresis chamber, in which the lower reservoir contained 0.02 M NaOH (degassed) and the upper chamber 0.01 M phosphoric acid. An aliquot (30 µl) of the cell lysate was added to each tube. The sample was overlaid with 20 µl of the overlay buffer and the tube was filled with phosphoric acid. The gels were electrophoresed toward the cathode for 4-1/2 hr at 400 V. The gel was extruded into 5 ml of dissociation buffer consisting of 10% (w/v) glycerol, 5% (v/v) 2-mercaptoethanol, 2% SDS and 0.0626 M Tris-HCl (pH 6.8) and frozen at -70°C until the second dimension was run.

The second dimension was the Laemmli gel system described above. As the stacking gel was allowed to polymerize, the NEPHGE tube gel was thawed and equilibrated for 2 hr in dissociation buffer with one buffer change. Then 0.75% agarose in dissociation buffer was melted in a boiling water bath and the Laemmli gel was overlayed with 1 ml of agarose. The NEPHGE gel was placed on the hot agarose and the agarose allowed to solidify. The gel was then electrophoresed as described above. When the electrophoresis was completed the gels were silver strained (26).

RESULTS

Selection of lipid A binding proteins on sucrose gradients. Spleen cells or purified B lymphocytes were cultured with ^{125}I-lipid A (5 µg/ml) and Ph-LPS (25 µg/ml) for 24 hours. In all instances the ^{125}I lipid A served as a tracer for proteins which bound lipid A or Ph-LPS and the cold Ph-LPS was the mitogen source. Figure 1 shows the pattern

of radioactivity when ^{125}I lipid A only is fractionated on a sucrose gradient. Five separate sucrose gradient runs gave nearly identical patterns of radioactivity distribution. The ^{125}I lipid A was found in a broad band in the upper half (fractions #15-30) of the gradient and fraction #19 or #20 contained the greatest amount of radioactivity. If Ph-LPS (0.1 μg) was mixed with the ^{125}I lipid A there was no alteration in the distribution of the ^{125}I lipid A in the sucrose gradient (Figure 1).

When lysates of spleen cells which had been incubated with ^{125}I lipid A and Ph-LPS were fractionated on a sucrose gradient, a common pattern emerged. First, the broad band of ^{125}I lipid A was found in all gradient runs in the less dense half of the gradient like that seen for ^{125}I lipid A alone (Figure 2a versus Figure 1). In addition, two separate peaks of radioactivity could be demonstrated, one at fractions #4-6 and the other at fractions #10-13 (Figure 2a), although some variation in this pattern was seen. In some sucrose gradient runs the only radioactive peak occurred at fractions #4-6, and in other runs only the peak at fractions #10-13 was seen, while both radioactive peaks were apparent in other gradients. When sucrose gradient fractions of lysates from spleen cells from C3H/HeJ mice (Figure 2b) or B lymphocytes from C3H/HeN mice (Figure 2c) were examined, a nearly identical pattern was observed, with peaks of radioactivity at fractions #4-6 and #10-13, in addition to the lipid A band (fractions #15-30).

Characterization of proteins found in the ^{125}I-lipid A containing sucrose fractions. Once the pattern of distribution of ^{125}I lipid A had been determined, SDS-polyacrylamide gels were used to visualize the proteins found in the radioactive peaks. The fractions examined

Figure 1. Distribution of ^{125}I lipid A in a sucrose gradient. The radioactivity of each fraction is expressed as a percent of the total radioactivity found in the gradient (cpm of a fraction-background cpm/total cpm of all fractions). Fraction #1 is the most dense sucrose and fraction #30 is the least dense sucrose. ^{125}I lipid A alone, ^{125}I lipid A alone, ^{125}I lipid A + Ph-LPS.

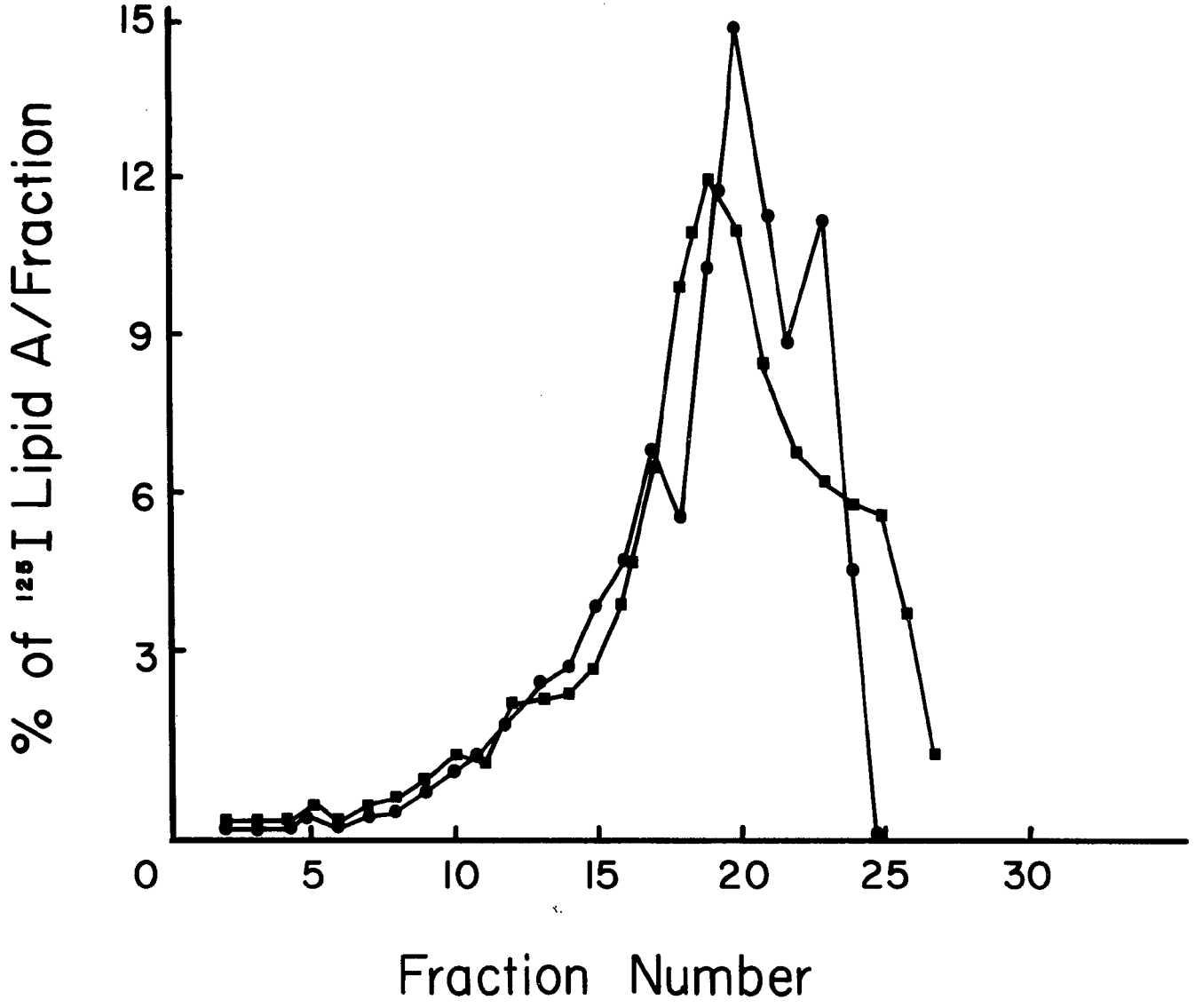
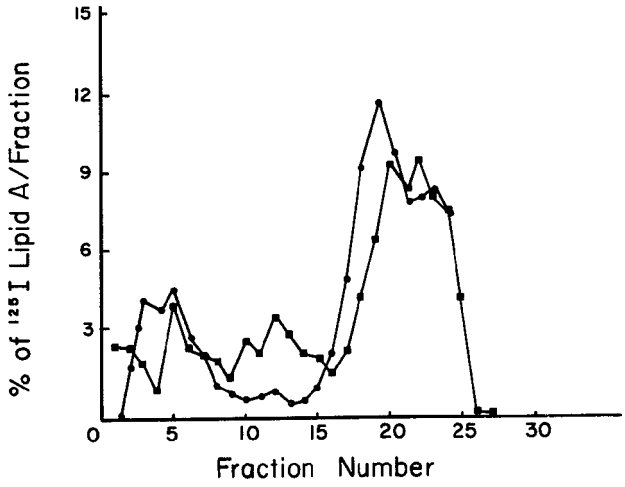

15
12
9
6
3
0
5
10
15
20
25
30
% of ^{125}I Lipid A/Fraction
Fraction Number

Figure 2. Distribution of ^{125}I lipid A plus cellular lysates in a sucrose gradient. Lysates from C3H/HeN spleen cells (a), C3H/HeJ spleen cells (b) and C3H/HeN B cells (c) which have been cultured with ^{125}I lipid A (5μ g/ml) and Ph-LPS (25 μg/ml) for 24 hours and then fractionated on a sucrose gradient. Each panel shows two independent fractionations of the lysates from these cells. The ordinate is a percent of the total radioactivity found in the sucrose gradient (cpm of the fraction-background cpm/total cpm of all fractions). The abscissa is the fraction number; fraction #1 is the most dense sucrose and fraction #30 is the least dense sucrose.


% of ^{125}I Lipid A/Fraction
15
12
9
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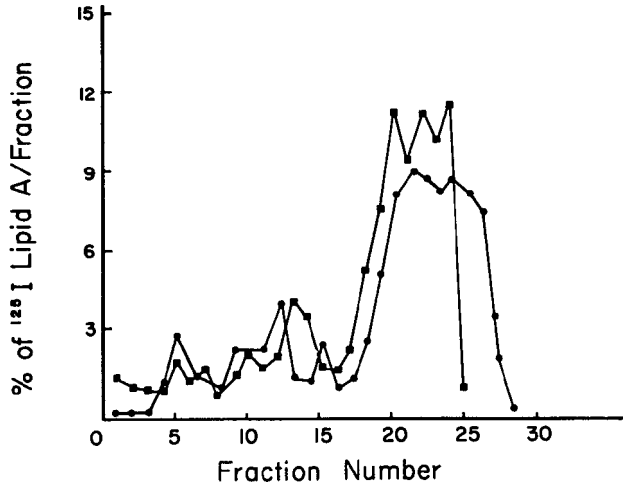
10
15
20
25
30
Fraction Number


% of ^{125}I Lipid A/Fraction
15
12
9
6
3
0
5
10
15
20
25
30
Fraction Number

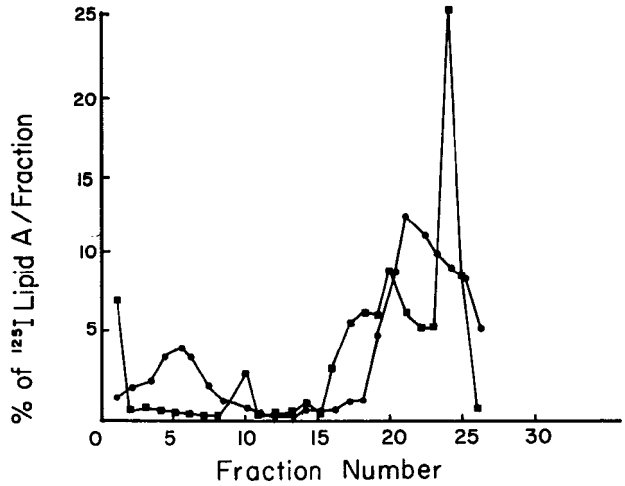
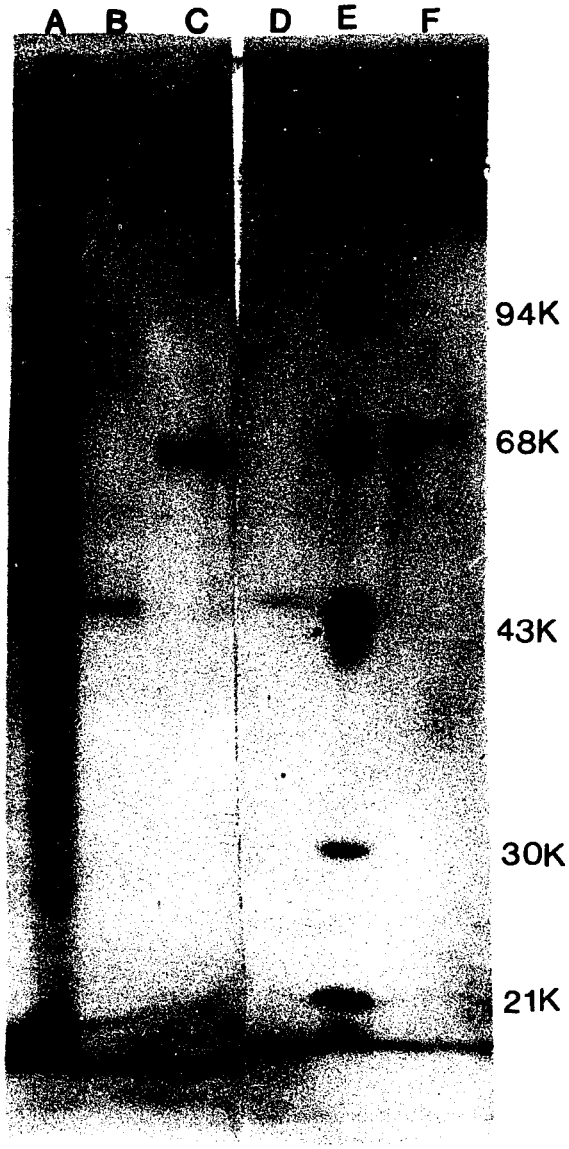

% of ^{125}I Lipid A/Fraction
25
20
15
10
5
0
5
10
15
20
25
30
Fraction Number

Figure 3. Silver stained SDS-polyacrylamide gels of fractions from sucrose gradients. Twenty-five µl of a fraction from the sucrose gradient was mixed with 25 µl of dissociation buffer, heated 2 minutes in a boiling water bath and 40 µl was added to a 10% SDS-polyacrylamide gel for electrophoresis. Figure 3a: Lane A = whole cell lysates, Lane B = fraction #13 from C3H/HeJ spleen cell lysates, Lane C = fraction #20 from C3H/HeJ spleen cell lysates, Lane D = fraction #13 from C3H/HeN spleen cell lysates, Lane E = molecular weight standards, Lane F = fraction #20 from C3H/HeN spleen cell lysates. Figure 3b. Lane A = fraction #5 from C3H/HeJ spleen cell lysates. Lane B = fraction #5 from C3H/HeN spleen cell lysates, Lane C = molecular weight standards.


A
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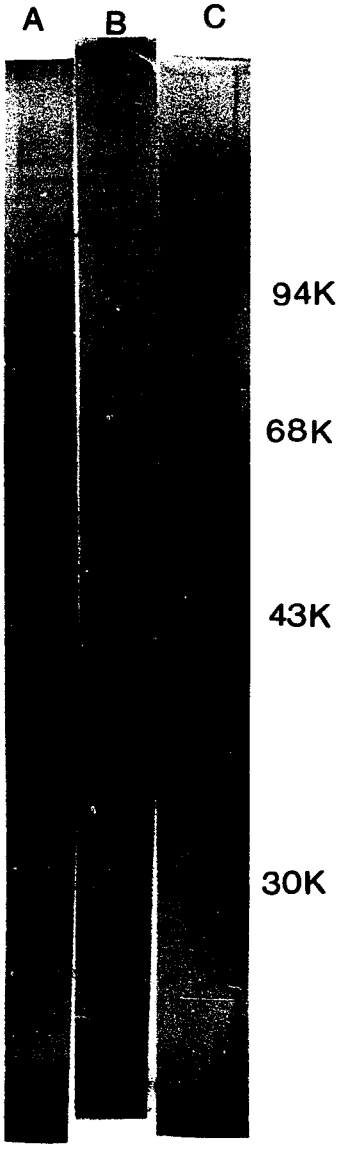
C
D
E
F
94K
68K
43K
30K
21K


A
B
C
94K
68K
43K
30K

were fractions #5, #13, and #20 from sucrose gradients of cell lysates and fraction #20 from sucrose gradients of ^{125}I lipid A. Figure 3a shows the electrophorecic pattern or fractions #13 and #20. Lanes B and D were fraction #13 from sucrose gradients of lysates from C3H/HeJ and C3H/HeN spleen cells, respectively. The protein seen in lanes B and C has a molecular weight of 43-45,000 daltons and comigrates with a major band which is seen when lysates of whole cells were electrophoresed into the gel (lane A). Lanes C and F were from fraction #20 of the sucrose gradients from spleen cell lysates from C3H/HeJ and C3H/HeN mice, respectively. The protein in lanes C and F demonstrated a wide range of molecular weights from 60,000-68,000 daltons. Although a single protein band was exhibited (Figure 3a, lanes C and F), many repeats of the experiments suggested that a lower molecular weight, 60,000-62,000 daltons was more accurate and that there were two molecules 60,000 daltons and 62,000 daltons, in fraction #20 (see below). It should be noted that no major protein bands were seen in the whole cell lysate in the 60-70,000 molecular weight range, so the sucrose gradient has served to enrich for these proteins. Figure 3b showed that both the 43-45,000 dalton protein and the 60,000 dalton and 62,000 dalton proteins (seen in lanes A and B) could be found in fraction #5 of sucrose gradients of spleen cell lysates from C3H/HeN and C3H/HeJ mice. In addition, several other faint bands could be identified.

To determine if ^{125}I lipid A remained associated with any of these proteins, the silver stained gels were dried and x-ray film was exposed to the gel. Figure 4 shows the autoradiograph of the silver strained gels. Only the 60,000 dalton and 62,000 dalton proteins bind ^{125}I lipid A in an SDS-polyacrylamide gel while the 43-45,000 dalton protein

did not. The autoradiograph showed the 60,000 and 62,000 dalton molecules as separate proteins as was was previously demonstrated when fraction #5 was electrophoresed (Figure 3b, lanes A and B). In addition, a faint band of radioactivity was seen at a molecular weight of 28,000 daltons in the same lane with the 60,000 and 62,000 dalton proteins. The fact that the 60,000 and 62,000 dalton molecule bind lipid A may account for the variability in the molecular weight. Ph-LPS has a wide range of molecular weights and the form of Ph-LPS bound to the 60,000 and 62,000 dalton proteins could alter the apparent molecular weights of the molecules.

In our hands, we have never been able to detect the lipid A molecule by silver staining. Ph-LPS, however, did silver stain and gave multiple bands in the gel. An autoradiograph of the gel in which ^{125}I lipid A has been electrophorsed showed radioactivity at the lower edge of the gel (Figure 4). If ^{125}I lipid A was mixed with Ph-LPS, spun on a sucrose gradient, fractionated and the peak fraction of radioactivity electrophoresed into a gel, the autoradiograph again showed only radioactivity at the lower edge of the gel (data not shown), even though multiple bands of Ph-LPS could be seen on the silver stained gel.

Two-dimensional Gel Autoradiographs. Autoradiographs of two-dimensional gels of proteins from cells stimulated with ^{125}I-lipid A were used in an attempt to obtain a relative pI of the lipid A binding protein. Figure 5a is a photograph of the two-dimensional gel which has been silver stained. There were over 200 proteins discernible in the gel. Figure 5 is the autoradiograph of the same gel. As can be seen, unlike the one dimensional gel in Figure 4, there is no association between any protein and the ^{125}I-lipid A so the ^{125}I lipid A did

Figure 4. Silver stain and autoradiograph of SDS-polyacrylamide gels of fractions from sucrose gradients. The first lane (left lane) is molecular weight standards, then two lanes of whole cell lysates, the fraction #20 from a sucrose gradient of C3H/HeJ spleen cell lysates. These four lanes are silver stained. The final two lanes are autoradiographs of fraction #20 and ^{125}I lipid A only, respectively.

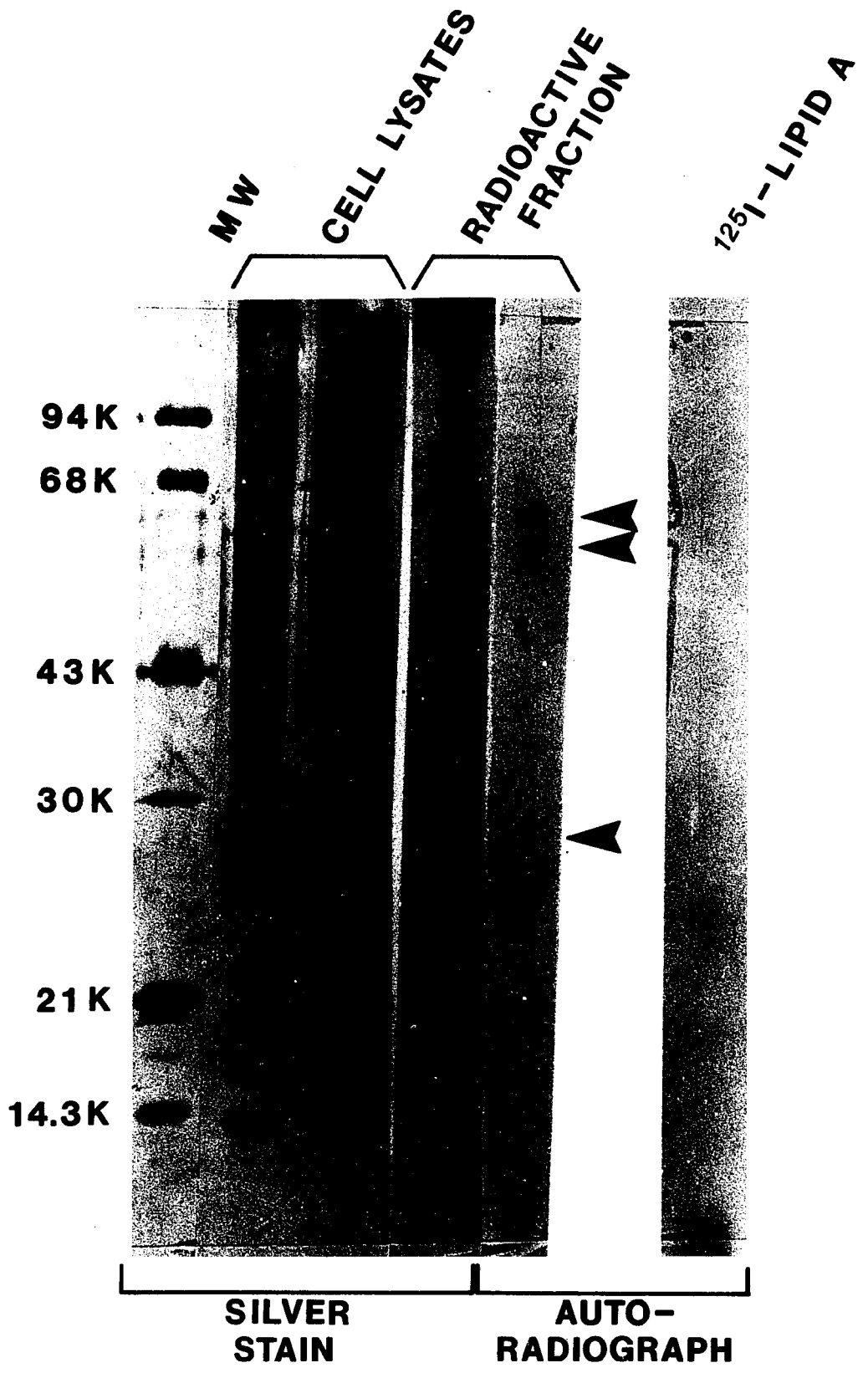
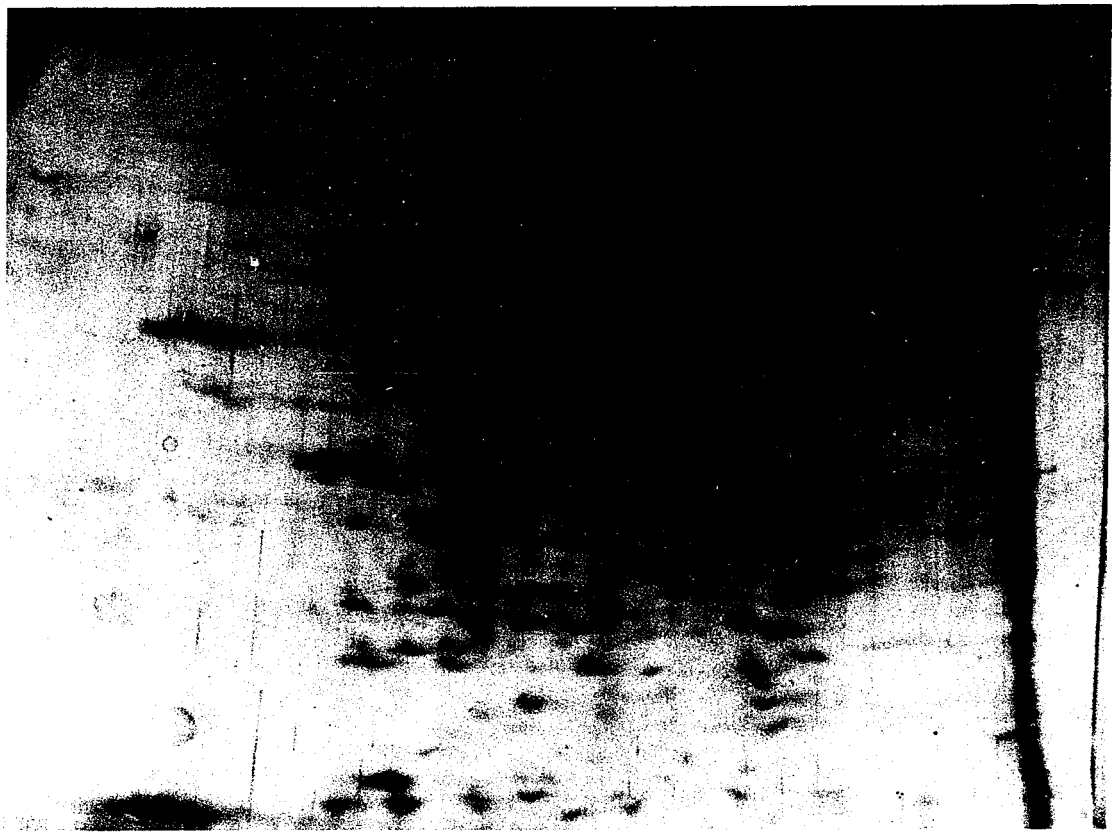
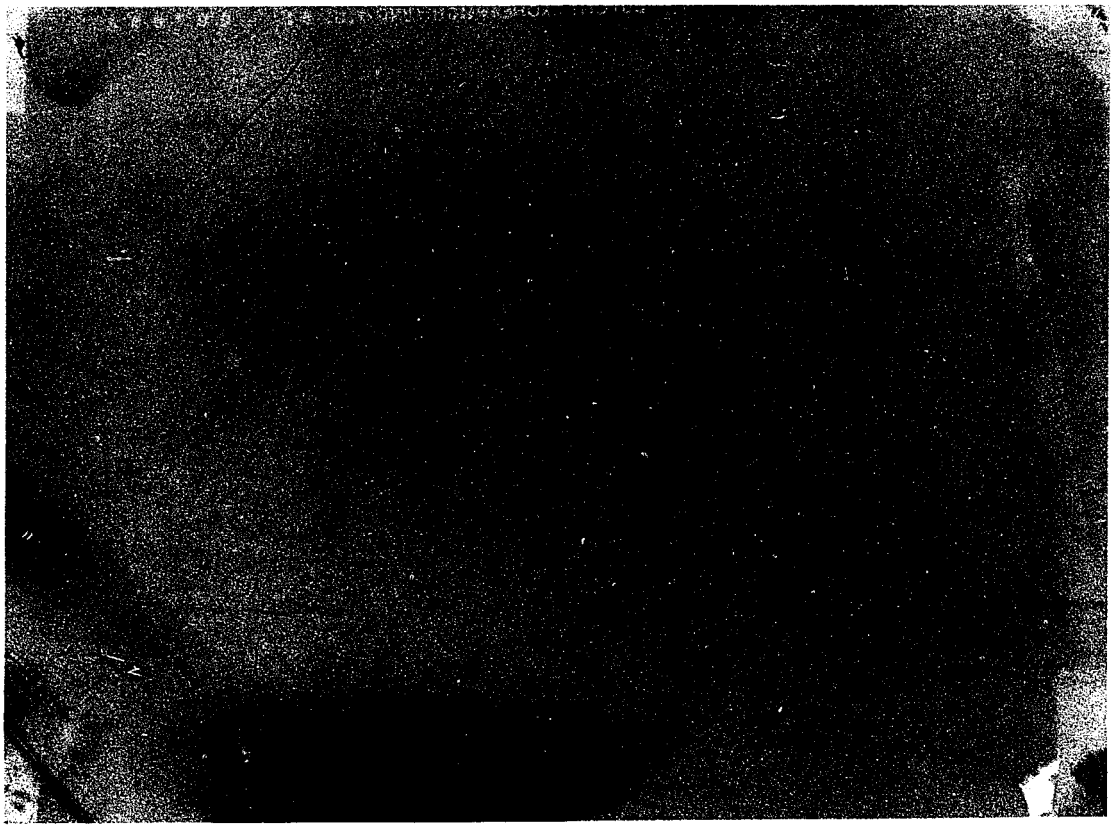

MW
CELL LYSATES
RADIOACTIVE FRACTION
^{125}I-LIPID A
94K
68K
43K
30K
21K
14.3K
SILVER STAIN
AUTO-RADIOGRAPH

Figure 5. A silver stained two-dimensional gel representing the proteins from spleen cells and an autoradiograph of that gel. Panel A is the two-dimensional gel of proteins from C3H/HeN spleen cells which were incubated for 24 hours with ^{125}I lipid A and Ph LPS. Panel B is the autoradiograph of that gel.





not remain bound to the protein in a two dimensional gel system.

DISCUSSION

In spite of efforts by many groups to identify the LPS "receptor," the nature of this molecule has remained elusive. At least three groups have implicated Ia as an LPS binding protein. Yokoyama et al. (18) eluted seven proteins from a sepharose column to which LPS was attached. These were 73,000, 62,000, 42,000, 32,000, 24,000, 23,000 and 12,000 daltons in molecular weight. They could immunoprecipitate the 42,000 molecule with either an alloanti-H-2^k or an alloanti-H-2^d antisera from the column eluate. Anti-Ia immunoprecipitated two molecules of 32,000 and 25,000 in molecular weight. They could also inhibit LPS stimulated mitogenesis with the anti-H-2 antisera. In this regard, Neiderhuber et al. (14) used anti-Ia to suppress the proliferative response of LPS. In these studies, anti-Ia plus complement removed the cells which responded to LPS. Preincubation of cells with the anti-Ia blocked the ability of LPS to induce proliferation and anti-Ia alone failed to induce proliferation which suggests that aggregation of Ia molecules on the cell surface is not sufficient to trigger a mitogenic response. Finally, Wolfert et al. (19) have shown that LPS aggregates at least one other molecule with Ia on the cell surface. They used a photoreactive crosslinking agent, dithiobisphenylazide, to complex LPS with the cell surface molecules. They demonstrated that Ia was linked to an LPS complex by immunoprecipitating the complex with anti-Ia. The molecular weight of this complex was 95,000-100,000 daltons, which implied that another molecule besides LPS and Ia was

present and it was suggested that this might be fragment of IgD.

Kearney et al. (10,11) have shown that incubation of high doses (100 μg/ml) of anti-IgM with adult murine B lymphocytes can block LPS stimulated differentiation into antibody secreting plasma cells, while 300-fold less anti-IgM could suppress LPS activated B cell differentiation in newborns. They suggested that removal of membrane IgM (mIgM) by endocytosis was a key factor in this suppression. One might infer from this study that mIgM is necessary for LPS stimulated mitogenesis. Thus, at least three B cell surface molecules have been linked either directly or indirectly to LPS stimulated mitogenesis, H-2 (45,000 daltons), Ia (58-60,000 daltons) and mIgM (185,000 daltons).

In the present work three molecules have been identified which bind lipid A, 60,000 and 62,000 dalton proteins which are expressed as a doublet on an SDS-polyacrylamide gel and a 28,000 dalton molecule. The strong association between these molecules and lipid A was somewhat surprising. Lipid A contains a diglucosamine in a β 1-6 linkage (30). Attached to the diglucosamine are two phosphate groups and five fatty acid chains. The five fatty acid chains make this molecule highly hydrophobic which led us to think that the interaction between lipid A and B cell surface molecules would be a hydrophobic interaction and would be unstable in detergent. In the preliminary experiments in which we tried to identify the lipid A binding protein, two procedures were used; in the first method the cells were treated with 2% NP-40 to dissociate the membrane, while in the second, the cells were frozen and thawed five times. No difference was seen between these two protocols in the distribution of ^{125}I lipid A in the sucrose gradient profiles, so it was assumed that the interaction between the cellular molecule

and ^{125}I lipid A was stable in detergent and NP-40 was employed for the remainder of the study.

The fact that the 62,000, 60,000 and 28,000 dalton proteins can maintain an interaction with lipid A in the presence of 2% NP-40, 2% SDS and 5% 2-mercaptoethanol would suggest a very stable association between each of these three molecules and lipid A. Since the association is not maintained in a two-dimensional gel system, the strong interaction between lipid A and the 62,000, 60,000 and 28,000 dalton molecules should be considered to be non-covalent. The two-dimensional gel system could have caused the dissociation by pH or by high (9M) urea.

It should also be pointed out that the 28,000 dalton molecule is not routinely seen in the gels. Two explanations could account for this. First, the 28,000 dalton protein could be a separate binding protein which is expressed in low density on the cell surface. The amount of protein which was added to the sucrose gradients was not constant. After lysis of the spleen cell cultures which had been incubated for 24 hours with ^{125}I lipid A, the lysates were adjusted to 50,000 cpm/0.1 ml and 0.1 ml was added to the sucrose gradient. The number of cells in a 0.1 ml aliquot was variable since the specific activity of each batch of ^{125}I lipid A varied. In a given sucrose fraction there could be a tenfold difference in the amount of protein in different experiments. If a particular fraction contained a greater concentration of protein, a molecule expressed less frequently on the cell surface is more likely to be identified. The second possibility, is that the 28,000 dalton protein is a cleavage product or a subcomponent of the 60,000 and 62,000 dalton proteins. If it is a cleavage product,

it is cleaved at a low rate. If it is a subcomponent, it may not be easily dissociated, especially if association with lipid A stabilizes the components of the 60,000 and 62,000 dalton molecules. The experiments performed here cannot distinguish between these two alternatives.

The 60,000 and 62,000 dalton proteins can be discussed in several ways: 1) it is a single molecule, 2) it is two separate molecules and 3) one molecule is a degradation product of the other. If it is a single molecule, one would have to propose selective binding of two separate sizes of Ph-LPS to account for the appearance of the doublet. Recently, Jacobs et al. (31) have demonstrated that B lymphocytes first bind the lower molecular weight fractions of LPS. In addition, one would have to propose that at least two molecules are bound to the 60-62,000 dalton protein, one ^{125}I lipid A molecule and the other, either lipid A (lowest molecular weight component of LPS) or the lowest molecular weight fraction of Ph-LPS. This would give rise to two molecules of separate molecular weights and might account for a smearing effect which is seen occasionally with this molecule. The second possibility is that there are two separate molecules. Both molecules bind ^{125}I lipid A, but because they have similar molecular weights they are not always resolved on the gel. The third possibility is that the 60,000 dalton protein is a cleavage product of the 62,000 dalton protein. Even though mitogenesis should proceed at the same rate, perhaps 24 hours is a critical time point and the cleavage of the 62,000 dalton protein may only be beginning and thus the 60,000 dalton molecule cannot always be identified. Again the experiments performed in this work do not allow one to decide among these alternatives. In any case, it is

clear that at least two molecules 60-62,000 daltons and 28,000 daltons bind lipid A with high affinity.

The 43-45,000 dalton protein may be a lipid A binding protein which is easily dissociated from the lipid A or alternately, it could be a bystander molecule which co-isolates with a second undetectable lipid A binding protein. Again, further investigation will be necessary to determine which hypothesis is correct.

Another question which needs to be addressed is the variability of the patterns or profiles of the sucrose gradients. Why are radioactive peaks not always found at both fractions #4-6 and #10-13 in all sucrose gradient fractionations? The suggestion of Wolfert et al. (19) that LPS crosslinks molecules on the surface of B lymphocyte allows the most reasonable explanation. If the 60,000 and 62,000 dalton proteins are the major binding proteins for lipid A and a third molecule is involved in aggregating event, then depending on the stability of the interaction, one might see three different profiles. The 60,000 and 62,000 dalton molecules will always be seen in fraction #20 of the sucrose gradient of cell lysates. This is the most stable interaction as evidenced by stability in an SDS-polyacrylamide gel. If the third molecule is the 43-45,000 dalton molecule or an undefined comigrating molecule, this is the least stable interaction i.e., lipid A bound to the third molecule only. The appearance of radioactivity in fraction #4-6 could be due to a stable interaction of the of 60,000 and 62,000 dalton protein with another molecule, possibly the 43-45,000 dalton molecule. The presence of radioactivity in this fraction is dependent on the stability of the interaction of ^{125}I lipid A with a molecule which has a low affinity for lipid A, i.e., 43-45,000 dalton protein.

In addition, one would have to propose that the aggregated molecule undergoes a conformational change to give a three molecule complex, lipid A, 60,000 or 62,000 dalton protein and a third molecule which yields a more compact and dense molecule.

Finally, the fact that it is possible to isolate the lipid A binding protein from spleen cells of both C3H/HeN and C3H/HeJ mice would lead to two conclusions. First, previous studies with spleen cell from these mouse strains (17) have indicated that there is no difference in the binding of lipid A to the cells of either mouse strain. Therefore, it is not surprising that identical lipid A binding proteins were isolated from these strains. Second, binding of lipid A to these molecules is not sufficient to trigger mitogenesis and would suggest that: 1) these proteins are the LPS "receptors" and the molecule from the C3H/HeJ mouse has a mutation in the molecule which does not affect binding of lipid A to the B cell but does affect the triggering of mitogenesis or 2) there is another molecule(s) which has yet to be isolated which controls the mitogenic signal for murine B lymphocytes.

Given that the signal for proliferation and differentiation of B lymphocytes seem to be independent (32) and that at least three signals are necessary to stimulate differentiation (33), it is entirely possible that these two molecules represent a single set of signals and that a second LPS "receptor" remains to be identified. Thus, even though three lipid A binding proteins have been identified, others may also be found.

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Comparison of Proteins from Lipopolysaccharide-stimulated and from Non-stimulated Murine B Lymphocytes: Computer-assisted Densitometric Readings

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ABSTRACT

This study used two-dimensional gel electrophoresis, silver staining, and computer-assisted densitometry to examine the proteins from murine B lymphocytes which had been stimulated with lipopolysaccharide (LPS). No qualitative or quantitative changes were found in the identifiable proteins from B cells which had been cultured for 24 hours with LPS when compared with the proteins from B cells which had been cultured for 24 hours without LPS. Some of the limitations of the system which was employed in these studies were also discussed.

INTRODUCTION

Mitogen-induced cell proliferation is commonly used in cellular immunology; however, the subcellular changes which occur are still poorly understood. Mitogens are substances, usually of plant or bacterial origin, that may mimic cell-derived mediators involved in cell proliferation. Lipopolysaccharide (LPS) is a major component of the outer cell wall of Gram-negative bacteria and is a potent B cell mitogen that activates a subset of murine B lymphocytes which are intermediate in maturity (Bona et al., 1975; Gronowicz and Coutinho, 1975). B cells from the C3H/HeJ mouse strain are hyporesponsive to phenol-water extracted LPS (Ph-LPS), but respond in a normal manner to butanol-water extracted LPS (Bu-LPS) (Sultzer and Goodman, 1976). The latter preparation contains a protein component which is mitogenic for murine B cells. The syngeneic C3H/HeN mouse strain possess B cells which are fully responsive to both forms of LPS. The inability of B lymphocytes of C3H/HeJ mice to respond to Ph-LPS is due to a single autosomal gene defect on the fourth chromosome (Watson and Riblet, 1975). The nature of the defect remains undefined, but it is known that this defect does not impair the binding of LPS to B lymphocytes of C3H/HeJ mice (Gregory et al., 1980).

It is usually assumed that in the course of mitogen-induced stimulation of B lymphocytes, a number of proteins are newly synthesized and increase in density (or are suppressed). In this study, proteins from untreated and LPS stimulated B lymphocytes of C3H/HeJ and C3H/HeN mice were compared by electrophoresis in a two-dimensional gel system. The densitometric readings of one-third of the identifiable cellular

proteins from B lymphocytes was determined in a computer-assisted system.

MATERIALS AND METHODS

Preparation of B Lymphocytes. Spleens were removed aseptically from groups of ten 7-12 week old C3H/HeN mice (obtained from the Core Facility for Immunocompromised Mice, Comprehensive Cancer Center at UAB) and C3H/HeJ mice (Jackson Laboratories, Bar Harbor, ME). Single cell suspensions were obained by processing spleens through a 60 gauge stainless steel mesh. The cell suspension was washed once with RPMI 1640 medium (GIBCO, Grand Island, NY). Single cell suspensions were treated with Tris-ammonium chloride solution (Boyle, 1968) for 5 min at 37°C to lyse red blood cells, washed once in RPMI 1640 and adjusted to 14 x 10^4 cells/ml in phosphate-buffered saline (PBS; 0.14 M NaCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl) containing 5% fetal calf serum (FCS) (HyClone, Logan, UT).

Splenic B cells were purified by panning spleen cells (Wysocki and Sato, 1978). Goat anti-mouse immunoglobulin (5 ml; 25 μg/ml) in 0.05 M Tris (pH 9.5) was added to Petri dishes (100 x 25 mm; Falcon 1029). The plates were gently rotated to distribute the reagent over the entire surface of the plate, incubated for 40 min at room temperature, and then washed five times with PBS and once with PBS containing 1% FCS. Three ml of the spleen cell suspension was added to each Petri plate and the plates were incubated for 70 min at 4°C. After 35 min of the 70 min incubation, the plates were gently swirled to insure even coating of the cells onto the plate. Each plate was washed five times with PBS containing 1% FCS, followed by vigorous pipetting

to remove the cells which were attached to the Petri plate.

A mitogenesis assay was used to determine the purity of the B lymphocyte population. Purified B lymphocytes or whole spleen cells were adjusted to 5 x 10^6 cells/ml in RPMI 1640 supplemented with L glutamine, 100 u./ml penicillin and 100 μg/ml streptomycin (GIBCO). Aliquots of the appropriate cell preparation (5 x 10^5 cells/0.1 ml) were dispensed into 96-well flat bottom microtiter plates (Linbro Chemical Co., McClean, VA) and were stimulated with phytohemagglutinin (PHA; 2 μg/ml; Burroughs-Wellcome, Triangle Park, NC), Bu-LPS (25 μg/ml) or Ph-LPS (25 μg/ml) (Morrison and Leive, 1975). The plates were incubated at 37° C in a humidifed atmosphere of 5% CO_2 in air. Cultures were pulsed with ^{3}H-thymidine (0.5 μCi/well; New England Nuclear [NEN], Boston, MA) for the last 6 hr of a 48 hr incubation, and cells were harvested with a MASH II (Microbiological Associates, Walkersville, MD) onto glass filter paper. Paper discs were dried and placed into mini vials (Wheaton, Millville, NJ) and resuspended in 3 ml of Scint-0 (Packard Instrument Co., Downers Grove, IL). The amount of radioactivity was determined in a Beckman LS 8000 scintillation counter. B lymphocyte preparations were assessed in two ways. First the ability of B lymphocytes to respond to the B cell mitogens, Bu-LPS and Ph-LPS, was determined. The B lymphocyte cultures from both the C3H/HeJ and C3H/HeN mouse strains were responsive to Bu-LPS, but only the B lymphocytes from C3H/HeN mice responded to Ph-LPS. Purity of B cell preparations was assessed by stimulation with PHA and compared with non-mitogen treated cultures (Table 1).

Cells to be analyzed in the two-dimensional gel system were adjusted to 5 x10^6 cells/ml in RPMI 1640 and then dispensed into a

24-well culture plates at a volume of one ml per well. One series of cultures were untreated, while a second series was stimulated with 25 μg (25 μl of 1 mg/ml) of Ph-LPS. Plates were incubated for 24 hr at 37°C in 5% CO_2. At the end of 24 hr, the cells were harvested with a Pasteur pipette and washed twice with PBS. The cells were resuspended in 10 ml of PBS, layered on Ficoll-hypaque (Boyum, 1968) and centrifuged at 1500 rpm for 20 min. The cells at the interface were removed, washed once in PBS, and counted. This procedure yielded greater than 95% viable cells and eliminated artificial protein spot changes resulting from dead cell contamination (data not shown).

Preparation of two-dimensional gels. B lymphocytes from Ficoll-hypaque gradients were adjusted to 1 x 10^7 cells/tube and centrifuged at 200 x g. The supernatant was removed and 100 μl of lysis buffer [9.5 M urea; 2% (w/v) Nonidet P-40 (NP-40); 2% Ampholine, LKB pH 3.5-10; and 5% 2-mercaptoethanol] was added to the 10^7 cells (1 x 10^6 cells/10 μl). These preparations were stored at -70°C until used in the first dimension of the two-dimensional gel.

The first dimension was a nonequilibrium pH gel electrophoresis (NEPHGE; O'Farrell et al., 1977). The gels consisted of 9.2 M urea, 2% (w/v) NP-40, 4% acrylamide/bisacrylamide, 2% Ampholines (pH 3.5-10), 0.87 mM ammonium persulfate and 12 mM N,N,N',N'-tetramethylethylenediamine (TEMED) were poured into 130 mm x 2.5 mm tubes to a height of 12 cm and then overlayed with a solution of 8 M urea and 1% ampholines. After polymerization (1 hr), the overlay buffer was replaced with the lysis buffer and allowed to equilibrate for 1 hr. The tubes were placed in an electrophoresis chamber in which the lower reservoir contained 0.02 M NaOH (degassed) and the upper chamber 0.01 M phosphoric

acid. An aliquot (30 µl) of the cell lysate was added to each tube, overlayed with 20 µl of the overlay buffer and the tube was filled with phosphoric acid. The samples were electrophoresed toward the cathode for 4-1/2 hr at 400 V. The gel was extruded into 5 ml of equilibration buffer consisting of 10% (w/v) glycerol, 5% (v/v) 2-mercaptoethanol, 2.3% (w/v) sodium dodecyl sulfate (SDS) and 0.0626 M Tris-HCl (pH 6.8) and stored at -70°C.

The second dimension was a Laemmli gel system (Laemmli, 1970). The resolving gel (1% acrylamide/bisacrylamide, 2% SDS and 0.375 M Tris-HCl, pH 8.8) was poured (0.7 mm thick) and aged overnight. A 3% acrylamide stacking gel which contained 0.1% SDS and 0.125 M Tris - HCl (pH 6.8) was layered on the resolving gel. As the stacking gel was allowed to polymerize, the NEPHGE gel was thawed and equilibrated for 2 hr in equilibration buffer with one buffer change. Then 0.75% agarose in equilibration buffer was melted in a boiling water bath and the Laemmli gel was overlayed with 1 ml of the agarose. The NEPHGE gel was placed on the hot agarose and the agarose was allowed to solidify. The gel was electrophoresed at 4°C at 15 mA for approximately 4 hr or until the bromphenol blue dye reached the bottom edge of the gel in a Tris glycine buffer (0.02 M Tris, 0.19 M glycine and 2% SDS, pH greater than 8). The gel was removed, silver stained (Merril et al., 1980), and photographed (4 inch x 5 inch color transparencies).

Computer Analysis. The computer system used to analyse these gels has been previously described (Goldman et al., 1982). A set of three gels was used for each group, e.g., C3H/HeN or C3H/HeJ B lymphocytes cultured without LPS and C3H/HeN or C3H/HeJ B lymphocytes cultured with LPS. Photographs of the gels were digitized with a 1000HS scanning

microdensitometer (Optronic, Chelmsford, MA). The computerized densitometric readings were performed by using a PDP 1/60 computer (Digital Equipment, Maynard, MA) which was equipped with an IP5000 image processor (DeAnza Systems, San Jose, CA) with three 512 x 512 x 8 bit image arrays. Of 306 identifiable protein spots, 100 were randomly chosen for analysis. Each density measurement was operator assisted and a single protein was measured on all 12 gels before proceding to the next protein. The measurement area was a large polygon with a smaller inner polygon. The inner polygon could be varied in size to accommodate the protein spot, but was not varied for any one set of 12 readings. The outer polygon measured background density which was computed by the modal density of the pixels (picture elements) contained in the outer polygon. The protein densities were calculated as average density within the inner polygon-background density x area of the inner polygon (units = optical density x mm^2). The densities for each gel were normalized against a reference gel. This calculation used the sum of the densities of the 100 proteins to determine the normalization factor.

Analysis of Qualitative Protein Changes. Two approaches were used to determine qualitative changes in the protein populations. In the first system, the two-dimensional gels were compared over a light box. Careful visual inspection of each section of the gels would have allowed any differences between the gels to be determined. In the second method of analysis (submitted for publication), a stereoscope viewer (Model K10 precision mirror stereoscope, Ben Meadows Co., Atlanta, GA), colored acetate sheets, a light box and photograph negatives of pictures of two-dimensional gels (Polaroid 55

positive/negative film) were used to determine qualitative differences. The stereoscope was placed on a light box. Under the left mirror of the stereoscope, a photographic negative of a two-dimensional gel was placed on a sheet of red acetate. Under the right mirror of the stereoscope, a photographic negative of the second two-dimensional gel was placed on a sheet of yellow acetate. In the superimposed image, the common protein spots appeared orange while unique protein spots would remain red or yellow, depending upon the gel where the difference occurred.

RESULTS

Mitogenesis. The mitogenic response of whole spleen cells and purified B cell cultures is presented in Table 1. When Ph-LPS was used as a mitogen, only the B lymphocytes from the C3H/HeN mice responded, while both B lymphocyte and whole spleen cultures showed good ^{3}H-thymidine uptake when Bu-LPS was used as a mitogen. The T cell mitogen PHA failed to stimulate ^{3}H-thymidine uptake in the purified B cell populations. The responses of whole spleen cell cultures were included to assure that the T cell mitogen was indeed capable of stimulating T lymphocytes.

Two-dimensional Gel Analysis. Typical two-dimensional gels of C3H/HeN and C3H/HeJ splenic B lymphocytes are shown in Figure 1. There are 306 identifiable protein spots on these gels. A NEPHGE system was used in the first dimension so that as many basic proteins as possible could be visualized. Figure 2 displays scatter plots which compare densitometric readings of individual proteins from the groups. Densitometric reading of protein spots of Ph-LPS stimulated B lymphocytes

TABLE 1

Mitogenic Responses of Spleen Cell and Purified B Cell Cultures From C3H/HeN and C3H/HeJ Mice

Cell type and treatment	cpm[3]	E/C[4]
C3H/HeJ B cells		
Medium only	565 ± 214	--
Bu LPS (25 μg/ml)	9,159 ± 1,130	16.2
Ph LPS (25 μg/ml)	655 ± 85	0.9
PHA (2 μg/ml)	174 ± 50	0.3
C3H/HeN B cells		
Medium only	683 ± 107	--
Bu LPS	10,254 ± 1,130	15.0
Ph LPS	8,594 ± 901	12.6
PHA	536 ± 183	0.8
C3H/HeJ Splenic Cells		
Medium only	393 ± 71	--
Bu LPS	5,788 ± 1,619	14.7
Ph LPS	1,169 ± 395	2.9
PHA	8,273 ± 2,053	21.0
C3H/HeN Splenic Cells		
Medium only	919 ± 167	--
Bu LPS	9,203 ± 1,770	10.0
Ph LPS	7,031 ± 2,019	7.6
PHA	5,527 ± 1,737	6.0

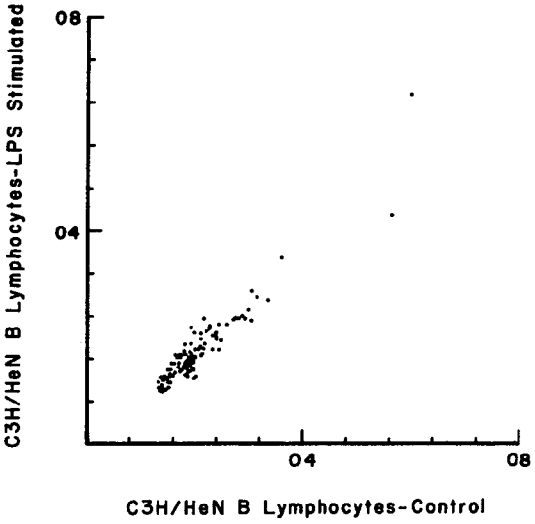
[1]Purified B lymphocytes or spleen cells were adjusted to 5 x 10^6/ml and dispensed into a 96-well culture plate at 0.1 ml/well.

[2]RPMI 1640 medium or the appropriate mitogen was added (0.1 ml volume) to each well. All cultures were performed in quadruplicate.

[3]Mean counts per minute (cpm) ± standard deviation.

[4]Experimental cpm/control (RPMI) cpm.

Figure 1. Representative two-dimensional gels of the proteins from murine B lymphocytes. The upper gel represents a two-dimensional separation of the proteins from LPS stimulated B lymphocytes from C3H/HeN mice, while the lower gel depicts proteins from LPS-stimulated B lymphocytes from C3H/HeJ mice.


C3H/HeN B Lymphocytes-LPS Stimulated
08
04
04
08
C3H/HeN B Lymphocytes-Control

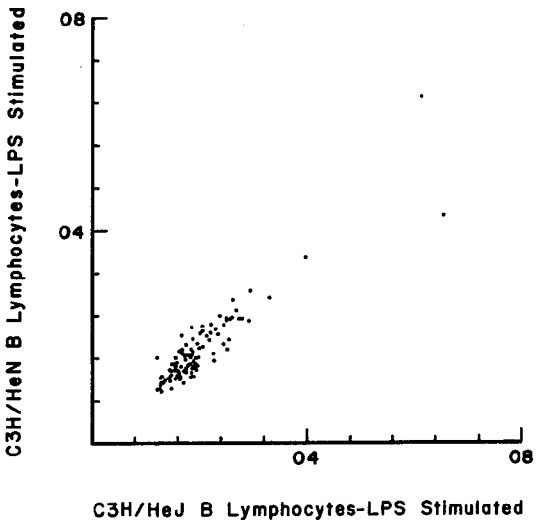
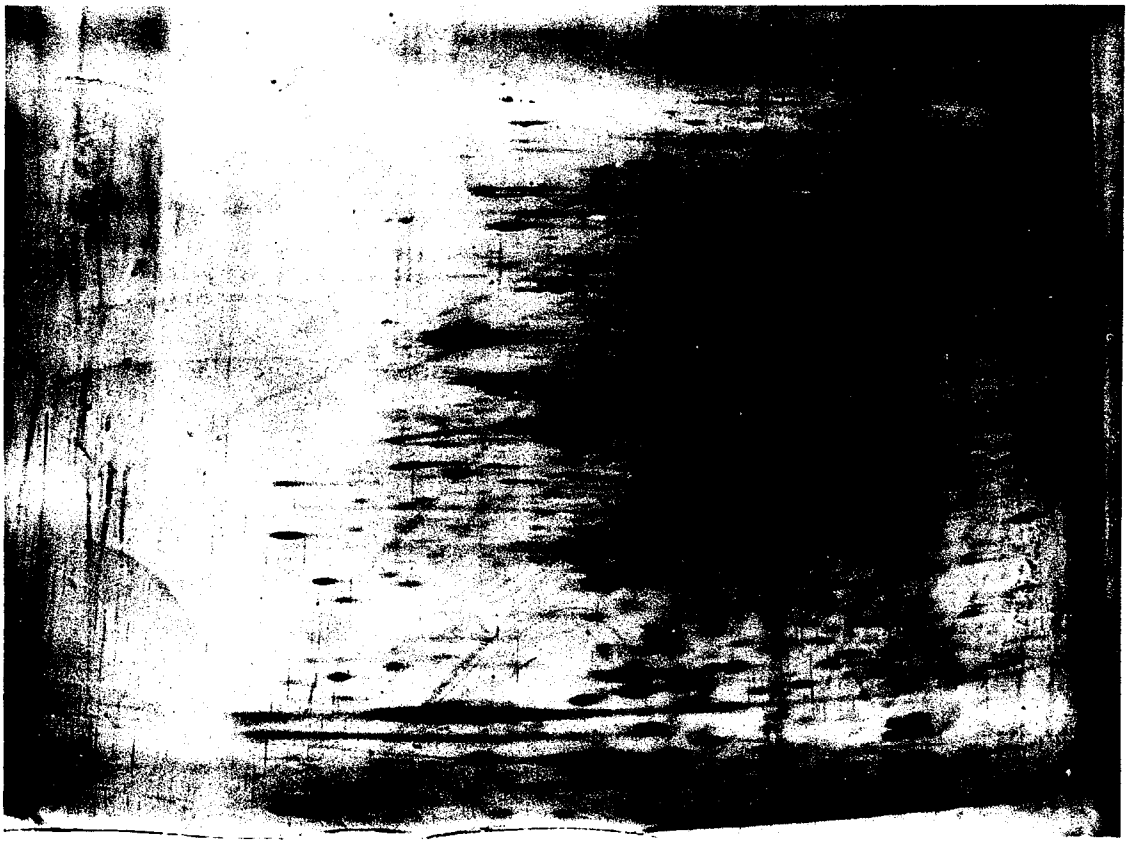

C3H/HeN B Lymphocytes-LPS Stimulated
08
04
04
08
C3H/HeJ B Lymphocytes-LPS Stimulated

Figure 2. A comparison of densitometric readings of the proteins from murine B lymphocytes. The axis are units = optical density x mm^2. On the left, the average densitometric readings of proteins from LPS-stimulated B cells from C3H/HeN mice are compared with the average densitometric reading of proteins from C3H/HeN control mice. On the right, the average densitometric reading of proteins from LPS stimulated B cells from C3H/HeN mice are compared with the average densitometric readings of proteins from LPS stimulated B cells from C3H/HeJ mice.



from C3H/HeN mice are compared with unstimulated cells from the same strain (Figure 2a) or with Ph-LPS stimulated B lymphocytes from C3H/HeJ mice (Figure 2b). No statistically significant differences between groups were seen. The protein which had a reading of 7 units on the C3H/HeJ B lymphocyte LPS stimulated axis and a reading of 4 units on C3H/HeN B lymphocyte LPS stimulates axis appears to be different. Readings in excess of 4 units were considered invalid since more than half of the densitometric readings were saturated. When the number of units were calculated an artificial number was generated which may or may not correctly reflect the densitometric reading. The scatter appears to be wider when C3H/HeJ lymphocytes were compared with C3H/HeN lymphocytes, but there were no statistical differences. The tightness of fit observed when the C3H/HeN groups were compared with each other may be due to the fact that these cells came from common cell pools for each experiment. The most surprising finding was that cells from LPS responsive C3H/HeN mice displayed no statistically significant increase in density of any protein analyzed. In addition, no consistent qualitative differences could be detected in the protein populations when B lymphocytes were stimulated with LPS.

DISCUSSION

The system used here is a quantitative means of detecting changes which occur in protein population from stimulated or nonstimulated cells at 24 hours. The two strains of mice used, i.e., C3H/HeJ and C3H/HeN, seemed ideal to study protein population changes which occur in B lymphocytes when these cells are stimulated with a mitogen since they are syngeneic in all respects except their ability to respond to

LPS. The time point of 24 hours was chosen because the LPS-stimulated B lymphocytes have activated a number of systems which include phosphorylation of nuclear proteins (Stott & Williamson, 1978), activation of a serine protease (Ku et al., 1981), and calcium ion influx (Diamanstein & Ulmer, 1975). This suggests that the machinery involved in LPS stimulation is functioning in the majority of LPS responsive cells. In a previous two-dimensional gel study which examined protein sythesis in PHA stimulated T lymphocytes (Lester et al., 1981), it was concluded that 24 hour, 48 hour and 120 hour gel patterns were unchanged. Visual inspection of two-dimensional gels of B lymphocytes which were stimulated with LPS for 48 hours with gels from cells which were stimulated for 24 hours with LPS, led us to conclude that there were no major differences among the 306 identifiable proteins from B lymphocytes which had been incubated with LPS for 48 hours (data not shown).

The fact that no protein spots appeared or disappeared was not surprising. Two previous reports, one which examined PHA stimulation of T lymphocytes (Lester et al., 1981) and the other which examined dividing and stationary nerve cell lines (Garrels, 1979), both failed to show qualitative changes. Our work with LPS stimulated B lymphocytes would support these findings. It may be that short-lived, small copy number proteins are highly effective in the activation of cells. Furthermore, the protein interaction with a cation may be more important in cell activation. It is becoming clear that major changes in protein numbers do not occur in activated cell systems.

Of the 100 proteins which were analyzed by densitometric readings, none showed a statistically significant difference in density readings when the groups were compared. This was somewhat surprising since

others have recently shown that a 35,000 dalton protein is expressed in resting B cells, but disappears in proliferating cultures (Rahmsdorf et al., 1982). In addition, these workers reported that several new proteins were synthesized in LPS stimulated cultures as determined by ^{35}S incorporation into proteins and autoradiography. Several possibilities exist for the discrepancy between our work and that of Rahmsdorf et al. (1982) and these include: 1) the time chosen to examine the cells-24 hours (this work) versus 30-72 hours, 2) the method of standardization, normalization against a reference gel (this work) versus standardization against internal proteins, and 3) the system used to identify proteins, silver stain (static protein population) versus autoradiographs (newly synthesized protein population).

It is felt that the analysis of our gels is a valid representation of cellular events at 24 hours of mitogenesis. The gels were electrophoresed in sets, i.e., a group of C3H/HeN and a group of C3H/HeJ mice constituted a set, and three sets were compared. Further, each set was a pool of 10 mice which would eliminate any individual variances. The cells were always analyzed at 24 hours and cultured in the absence of serum because serum itself may contain LPS or have LPS inhibitors. The absence of serum prevented examination of time points after 48 hours. As previously stated, visual inspection of gels from cells cultured for 48 hours with LPS showed no protein spot changes. By 24 hours, any protein changes which were directly activated by LPS should be apparent and later protein changes may be secondary in nature, i.e., proteins involved in proliferation which were activated by a cascade of earlier events. There is no recognized system for standardization of protein densities when comparisons of two-dimensional

gels are performed. A reference gel was chosen to normalize the proteins of all gels. In addition, the computer system employed would correct for an increase in density due to increased background density. Silver stained gels which were examined had several advantages. First, it is a sensitive system for protein identification which is not dependent upon the presence of a particular amino acid such as methionine (^{35}S methionine). Second, it would allow us to identify up or down regulation of proteins which change slowly over a long period of time. Third, it is highly reproducible.

The finding of no density changes between proteins of LPS stimulated cells and control cells was unexpected. The experimental design included three separate controls. Cells from both strains of mice served as controls, since these mouse strains are syngeneic. The C3H/HeJ mice are hyporesponsive to LPS so these gels also served as nonstimulated controls. The average variance for the protein densitometric readings of the LPS stimulated C3H/HeN B lymphocytes was 0.056 and in general readings of the protein spots from the three gels from this group had good correlation. Failure to see differences between groups could be accounted for in several ways. First, culture conditions were identical for control and LPS stimulated cells, i.e., the control cells were incubated in the same media and for the same length of time as the mitogen stimulated culture. This would prevent alterations which were strictly due to the stress of culture. Second, the dead cells were removed after the cells were cultured and prior to electrophoretic separation. Previous work in our laboratory (data not shown) had suggested that artificial protein changes occur, i.e., selective disappearance of protein spots, if this step is not taken.

Third, only one-third of the splenic B lymphocytes respond to LPS, while the remaining cells would be expected to remain unchanged. Thus, relatively small alterations in this system would be difficult to detect.

Although the technology used in this work is the best system which is presently available, one should view these findings from these systems with caution. It is difficult to examine every protein spot in these gels and thus a number of protein spots were not examined. It may be that a change in the level of expression of a single protein may be sufficient to trigger mitogenic events and that key molecules were not analyzed. Further, even though silver stained gels were extremely sensitive, the major identifiable proteins may be structural or maintenance-type proteins rather than regulatory proteins. It has been suggested that a mammalian cell has as many as 10,000 proteins (Duncan & McConkey, 1982). If this is the case, only 3% of the total cellular proteins can be identified on these gels. A protein population of 10,000 unique molecules might be extreme. Recent work with HeLa cells (Bravo & Celis, 1982), fibroblasts (Leavitt et al., 1982) and leukemia cells (Hanash et al., 1982) showed 1,357 proteins, greater than 1,000 proteins, and 800 proteins, respectively. If these are more realistic numbers then only 22-38% of the cellular proteins were displayed on a gel and 7-12% of the proteins were examined. It is probable that a low copy number molecule could be a regulatory molecule. In addition, it is known that new molecules are synthesized in response to mitogens; for instance, Pc.1 is expressed on the surface of plasma cells but not B lymphocytes (Ahmed et al., 1978) and that expression of Ia can be induced on the surface of T lymphocytes with T cell

mitogens (Winchester & Kunkel, 1979). Neither this study with B lymphocytes nor that of Lester et al. (1981) with T lymphocytes was able to detect the appearance of new molecules in two-dimensional gels, which suggests that even though computer analysis of two-dimensional gels is "state-of-the-art" in gel analysis, it may not be able to detect subtle regulatory changes in cells.

In this system the absolute protein densitometric reading per protein per cell does not change perceptibly in the identifiable proteins at 24 hours of LPS stimulation. It is possible that slight changes in ratios of proteins or interaction of a constitutive protein with a cation are more important initial events in mitogenesis. This does not preclude protein changes at later times which may be directly or indirectly related to LPS stimulation or changes in proteins which cannot be detected with this system.

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SUMMARY

The work described in this text examined two separate aspects of the same system, i.e., the B lymphocyte response to lipopolysaccharide (LPS). LPS is a potent B lymphocyte mitogen which stimulates both proliferation and differentiation of a subset of B lymphocytes. The subset comprises one-third of the total mature B cell subpopulation and can be characterized by the expression of sIgD, Ia, Lyb 3, and Lyb 5, as well as the ability to respond to TI-2 antigens. Even though the specific B cell subpopulation which responds to LPS has been identified, and certain LPS-triggered biological events including phosphorylation of nonhistone chromosomal proteins (1), calcium ion influx (2), activation of cGMP (3,4), and membrane depolarization (5) have been elucidated, most of the molecular mechanisms involved in this reaction(s) remain obscure. Two specific questions are addressed in the present work. What changes occur in the total protein population of the B lymphocyte when they are stimulated with LPS? Can specific LPS binding proteins, potential LPS receptors, be identified?

To address the first question, which concerns changes in the protein pool of LPS stimulated cells, silver-stained, two-dimensional gels of the proteins from B lymphocytes were examined with computer-assisted densitometry. B lymphocytes from the LPS responsive, C3H/HeN mouse strain, or LPS hyporesponsive, C3H/HeJ mouse strain were cultured with or without LPS. Two-dimensional gels of the proteins from culture lysates were subjected to densitometric analysis. The surprising result was that neither qualitative nor quantitative differences in proteins could be detected between these groups. Since previous work (6) with

mitogen stimulated T lymphocytes had also revealed no qualitative changes, it can be concluded that mitogen-induced triggering of either T or B cells does not result in significant changes in cell proteins.

The failure of the proteins from LPS-stimulated B lymphocytes to express quantitative changes was far more puzzling. It was concluded that three sets of conditions might account for this finding. First, control cells were treated in the same manner as cells cultured with LPS except for addition of mitogen. It may be that failure of others to treat the cell cultures identically could cause protein changes which are totally dependent on culture conditions and have nothing to do with the presence or absence of mitogen. Second, after the cells were cultured and prior to lysis of the cells to release proteins, all dead cells were removed. Early work on this project showed that if the dead cells were not removed, a number of protein changes were detectable. If, on the other hand, the dead cells were removed, these changes no longer existed. Finally, the fact that only one-third of the splenic B lymphocyte population responds to LPS may account for the inability of this system to detect protein changes. It is concluded that during the first 24 hours of culture of B lymphocytes with LPS there are no detectable qualitative or quantitative protein changes. It is suggested that changes in protein form (phosphorylation), interaction of a cation with a constitutively produced protein, or relatively small changes in the ratios of two different proteins could be more important early events in mitogenesis.

Further support for the general stability of the protein population of lymphocytes is garnered from work presented in the appendix of this text. It was shown that T lymphocytes and macrophages have only

a small number (6%) of proteins which are unique, i.e., not found in B lymphocytes. This finding is supported by previous work comparing the proteins from lymphoid cell lines (7), as well as by work which examined unique mRNA between T and B cell tumor lines (8). Thus, if cells with common stem cells, but dramatically different functions, fail to express substantial protein differences, it is not surprising that B lymphocytes in either proliferative or resting states fail to express any detectable differences.

In the second part of this work two lipid A binding proteins were characterized. Lipid A, as previously discussed, is the portion of the LPS molecule which is responsible for most of the biological effects of LPS. The three proteins isolated are 60,000, 62,000 and 28,000 daltons in molecular weight. The 60,000 and 62,000 molecular weight protein binds lipid A strongly. The association is maintained in an SDS-polyacrylamide gel but can be broken in a two-dimensional gel. The 28,000 dalton protein co-isolates with the 60,000 and 62,000 dalton proteins and is either a protein expressed in low density on the cell surface or is a degradation product of the 60,000 and 62,000 dalton protein. A fourth protein, 43-45,000 daltons, co-isolates in a sucrose gradient fraction with increased levels of ^{125}I lipid A, the radioactive tracer. Although these proteins are always detectable in separate fractions when lymphocytes were incubated with ^{125}I-lipid A, then lysed and fractionated on a 5-30% sucrose gradient, it is possible to co-isolate these molecules. These results suggest that lipid A may cross-link these molecules on the cell surface. This possibility is supported by previous work (9) which used a photoreactive crosslinking agent to identify molecules on the surface of lymphocytes which bind LPS. In

that study, it was found that LPS complexes a molecule which has a molecular weight of 95-100,000 daltons. This molecule could be immunoprecipitated with anti-Ia. Ia, however, has a molecular weight of 58-60,000 daltons which suggested that another molecule was involved in this complex.

The fact that this was not the first group to implicate Ia as an integral part of LPS-triggered mitogenesis (10,11) and the fact that one of the molecules described here has an identical molecular weight as Ia led to speculation on the nature of the "LPS receptor." The 60,000 and 62,000 dalton protein not only coincides with the molecular weight of Ia, but will occasionally comigrate with (or release) another protein which has a molecular weight of 28,000 daltons. The components of Ia are α and β chains which have molecular weights of 30,000 and 28,000 daltons, respectively. The 28,000 component of our system will also bind lipid A in an SDS-polyacrylamide gel which suggests that the 62,000 dalton protein is Ia and would further suggest that lipid A may bind to the β subunit.

It is difficult to pinpoint the 45,000 dalton binding protein. However, if one were to examine the known surface proteins of a B lymphocyte, a set of three proteins emerge as prime candidates; these are Lyb 2, Lyb 4, and Lyb 6. The genes which code for these three proteins map within 4 units of each other and lie between the centromere and Mup-1 on the fourth chromosome of the mouse. All three proteins have reported molecular weights which vary from 40-45,000 daltons (12,13). A monoclonal anti-Lyb 2 can inhibit *in vitro* antibody responses to T dependent antigens and TI-2 antigens, but not TI-1 antigens (14). Further anti-Lyb 2 can cause a proliferative effect in the Lyb 5^+

subset of B lymphocytes (13). Little is known about Lyb 6 except that its molecular weight is 45,000 and it is present on both Lyb 5^+ and Lyb 5^- cells. Anti-Lyb 4, like anti-Ia, can block mixed lymphocyte reactions at the stimulator cell level (16). This led the authors of that work to speculate on an association between Ia and Lyb 4. Perhaps the strongest evidence in favor of Lyb 4 as an LPS binding protein comes from genetic studies. Watson and Riblet (17) demonstrated that C3H/HeJ mice, which are hyporesponsive to LPS, have a single defect on the fourth chromosome which controls the LPS non-responsiveness. Howe et al. (18) have shown that Lyb 4 fails to map between Lyb-2 and Mup-1 on chromosome 4 in the C3H/HeJ mouse strain. Thus, Lyb 4, although expressed in C3H/HeJ mice, has undergone a gene translocation. If this is the case, a slight mutation in the base sequence during this translocation could lead to an alteration in the amino acid sequence of Lyb 4 which might make it incapable of responding to the LPS signal for mitogenesis, but allow the B lymphocyte to respond to the "natural" B cell signals.

What are the "natural" B cell signals which LPS appears to mimic? The stimuli for B cell proliferation and B cell differentiation appear to be independent signals (19). For instance, a number of antibodies to B cell surface antigens will cause proliferation without differentiation into an antibody secreting cell. These antisera include anti-IgM (20), anti-IgD (21) and anti-Lyb 2 (14). Other antisera, specifically anti-Ia (10), fail to induce proliferation. Aside from the fact that the proliferative and differentiative signals are separate, it is now clear that at least three sets of signals are necessary to stimulate proliferation and differentiation in the B lymphocyte (22,23); these

signals include: 1) a T helper specific signal which is Ia-dependent, 2) interaction between the cell surface immunoglobulin (sIg) and antigen, and 3) a nonspecific T helper cell factor termed B cell helper factor (BHF). Although most of the details of the three signals remain unresolved, it is generally thought that the specific T helper cell signal and antigen-sIg interactions trigger a proliferative response. The nature of the BHF remains in question, since it can only be shown to stimulate differentiation in the presence of antigen. Even though the specific T helper cell signal can be bypassed by LPS, it cannot trigger differentiation. Thus, we are no closer to dissecting the nature of the "LPS receptor" than before.

In view of the fact that two lipid A binding proteins, have been co-isolated and that this is corroborated by others (9), the following sequence of events can be proposed. LPS aggregates three molecules 60-62,000 daltons (Ia?) and 45,000 daltons (Lyb 4 ?). This sends a signal, which is most likely the T cell specific signal (Ia dependent). Since LPS can drive the B lymphocytes to differentiate, as well as proliferate, LPS must interact with other cell surface antigens. Evidence for this comes from three groups (9,11,24), each of which have isolated at least five LPS binding proteins. The system chosen for this work identifies only LPS binding proteins with high avidity for lipid A and may have excluded other proteins. Thus, the two molecules isolated here are probably only part of the LPS induced events, and other molecules will have to be defined before the complete system can be elucidated. Perhaps the differentiation signal is a combination of proliferative signals such that the simultaneous activation of two or three types of proliferative signals will trigger differentiation.

In summary, there are no discernible protein changes which occur in the first 24 hours when murine B lymphocytes are stimulated with LPS. Three lipid A binding proteins, 60,000, 62,000 and 28,000 daltons, can be detected. In addition, a 43-45,000 dalton molecule is found in a sucrose gradient fraction along with increased levels of ^{125}I lipid A. These proteins may aggregated by LPS on the cell surface.

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APPENDIX

A Stereoscopic Method for Comparison of Protein Spot Differences in Two-dimensional Gels

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Running Title: Comparison of two-dimensional gels

Subject Category: Special Techniques

Key Words: two-dimensional gels, B lymphocytes, T lymphocytes, macrophages, stereoscope viewer, and protein analysis

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Abstract: A stereoscopic viewer was used to compare protein spot differences in two-dimensional gels. Lysates of murine spleen cells or purified splenic B lymphocytes were electrophoresed in an O'Farrell two-dimensional gel system. The gels were silver stained and photographed. The photographic negatives of each gel were used in conjunction with colored acetate or photographic light filters and a stereoscope viewer to discern protein spots which were absent in B cells, but present in whole spleen cells and thus unique to T lymphocytes and/or macrophages.

INTRODUCTION

The advent of the O'Farrell two-dimensional gel (1,2) system has given cellular and molecular biologists a powerful tool for characterizing subcellular proteins or detecting protein changes within cells. Two-dimensional gels have been used to characterize a single protein or set of proteins (3), while other studies use gel analysis to detect protein changes within cells (4,5). In the latter case, two-dimensional gel analysis allows identification of a small number of protein changes (detected by protein spot appearance) in the midst of several hundred proteins. It is the analysis of these complex gels which can be simplified by the technique presented here.

A commercial stereoscopic viewer was used to compare protein spots in a two-dimensional gel of the total proteins from a spleen cell preparation (T and B lymphocytes and macrophages) with a two-dimensional gel of the total proteins from purified splenic B lymphocytes. Using this technique, it was possible to detect proteins which were present only in the whole spleen cell preparation and were therefore specific for T lymphocytes or macrophages.

MATERIALS AND METHODS

Preparation of spleen cells

Spleens were removed aseptically from 7-12 week old C3H/HeN mice (obtained from the Core Facility for Immunocompromised Mice, Comprehensive Cancer Center at UAB). Single cell suspensions were obtained by passage of spleens through a sterile 60 gauge stainless steel mesh. The cell suspension was washed once in RPMI 1640 medium (Grand Island Biological Co., [GIBCO]; Grand Island, NY). Single cell suspensions

were treated with Tris-ammonium chloride solution (6) for 5 minutes at 37°C to lyse red cells, washed once in RPMI 1640 and adjusted to 14 x 10^6 cells/ml in phosphate-buffered saline (PBS; 0.14 M NaCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl), containing 5% fetal calf serum (FCS) (HyClone, Logan, UT). A portion of these spleen cells were used to purify B lymphocytes and the remaining cells were used for mitogenic assay (see below).

B lymphocyte purification

The method used to purify B lymphocytes was an adaptation of the procedure described by Wysocki and Sato (7). Goat anti-mouse immunoglobulin (5 ml; 25 μg/ml) in 0.05 M Tris (pH 9.5) was added to Petri dishes (100 x 15 mm; Falcon 1029). The plates were gently swirled to distribute the reagent over the entire surface of the plate and were then incubated for 40 min at room temperature followed by four washes with PBS and once with PBS containing 1% FCS. Three ml of the spleen cell suspension was added to each Petri plate. The plates with the cells were incubated for 70 min at 4°C. After 35 min of incubation, the plates were gently swirled to insure even coating of the cells onto the plate. After five washes with PBS + 1% FCS, the plates were then treated with 3 ml of pancreatin (GIBCO) for 5 min at 37°C in 5% CO_2. Five ml of PBS containing 1% FCS was added to each petri plate, the cells were rinsed from each plate by vigorous pipetting, pooled and washed once with RPMI 1640 medium. To insure that differences in protein patterns obtained with whole spleen cells and B lymphocyte preparations were not due to treatment conditions, whole spleen cells were also treated with pancreatin for 5 min at 37°C and washed with medium.

Mitogenic assay

A mitogenesis assay was used to determine the purity of the B lymphocyte population. Purified B lymphocytes or whole spleen cells were adjusted to 5 x 10^6 cells/ml in RPMI 1640 supplemented with L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (GIBCO). Aliquots of the appropriate cell preparation (5 x 10^5/0.1 ml) were dispensed into a 96-well flat-bottom microtiter plate (Linbro Chemical Co., McClean, VA) and were stimulated with the T cell mitogen phytohemagglutinin (PHA; 2 μg/ml; Burroughs-Wellcome, Triangle Park, NC), or the B cell mitogen butanol-water extract lipopolysaccharide (Bu-LPS; 40 μg/ml) (8). The plates were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. Cultures were pulsed with ^{3}H-thymidine (New England Nuclear, Boston, MA; 0.5 μCi/well) for the last 6 hr of a 48 hr incubation and the cells were harvested with a MASH II unit (Microbiological Associates, Walkersville, MD) onto glass filter paper. Paper discs were dried and placed into mini vials (Wheaton, Millville, NJ) and resuspended in 3 ml of Scint-O (Packard Instrument Co., Downers Grove, IL). The amount of radioactivity was determined in a Beckman LS 8000 scintillation counter. An experimental/control index (cpm of stimulated cells/cpm of RPMI control cells) of less than 2 in PHA stimulated cultures was taken as evidence for the presence of little or no T cell contamination in the B cell preparation (see Table I).

Two-dimensional gel preparation

B lymphocytes or spleen cells were adjusted to 10^8/ml and centrifuged at 2000 x g. The supernatant was removed and 100 μl of lysis buffer (9.5 M urea; 2% (w/v) NP 40; 2% ampholines, LKB 3.5-10; and 5% 2 mercaptoethanol) was added to the cell pellet (10^8 cells). The lysate

TABLE 1

^{3}H-Thymidine Uptake for Mitogen Stimulated

Spleen Cells and B Lymphocytes

	Splenocytes[1]		B Lymphocytes[1]	
	cpm[2]	E/C[3]	cpm	E/C
RPMI only	980 ± 179	--	185 ± 91	--
BU-LPS (25 μg/ml)	25,390 ± 246	26	53,953 ± 4746	291
PHA (2 μg/ml)	42,286 ± 8302	43	287 ± 103	1.5

[1]Cells adjusted to 5 x 10^{6}/ml and aliquoted in 0.1 ml fractions in 96 well plates.

[2]Mean counts per minute of quadruplicate cultures ± S.D.

[3]Experimental/Control = $\frac{\text{cpm of mitogen stimulated sample}}{\text{cpm of unstimulated sample}}$

was stored frozen at -70°C until used in the first dimension of the two-dimensional gel.

The first dimension was a nonequilibrium pH gel electrophoresis (NEPHGE) gel and consisted of 9.2 M urea, 2% NP-40, 4% acrylamide/-bisacrylamide, 2% (pH 3.5-10) ampholines, 10 µl of 10% ammonium persulfate/10 ml and 7 µl of N, N, N', N' -tetramethylethylenediamine (TEMED)/10 ml which was poured into a 130 mm x 2.5 mm tube to a height of 12 cm and then overlayed with a solution of 8 M urea and 1% ampholines. After polymerization (1 hr), the overlay buffer (9 M urea + 1% ampholines) was replaced with the lysis buffer and allowed to equilibrate for 1 hr. The tubes were placed in an electrophoresis chamber, in which the lower reservoir contained 0.02 M NaOH (degased) and the upper chamber 0.01 M phosphoric acid. An aliquot (30 µl) of the cell lysate was added to each tube. This was overlaid with 20 µl of the overlay buffer and the tube was filled with phosphoric acid. The gels were electrophoresed (cathode on the bottom and the anode on the top) for 4-1/2 hr at 400 V. The gel was extruded into 5 ml of dissociation buffer consisting of 10% (w/v) glycerol, 5% (v/v) 2 mercaptoethanol, 2.3% (w/v) SDS and 0.0626 M Tris-HCl (pH 6.8) and frozen at -70°C until the second dimension was run.

The second dimension was a Laemmli gel system (9). The resolving gel (10% acrylamide/bisacrylamide, 0.1% SDS and 0.375 M Tris -HCl, pH 8.8) was poured (0.7 mm thick) and aged overnight. A 3% acrylamide stacker gel containing 0.1% SDS and 0.125 M Tris-HCl, (pH 6.8) was layered on the resolving gel. As the stacking gel was allowed to polymerize, the NEPHGE gel was thawed and equilibrated for 2 hr in dissociation buffer with one buffer change. The Laemmli gel was then

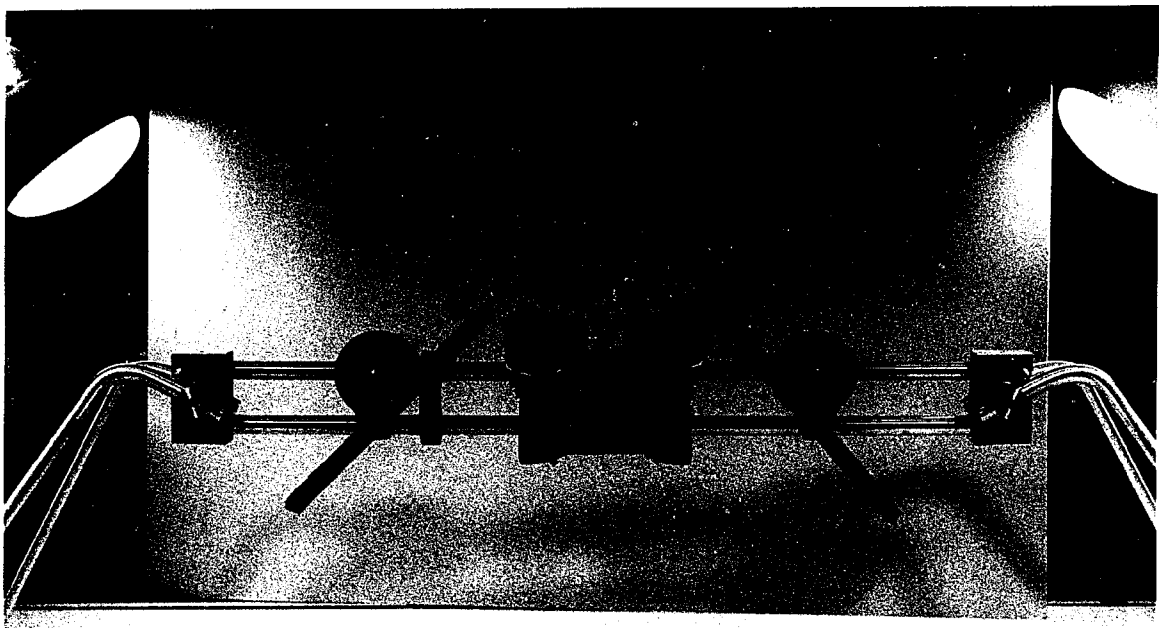
overlaid with 1 ml of 0.75% agarose and the NEPHGE gel was placed on this hot agarose and allowed to solidify. The gel was run at 4°C at 15 mA for approximately 4 hours or until the bromphenol blue dye reached the bottom edge of the gel in a Tris-glycine buffer (0.02 M Tris, 0.19 M glycine and 0.1% SDS). The gel was removed, silver stained (10) and photographed with Polaroid type 55 positive/negative film. The negatives were developed in 20% metabisulfate for 1 min, then washed in distilled water and air dried.

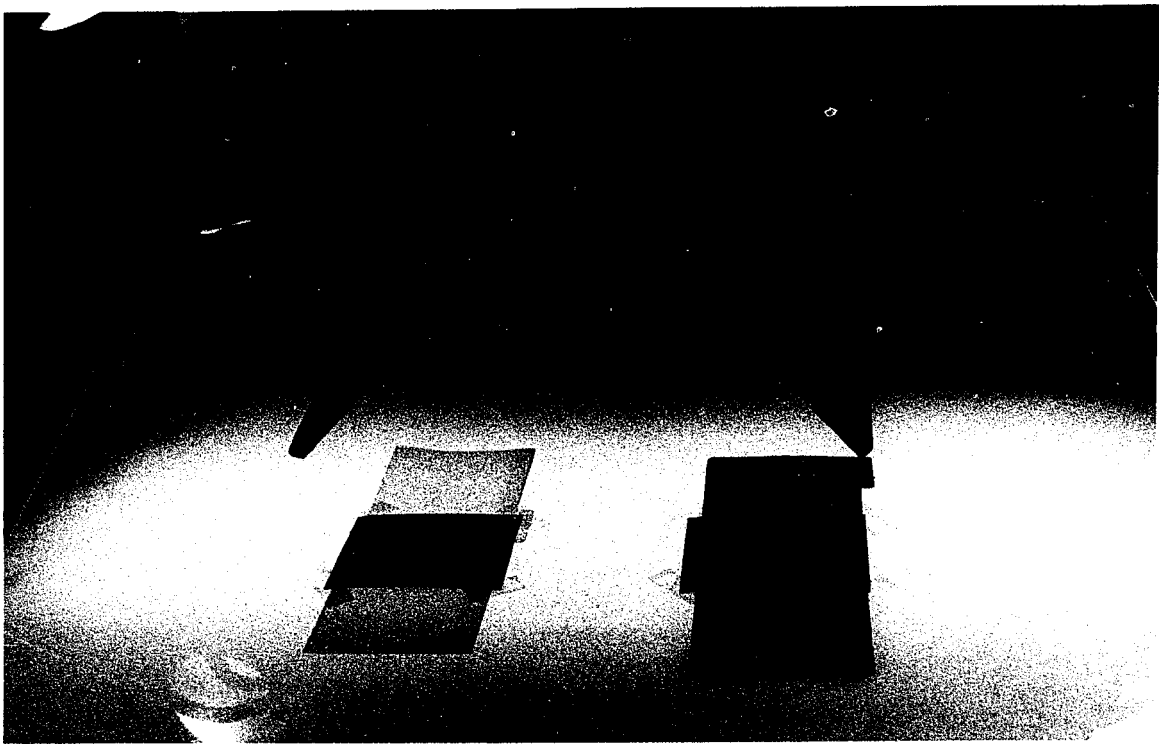
Stereoscope arrangement

The stereoscope viewer (Model K10 precision mirror stereoscope, Ben Meadows Co., Atlanta, GA) is depicted in Figure 1. The stereoscope was placed over a light box. One negative with a piece of red acetate under it was placed on the right and the second negative with a piece of yellow acetate was placed on the left. While the right negative remained stationary, the left negative was moved so that a key protein (actin) coincided. When this happens, the identical spots (coinciding spots) were orange, whereas spots unique on the right gel remained red and those on the left gel remained yellow.

A more expensive variation of this system employs light filters. A cyane filter (Kodak CC40C) was placed under each negative. The right eye piece contained a magenta filter (Kodak CC40M) and a yellow filter (Kodak CC40T) was placed in the left eye piece. The right film was purple when viewed alone and the left film was green; coinciding spots were "white."

Figure 1. Arrangement of stereoscope viewing system. A stereoscope viewer (1A) is placed on a large light box for visualization of gel sets. Figure 1B shows the placement of photographic negatives with two sheets of colored acetate placed under each negative. While one negative remains stationary, the other negative is moved until the images appear superimposed. It is then possible to detect spots common to both gels and spot unique to a single gel by color (see Materials and Methods).



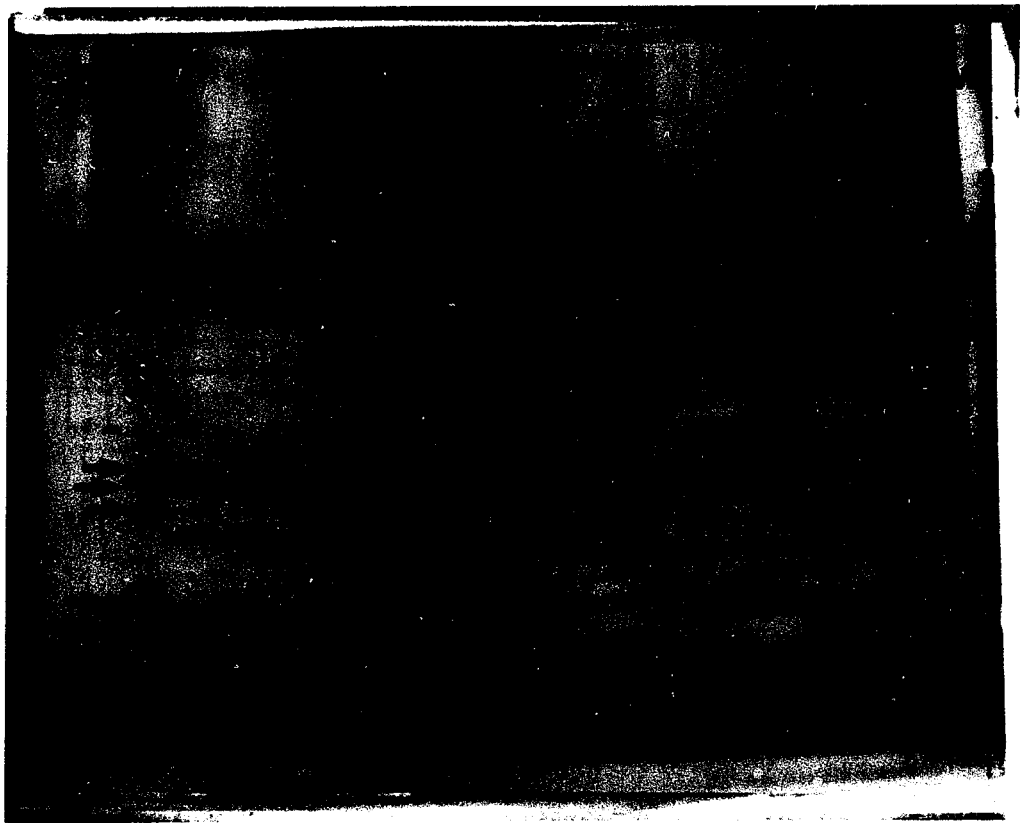


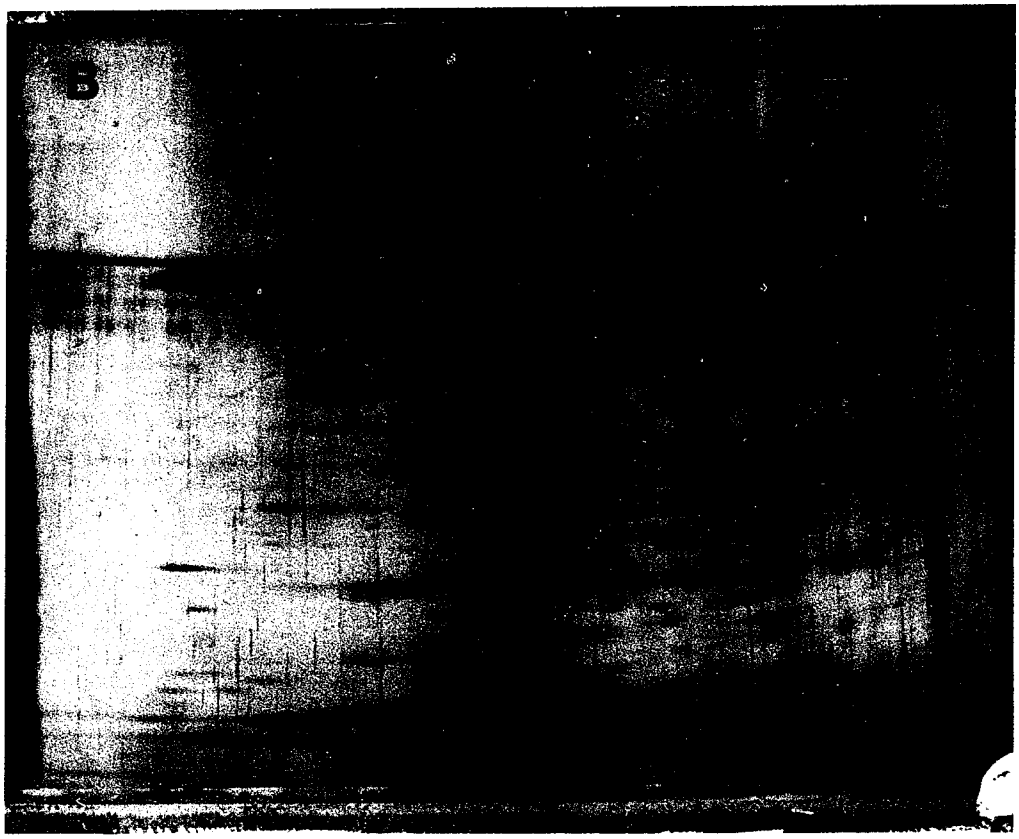
RESULTS AND DISCUSSION

A two-dimensional gel of proteins from a spleen cell preparation which includes T lymphocytes, B lymphocytes and macrophages was compared with a two-dimensional gel of proteins from purified B lymphocytes. Using the stereoscope viewer and red acetate under the negative of the two-dimensional gel of the spleen cell proteins and yellow acetate under the negative of the two-dimensional gel for the B lymphocytes, a number of protein differences could be discerned. Spots which were the same for both gels were orange, while protein spots which were unique for T lymphocytes and/or macrophages were red. These spots are marked in Figure 2A. There are a total of 173 protein spots in Figure 2A; of these 11 (6%) are unique. Davis et al. (10) compared the total in mRNA from B and T cell lines and have shown that only 2% of the message is unique to either T or B cells. Gemmell and Anderson (11) have shown with autoradiographs of two-dimensional gels that 4 out of 120 proteins (3%) are unique for human peripheral blood lymphocytes when compared with monocytes. The monocytes showed 7 unique proteins out of 120 (6%). These studies are consistent with the small number of protein differences seen between the two gels presented here.

Since no two-dimensional gels are exactly the same, a number of adjustments may have to be made. We have found that gels which are electrophoresed on the same days match more precisely and require the fewest adjustments. Gels which are electrophoresed on different days do not match evenly; however, this problem can be compensated by alignment of a reference spot, such as actin (A on Figure 2). The gels were analyzed in quadrants with actin serving as the origin of the axis. In this manner, the spots of any section were analyzed for

Figure 2. Comparison of two-dimensional gels from spleen cells and B cells. The gel to the top (2A) is a silver stained, two-dimensional gel of proteins from the entire murine spleen cells population. The bottom gel (2B) is a silver-stained, two-dimensional gel of the proteins from purified, splenic B lymphocytes. A=actin. The arrows indicate the spots which are unique to T cells and/or macrophages.





differences. Although this procedure requires an extra one or two minutes, the method is still rapid. An alternate method uses the photographic light filters described in the Methods section. Although it is a matter of personal preference, the colored acetate appears to be the easier for visualization between the two systems. This may be due to the intensity of the color with the acetate. Also from individual to individual, different colors of acetate were more suitable, i.e., some people preferred yellow and blue, while others preferred blue and red. This probably relates to how individuals perceive color, but the system is easy to change so that the person scanning the gels is comfortable with the system.

The technique described here employs a stereoscope viewer, colored acetate and photographic negatives to compare two-dimensional gels. It allows a rapid screening of complex gel systems which is not as tedious as spot-to-spot comparison, nor as expensive as the computer systems which are available for two-dimensional gel analysis.

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