RAT LYMPHOCYTE SURFACE IMMUNOGLOBULIN D

John H. Ruddick

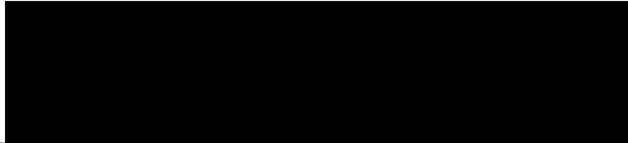
A THESIS

Presented to the Department of Microbiology and Immunology
and the Graduate Division of the University of Oregon
Health Sciences Center in partial fulfillment of the
requirements for the degree of

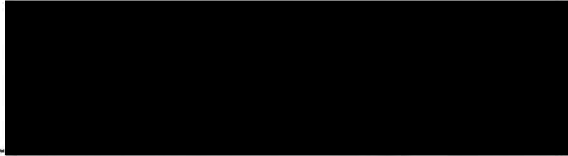
Master of Science

December, 1976

APPROVED:



Professor in charge of thesis



Chairman, Graduate Council

ACKNOWLEDGEMENTS:

I gratefully appreciate the continued support and direction given to me by my advisor, Dr. Gerrie A. Leslie, his comments and ideas were helpful and often enlightening. I also extend my gratitude to Drs. Denis R. Burger, and Marvin B. Rittenberg for their criticisms and positive comments, as well as to Drs. Vali Kermani-Arab and Michael I.Luster for their comments, aid and moral support. Lastly, I thank Ms. Gayle Teramura and Ms. Patricia McDaniels for their technical aid and friendship, and Ms. Manon Ruddick for her longsuffering aid in the preparation of this manuscript.

TABLE OF CONTENTS

Title Page	i
Approval Page	ii
Acknowledgements	iii
Table of Contents	iv
List of Tables	vii
List of Figures	viii
Introduction	1
Statement of the Problem	
Human IgD	3
IgD as a Cell Surface Ig	4
Phylogeny of IgD	6
Characteristics of the Murine Candidate for IgD	8
Materials and Methods	13
Animals	13
Media and Buffers	13
Other Reagents	16
Preparation of Human IgD	17
Antisera:	18
Chicken anti-human δ	18
Rabbit anti-rat IgM	19
Goat anti-rabbit gammaglobulin	19

TABLE OF CONTENTS (cont.)

Materials and Methods

Antisera:

Anti-chicken L chain reagents 20

Rabbit anti-rat L chain 20

Immunodiffusion Studies 20

IgD Radioimmunoassay 21

Cell Suspensions 22

Cell Surface Iodination 23

Co-precipitation of Labelled Surface Ig 24

Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis (SDS-PAGE) 24

Preparation of Rabbit Anti-rat Membrane IgD 25

In Vivo Administration of Anti-δ and Anti-μ Antibody 27

Preparation of Immunoadsorbants 29

Results 30

Chicken Anti-δ Antibody Specificity 30

Immunodiffusion Studies 31

IgD Radioimmunoassay 32

Anti-L Chain Precipitation 34

Immunoprecipitation of Rat Cell Surface IgD and IgM 35

Specificity of Chicken Anti-δ Precipitations: 39

Adsorption studies 39

Reprecipitation studies 41

Tissue Distribution of IgD bearing Cells 46

TABLE OF CONTENTS (cont.)

Ontogenic Emergence of IgD as a Cell Surface Ig	48
Genetic Studies	48
Reelectrophoresis of δ chain Peak	50
Molecular Weight of Unreduced Cell Surface IgD	52
Precipitation of Cell Surface IgD with Rabbit Anti-rat Membrane IgD	54
Anti-μ and Anti-δ *In Vivo* Administration	57
Discussion	62
Specificity of Chicken Anti-δ	63
Rat Cell Surface Ig	63
Rat Cell Surface IgD	64
Tissue Distribution and Ontogeny of Rat IgD	71
Anti-δ and Anti-μ Administration	74
Summary and Conclusions	80
Speculation	81
References	86

LIST OF TABLES

TABLE

I Anti-μ and Anti-δ Administration In Vivo: Injection Schedule and Dosage of Anti-globulin Reagents.

II Radioimmunoassay for IgD in the Sera of Various Species

III Tissue Distribution of IgD Bearing Lymphocytes in the Rat

Ontogeny of Cell Surface Immunoglobulins in the Rat.

V Cell Surface IgD and IgM: Relationship to Strain Variation and Ag-B Loci.

VI Serum Immunoglobulin Levels and Cell Surface IgD and IgM Patterns Following Antiimmunoglobulin Administration In Vivo

LIST OF FIGURES

Figure:

1 SDS-PAGE Electrophoretogram: Anti-L Chain Precipitated Labelled Rat Cell Surface Ig.

2 SDS-PAGE Electrophoretogram: Anti-δ and Anti-μ Precipitated Rat Splenocyte Surface Ig.

3 SDS-PAGE Electrophoretogram: NCS and NRabS Controls.

4 SDS-PAGE Electrophoretogram: Effects of Absorption of Chicken Anti-δ With Purified Human IgD or Rat IgM.

5 SDS-PAGE Electrophoretogram: Effects of Absorption of Chicken Anti-δ With Fetuin.

6 SDS-PAGE Electrophoretogram: a) Anti-δ and Anti-μ Reprecipitation of an IgD Depleted Labelled Rat Splenocyte Lysate. b) Anti-δ and Anti-μ Reprecipitation of an IgM Depleted Labelled Rat Splenocyte Lysate.

7 SDS-PAGE Electrophoretogram: Reelectrophoresis of Rat Splenic δ and μ Chains Isolated by Electrophoresis.

8 SDS-PAGE Electrophoretogram: Intact, Unreduced Rat Splenocyte Surface IgM and IgD.

9 SDS-PAGE Electrophoretogram: Precipitation of Rat Lymphocyte IgD with Rabbit Anti-rat Membrane Bound IgD.

10 Histological Changes in Anti-δ or Anti-μ Treated Rat Spleens as Compared to Control (Untreated) Animals.

11 SDS-PAGE Electrophoretogram: Anti-δ and Anti-μ Precipitation of a Labelled Rat Thymocyte Lysate.

12 Hypothetical Working Model for B Cell Differentiation and stimulation by Antigen.

LIST OF ABBREVIATIONS

NCS	Normal Chicken Serum
Ig	Immunoglobulin
FACS	Fluorescence-activated Cell Sorter
N Rab S	Normal Rabbit Serum
NRS	Normal Rat Serum
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
C^1	Complement
PMSF	Phenyl Methyl Sulfonyl Fluoride
OD_{280}	Ultraviolet Absorbence at 280 NM
EACA	Epsilon Amino Caproic Acid
PEG	Polyethylene Glycol
IgY	Avian Equivalent of Mammalian IgG
MW	Molecular Weight
CPM	Counts per minute
C anti-δ	Chicken anti-δ
V-gene	Gene Coding for Immunoglobulin Variable Region
C-gene	Gene Coding for Immunoglobulin Constant Region
CH	Constant Heavy Chain Region
VH	Variable Heavy Chain Region

INTRODUCTION

Statement of the Problem:

It has been amply demonstrated by Rowe, Hug, Forni & Pernis (1973), Pernis, et al., (1974) and Winchester, Fu, Hoffman, & Kunkel (1975), that a large proportion of human peripheral blood lymphocytes bear IgD on their surface and may often bear IgM concommitantly. The observation that the percentage of IgD bearing cells in chronic lymphocytic leukemia patients is significantly elevated (Kubo, et al., 1974; Preudhomme, et al., 1974) and that these cells simultaneously bear IgD and IgM isotypes with shared idiotypic determinants (Fu, Winchester, Feizi, Walzer, & Kunkel, 1974; Salsano, et al., 1974) has suggested that IgD may play a role in lymphocyte differentiation. Investigations involving the role of cell surface IgD have been limited due to the inflexibility and ethical considerations involved with the use of the human as a research model, and the lack of a detectable IgD homologue in an experimental species other than primates (Neoh, et al., 1973; Leslie & Armen, 1974; Martin, Leslie & Hindes, 1976; Martin, 1976). Recently, however, Melcher, et al., (1974) and Abney & Parkhouse, (1974) have detected a murine cell surface immunoglobulin with properties similar to human IgD (Spiegelberg, 1972). While considable characterization of this isotype has continued, primary

criteria for heavy chain homology (i.e. amino acid sequence homology or antigenic cross reactivity) with human IgD has not been established. For this reason, this thesis was designed to investigate the possible existance of an antigenically cross reactive IgD homologue in a manipulable experimental animal and to characterize its nature and evaluate certain of its biological functions.

<u>Human IgD</u>

IgD was originally defined by Rowe and Fahey, (1965,a) as an atypical myeloma protein and later detected in normal human sera (Rowe and Fahey, 1965,b). The novel isotype did not react with anti-γ, α or μ antisera but was reactive with anti-L chain antisera and could be reduced to H and L chains by 2-mercaptoethanol. Two characteristics of IgD has hindered its research, 1) the concentration of IgD in normal sera is low, averaging 30 μg/ml (Rowe & Fahey, 1965,b) and 2) it is a labile immunoglobulin which fragments readily during purification (Spiegelberg, 1972). The reported molecular weight varies from 172,000 (Spiegelberg, et al., 1970) to 200,000 (Saha, et al., 1970), while the δ chain molecular weight has been calculated to be ~ 70,000 daltons, (Perry & Milstein, 1970; Leslie, Clem & Rowe, 1971). Mass ratio analysis has suggested an H_2L_2 conformation of the intact immunoglobulin (Spiegelberg, 1972) and the carbohydrate content is estimated at 12% (Perry & Milstein, 1970). Concentrations of IgD in normal human sera range from undetectable to greater than 400 μg/ml and vary with age (Leslie, et al., 1975). IgD has generally not been detected in body fluids other than serum or cerebro-spinal fluid (Nerenberg & Prasad, 1975), however, using a sensitive radioimmunoassay it has been quantified in saliva and amniotic fluid (Leslie & Teramura, 1976). Since IgD can be detected on the surface of lymphocytes (Fahey et al., 1968, Van Boxel, et al., 1972), these observations have led to the suggestion that IgD may function as a receptor for lymphocyte differ-

entiation (Vitetta and Uhr, 1975,a). This concept however does not explain the presence of IgD antibody activity in serum directed against penicillin (Gluick, et al., 1969), bovine gamma globulin (Heiner and Rose, 1970), insulin (Lertora, et al., 1975;Devey, et al., 1970), or measles virus, (Luster, et al., 1976). These antigen binding properties have all been detected by indirect methods, probably a reflection of the low concentrations of IgD in serum. IgD does not exhibit other biological functions such as homocytotropic activity (Ovary, 1969), classical complement fixation (Henney, et al., 1969), or neutrophil degranulation (Henson, et al., 1972), although it has been reported to fix the later components of complement (Konno, et al., 1975).

IgD as a cell surface Ig

As previously stated, IgD occurs as the predominant class of cell surface immunoglobulin in the human (Rowe, Hug Faulk, McCormick, and Gerber, 1973) often in conjunction with IgM (Rowe, Hug, Forni, & Pernis, 1973). The average percentage of IgD bearing peripheral blood lymphocytes has been studied by rosetting or by immuno-fluorescence and has been found to range from less than 0.5% (Litwin, 1972) to 6% (Peissens, et al., 1973) although δ chain is by far the predominant heavy chain class on newborn lymphocytes (Rowe, Hug, Faulk, McCormick & Gerber, 1973). These findings, suggestive of the importance of IgD as a receptor, imply that IgD may be a predecessor of other surface-bound immunoglobulins, or

may be involved in differentiation of B cells to plasma cells secreting these classes. The possibility of a role in differentiation is augmented by findings of Fu, Winchester and Kunkel (1974) who noted that chronic lymphocytic leukemias have up to 89% IgD bearing cells and that, in some cases, up to 99% of these cells also bear IgM, suggestive of a differentiative defect at or near the time of appearance of IgD as a surface Ig. That this differentiation pattern was occurring in the monoclonal proliferation of the leukemic cells was confirmed by Pernis, et al., (1974) and Fu, Winchester, Feizi, Walzer & Kunkel, (1974) who showed that in leukemic cells concurrently bearing IgM and IgD, both surface Ig isotypes had similar antibody activity or idiotypic specificity, respectively. Despite early speculation that surface IgD precursed the appearance of other cell surface isotypes, Gupta, et al., (1976) demonstrated μ bearing cells in the human fetal liver as early as 13 weeks gestational age, whereas δ bearing cells were not apparent even at 17 weeks, implying that IgM - IgD cells are observed in the course of differentiation of their surface isotypes from IgM to IgD rather than the reverse sequence. This differentiative pattern is of interest since it appears to diverge from the exclusion theory of H chain expression restriction by allowing the apparent simultaneous synthesis of IgD and IgM, although double producing cells still appear to be restricted to a single L chain type (Fu, Winchester, & Kunkel, 1974). Numerous other studies have correlated altered IgD bearing cell frequencies with various disease states

such as allergic contact and atopic dermatitises (Cormane, et al., 1974), chronic lymphocytic leukemia (Kubo, et al., 1974), and malignant melanoma (Malka, et al., 1974), the latter suggesting that the increased level of IgD bearing cells in this disorder could be due to a compensatory IgD bearing cell proliferation due to a fall in T lymphocyte function.

Phylogeny of IgD

Neoh, et al., (1974) have assessed the plasma of 90 species of mammals, including 24 primate species, for the presence of IgD by double immunodiffusion. Their findings, furthered by Leslie and Armen, (1974), suggested that virtually all primates had detectable serum IgD as did certain canine species, artiodactyls (Bighorned & Barbary sheep; gnu, cow, wild boars, etc.). Guinea pigs, when infected with Lieshmania enriettii , also showed detectable IgD. The detection of non-primate humoral IgD required the use of avian antisera to human myeloma IgD in accord with the findings of Orlans and Fienstein, (1971), that antisera raised in chickens was especially cross reactive between immunoglobulin classes from various mammalian species. More recently, Martin, Leslie and Hindes (1976) have described IgD on the surface of lymphocytes from several non-human primate species by immunofluorescence. In these studies, 0-28% (means 1-8%) of the peripheral blood lymphocytes from individual animals stained with anti-human IgD whereas 0-35% (means 3.5-13%) stained with anti-human IgM.

Further studies by Martin & Leslie, (1976) have indicated that bi-staining cells are observable in the Rhesus monkey with fluorescein labelled anti-human δ and rhodamine labelled anti-human μ antisera and that the respective receptors cap independently of each other as was the case with human cord blood lymphocytes (Knapp, et al., 1973).

Melcher, et al., (1974) and Abney and Parkhouse, (1974), concurrently described the presence of an IgD-like molecule detectable by radio-electrophoresis on the surface of mouse lymphocytes that is precipitable from a lactoperoxidase-catalyzed ^{125}I labelled murine lymphocyte lysate with anti-mouse L chain antisera. This system, which will be described in the next section, has been compared to the human system as studied by Finkelman, et al., (1976). In this study Guinea pig and rabbit lymphocytes were also studied and it could not be concluded that a surface immunoglobulin similar to the murine IgD-like molecule existed in these species, although the finding of Jones et al., (1974), that rabbit IgA precursor cells, while bearing immunoglobulin, do not bear IgA or IgM suggests the presence of IgD in this species. Finkelman's study also concluded that the murine IgD candidate differed from human IgD in that its δ chains migrated considerably faster than human δ chains on 10% polyacrylamide-SDS gels.

Characteristics of the Murine Candidate for IgD.

The mouse model for IgD described above has been extensively defined, although to date, primary criteria for homology of murine and human δ chains has not been obtained. The murine IgD-like molecule (hereafter referred to as murine IgD for the sake of clarity), is immuno-precipitable with anti-mouse L chain antisera, but not with antisera against mouse μ, δ, or α chains (Vitetta & Uhr 1975,b) and is susceptible to "spontaneous" proteolysis, (Melcher, et al., 1974). Murine IgD appears to exist as a tetramer of approximately 178,000 daltons containing covalently bound H and L chains in an H_2L_2 conformation (Melcher and Uhr, 1975), although Abney and Parkhouse (1974) have suggested that H-L dimers may also exist. This form may be the result, however, of cleavage of the H_2L_2 form. The δ chain is observed primarily as a 66,000 dalton peak on 10% poly-acrylamide-SDS gels although one or two additional shoulders are often apparent at 59,000 and 53,000 daltons, respectively (Melcher and Uhr, 1976). Membrane bound μ chain under the same conditions had an apparent molecular weight of 74,000 daltons. This difference in mobility is opposed to the finding, in the human, by Finkelman, et al., (1976), that μ and δ chains coelectrophoresed under these conditions. Murine IgD can be shown to be synthesized by the cells on which it resides by stripping of the immunoglobulin from the cells with papain followed by subsequent resynthesis of the immunoglobulin (Vitetta & Uhr, 1975,b). It is not detectable ontogenically until after

IgM receptors are present. It is of interest that this ontogenic emergence of both IgM and IgD receptors occurs in the absence of intentional antigenic insult in germ-free or athymic (nu/nu) animals (Vitetta, Melcher, McWilliams, Lamm, Phillips, Quagliata & Uhr, 1975), implying both antigen and thymus independent development. Using the fluorescence activated cell sorter (FACS) Scher, Sharrow, Wistar, Asofsky, & Paul, (1976) have confirmed this developmental scheme by detection of a population of cells bearing low levels of IgM but fluorescing brightly with anti-Ig antisera. These results suggest a continuum of IgM and IgD bearing cells varying from those bearing only IgM to cells bearing much greater amounts of IgD than IgM, with cells exclusively expressing IgD comprising only a very minor percentage of lymph node or splenic lymphocytes.

A recent series of investigations (Finkelman, et al., 1975; Scher, et al., 1975; and Scher, Sharrow, and Paul, 1976), has provided evidence for an X-linked genetic defect in CBA/N mice that results in their inability to respond to a series of thymus independent antigens (Amsbaugh, et al., 1972). This defect may be linked to their finding that these animals have a decreased IgD/IgM surface immunoglobulin ratio when compared to normal BALB/c controls. IgD bearing cells in normal mice, are observed as cells fluorescing with a low to intermediate intensity using the FACS

with fluoresceinated anti-μ antisera and correspond to a distinct, mature subclass of B lymphocytes (Scher, Sharrow & Paul, 1976), and may be involved in B cell memory (Strober, 1975). This association of IgD with memory cells is furthered by Goodman, Vitetta, Melcher and Uhr (1975) who have shown IgD only on small, but not on large, lymphocyte populations in the spleen.

Genetic polymorphism (allotypy) of IgD has been recently described by Goding, et al., (1976), who noted that alloantisera raised in C57BL/6 mice against CBA, or BALB/c spleen cells, could precipitate an IgD-like molecule from ^{125}I labelled donor strain splenocyte lysates in addition to H-2 and Ia antigens. These antisera also precipitated an Fc receptor protein from these cells, but precipitated only H-2 antigens from donor thymocytes. However when congenic strain-derived spleen cells were labelled, the C57 anti-CBA anti-serum did not precipitate δ or L chain material in the case of B10, BR cells or H-2 or Ia material in the case of C57·Ig^{e} cells. These findings suggest that true polymorphism exists among δ chains in these 2 congenic pairs, since in this particular genetic back-ground (C57), δ chain can be found in 1 congenic strain (C57·Ig^{e}), but not the other (B10,BR). Immunoglobulin depletion of the labelled lysates removed the δ and L chain peaks and therefore, since the non-congenic anti - spleen cell sera failed to react with any known murine serum immunoglobulin (IgD is not detectable in murine sera (Abney, Hunter, Parkhouse, 1976)) or to cross-react

with alloantisera to Ig-1a, Ig-1e, Ig-3a or Ig-41, the alloantigenic determinant allele they have detected has been tentatively termed Ig-5a.

Mouse IgD has been further defined by the preparation of an antiserum directed against the membrane bound immunoglobulin (Abney, Hunter and Parkhouse, 1976). By immunoabsorbant depletion of Fc receptor and IgM from a Nonidet P40 lysate of 1750 BALB/c spleens, these researchers were able to isolate IgD by absorption onto a high capacity rabbit anti-mouse Fab immunoabsorbant. Following emulsification of the immunoabsorbant in complete Freund's adjuvant and injection into rabbits, an antiserum was prepared that, after appropriate absorptions, reacted only with cell surface murine δ chain. Use of this antiserum for indirect immunofluorescence resulted in percentages of stained cells ranging from 10.5% in peripheral lymph nodes to 26.8% in Peyer's patches. In conjunction with rhodaminated anti-mouse δ chain staining, from 1.5% (peripheral nodes) to 13.8% (spleen) of the cells were bistaining, results reflective of human IgD bearing cells.

A final segment of this model warranting discussion is the possible functional heterogeneity of cell surface IgM and cell surface IgD. Vitetta, Forman & Kettman (1975) have proposed that cells bearing only IgM or IgM and IgD concurrently, respond to LPS in a polyclonal response with subsequent IgM secretion, whereas cells bearing IgD

alone proliferate and undergo blastogenesis. "IgD only" cell populations were generated by depletion of IgM bearing cells with anti-μ and C' and therefore cells with high IgD/IgM ratios may have been spared due to incomplete cytotoxicity attributable to low IgM receptor density. Regardless, there appears to be receptor-related functional heterogeneity with mitogenic responses being related to the stage of B cell maturation. A later study by this same group (Cambier, et al., 1976) has shown that neonatal spleen cells (which bear only IgM) are tolerized _in vivo_ by a roughly 1000 fold lower concentration of the tolerogen $TNP_{17}HGG$ than are adult murine spleen cells (bearing both IgM and IgD). The mechanism for this difference is not known, but is consistant with the findings of Kearney and Lawton (1975,a & b) that LPS stimulation of either fetal lymphoid tissue or adult bone marrow cells resulted in a polyclonal response with subsequent IgM synthesis but failed to mount a proliferative response _in vitro_.

Materials and Methods

Animals. Outbred Sprague Dawley rats were selected from our colony. Animals were randomized with regard to sex, and, with the exception of ontogenic studies, were greater than 60 days of age. Donors of membrane IgD were retired breeding stock, obtained from Sprague-Dawley Labs.

F344 (Ag-B1); W/Fu (Ag-B2); Brown Norway (Ag-B3); Copenhagen (Ag-B4); August (Ag-B5); and M520 (Ag-B6) rats were obtained from the National Institutes of Health (Washington, D.C.) and were greater than six months of age. All rats were maintained in groups of 2-4 on Purina Chow Pellets (Ralston Purina Co., St. Louis, Mo.) and water ad libitum.

Media and Buffers.

1) Sephadex Buffer (Tris Buffered saline)

Stock Solution	
Trizma HCl	132.2 g.
Trizma Base	19.4 g.
Sodium Azide	100 g
Disodium EDTA	37.2 g.
Distilled H_2O	q.s. 1 l
pH	7.4

This solution is filtered through Whatman #1 paper prior to use.

Working Buffer

Stock solution	160 ml
NaCl	132.48 g.
Distilled H_2O	q.s. 16 l.
pH	7.4

2) Phosphate Buffered Saline (PBS)

NaCl	8.5 g.
Na_2HPO_4 (anhyd)	1.14 g.
KH_2PO_4 (anhyd)	0.270 g.
Distilled H_2O	q.s. 1 l.
pH	7.5

3) Immunoelectrophoresis Buffer

Sodium Barbital	108 g.
Sodium acetate (anhyd).	46.0 g.
0.1N HCL	1104 ml.
Thimersol (Lilly)	2 g.
Distilled H_2O	q.s. 4 l
pH	8.6

This buffer was stored as a 5X stock and diluted with distilled H_2O prior to use.

4a) Immunodiffusion Buffer

Sodium Barbital	6.98 g.
NaCl	6.0 g.
Thimersol (Lilly)	100 mg.
Distilled H_2O	q.s. 1 l.
pH	7.4

4b) High Salt Immunodiffusion Buffer

8.0 g. NaCl is added to 100 ml. of (4a) above

4c) Immunodiffusion Buffer with Polyethylene Glycol (PEG)

2 g. PEG is added to 100 ml. of (4a) above

5) NaSCN Buffer

Prepared as 4 M. stock in Sephadex Buffer (648.5 g/l.) and diluted to working concentrations with Sephadex Buffer.

6) PBS/BSA

1% Bovine serum albumen (Fraction V) in PBS - filtered through Whatman #1 paper

7) Sodium Phosphate Buffers

0.1 and 0.01 M phosphate buffers of various pH were prepared in distilled water as recommended by Williams & Chase, (1968).

8) Polyacrylamide Gel Chamber Buffer

$NaH_2PO_4 \cdot H_2O$	7.8 g.
$Na_2HPO_4 \cdot 7H_2O$	28.6 g.
Sodium dodecyl sulfate	2 g.
Distilled H_2O	q.s. 1 l.
pH	7.2

9) Polyacrylamide Gel Sample Buffer (reducing)

0.01 M phosphate pH 7.6 with 2% (W/V) SDS and 10% (V/V) 2 - mercaptoethanol (Sigma, St. Louis, Mo.)

10) Polyacrylamide Gel Sample Buffer (non-reducing)

0.01 M phosphate pH 7.6 with 2% (W/V) SDS and 8 M urea.

11) RPMI 1640 Medium

RPMI 1640 was purchased from Gibco (Grand Island, N.Y.)

Other Reagents

1) Acrylamide Solutions

1a) 10% acrylamide monomer

Acrylamide	22 g.
Bis.	0.6 g.
Distilled H_2O	q.s. 100 ml.

1b) 4.25 % acrylamide monomer

Acrylamide	10.6 g.
Bis.	0.276 g.
Distilled H_2O	q.s. 100 ml.

Acrylamide solutions were filtered through Whatman #1 paper and stored at 4 C in the dark.

Gels were photopolymerized following addition of:

22.5 ml Polyacrylamide Gel Chamber Buffer

20.25 ml Acrylamide Solution

2.25 ml $(NH_4)_2\ S_2O_8$ (10 mg./ml. in gel buffer)

0.067 ml. Temed

A water layer was added to the surface of gels prior to polymerization to minimize meniscus formation.

2) Immunodiffusion Agar

1% agarose in immunodiffusion buffer.

3) Immunoelectrophoresis Agar

1% agarose in immunoelectrophoresis buffer.

4) Hypaque-Ficoll (Terasaki, 1970)

9.0 g. Ficoll (Sigma) was dissolved in 100 ml. distilled water. 34 mls. of sodium metrizoate (50%) (Hypaque; Winthrop Labs.; New York, N.Y.) were added to 16 mls. distilled water. For use, 24 parts of 9% Ficoll were added to 10 parts of 34% Hypaque. Reagents were prepared fresh, prior to each use.

5) Trypan Blue Reagent for Cell Viability Determinations

0.05% trypan blue (Sigma) in PBS pH 7.2
Cells were diluted with an equal volume of trypan blue and both viable (non-staining) and dead (blue) cells were enumerated on a hemocytometer.

Preparation of human IgD. Human IgD was purified from a pool of normal human sera (NHS) containing 305 μg/ml IgD. The globulin fraction obtained following precipitation of the sera at 4 C with an equal volume of saturated ammonium sulfate (aqueous) was dissolved in 0.15M NaCl, 0.01M Tris buffer, pH 7.4, and filtered through a 2.5 X 100 cm Sephadex G-220 column (Pharmacia Fine Chemicals, Upsalla, Sweden). The IgD containing fractions were pooled and further fractionated on a DEAE cellulose column (10 X 2.5 cm)

equilibrated in 0.01 M phosphate buffer, pH 8.0. Following elution with the starting buffer, a linear gradient with a limit buffer of 0.1 M phosphate, pH 8.0, was applied. IgD eluting at a conductivity of 4,000 μmhos (approx. 0.02 M phosphate) was adsorbed onto a rabbit anti - IgD immunoadsorbent (Kermani-Arab, Burger & Leslie, 1976), and eluted with 3 M NaSCN (Stankus & Leslie, 1976). All buffers contained 0.01 M epsilon amino caproic acid. IgD from 5 pooled myeloma sera was prepared in a similar manner.

Antisera

Chicken anti-human δ : Chicken anti-human δ was prepared by hyperimmunization of adult White Leghorn hens with a pool of specifically purified IgD isolated from 5 different IgD myeloma sera and 2 normal human sera with elevated IgD levels. Birds were immunized 8 times over a one year period with each animal receiving a total of 5 mg. of protein in complete Freund's adjuvant (Difco, Detroit, Mich.). The antiserum was extensively absorbed (10-12 times) with pooled normal human serum containing less than 4 μg/ml IgD conjugated to Sepharose 4B (Pharmacia Fine Chemicals, Upsalla, Sweden) and with normal Sprague-Dawley rat brain tissue (4°C for 30 min). The specificity of the antiserum was determined by immunoelectrophoresis against normal human or rat sera and by immunodiffusion against purified rat and human serum proteins in 1.5 M NaCl-agarose gels (Leslie & Hattier, 1974). Specificity for δ chain was also determined by a modification

of the solid phase radioimmunoassay of Ceska & Lunkdvist, (1972), as developed by Leslie and Teramura, (1976). Chicken anti-δ was covalently coupled to filter paper discs (Munktell Paper Co., Grycksbo, Sweden) and the washed discs reacted with purified rat IgM, IgG NRS or human IgD. Following incubation for 2 hrs. at 37° C, the discs were washed and their ability to bind radiolabelled chicken anti-δ assessed. An elevation of counts bound to the discs was considered to indicate binding of the ligand by chicken anti-δ antibody.

Rabbit anti-rat IgM: Rat IgM was isolated by Sephadex G200 chromatography of specifically purified rat anti-group A streptococcal carbohydrate antibodies. This procedure, as well as immunoadsorbent columns and immunization schedules, have been described (Leslie & Clem, 1973; Stankus & Leslie, 1974). New Zealand White rabbits (David Robb, Inc., Sheridan, Ore.) received two injections of 1 mg. IgM in DFA, subcutaneously. Animals were bled 2 weeks following the last injection. Antisera were adsorbed with Sepharose immobilized rat IgG (prepared from rat gammaglobulin by DEAE cellulose chromatography) (Binaghi & Sarandon De Merlo, 1966).

Goat anti-rabbit gammaglobulin: Goat anti-rabbit gammaglobulin (GaRGG) was purchased from Calbiochem (LaJolla, California (lot # L 9933-11743)).

Anti-chicken L chain reagents: Goat anti-chicken L chain (GaCL) and rabbit anti-chicken L chain (RaCL) antisera were prepared as previously described (Leslie & Martin, 1973,b).

Briefly, IgY, H and L chains were separated by gel filtration in 8M urea-propionic acid following partial reduction of the intact immunoglobulin. Animals were immunized by subcutaneous injection of the purified proteins in CFA, followed by boosting 3 weeks later with antigen in incomplete Freund's adjuvant (IFA).

Rabbit anti-rat L chain: Rabbit anti-rat IgG with anti-L chain activity was purchased from Cappel Laboratories (Downington, Pa., Lot # 5981).

The specificity of all antisera was determined by double diffusion in gels and by immunoelectrophoresis against whole sera and the appropriate antigens.

Immunodiffusion Studies

High affinity chicken anti-δ was reacted against sera from various non-primate species by high salt double immunodiffusion. To increase sensitivity, oversized serum wells were multiply filled and polyethylene glycol was incorporated into the agarose. If further enhancement was desired, serum globulins were concentrated

ten fold by ammonium sulfate precipitation and the analyses repeated.

IgD Radioimmunoassay:

A specifically purified pool of human myeloma IgD was labelled with ^{125}I by the Chloramine T method of McConahey and Dixon, (1966), and the specific activity determined. Labelled IgD was diluted in PBS containing 1% BSA to minimize non-specific attachment of the label to reaction vessels. 10,000 cpm were employed and 0.01 M ε-amino caproic acid was included to prevent proteolysis of the IgD. Dilutions of anti-δ were titrated first against RaCL to determine the optimal proportions of these reagents needed to obtain maximum precipitation and then, maintaining this ratio, against 10,000 cpm of labelled IgD to determine the amount necessary to precipitate 50% of the ^{125}I counts.

30 or 100 μl of the test sera were incubated with C anti-δ at a 1/100 dilution in PBS/BSA for 1 hr. at 37 C. At this time, 10,000 cpm of labelled IgD was added and the assay reincubated for 1 hr. at 37 C. After this incubation, RaCL was added and the reaction put at 4 C overnight. The precipitate was centrifuged for 25 minutes at 4 C and 2000 g. and one half the total volume removed and counted. The tube, containing the other half of the supernatant plus the pellet (which was assumed to have negligible volume) was counted similarly. The percentage of counts precipitated was determined by the formula: $100 \left(\frac{P - S}{P + S}\right) =$ % precipitated cpms, in which P = one half the supernatant plus the pellet

and S = one half the supernatant. The relative percent inhibition was determined by dividing the percent precipitated counts from a sample by the percent precipitated counts in a control in which PBS/BSA was added in lieu of inhibitor, (i.e. Relative % inhibition = $\frac{\text{\% precipitated cpm (sample)}}{\text{\% precipitated cpm (control)}}$ X 100). The assay was titrated against dilutions of a W.H.O. standard IgD serum, a pool of NHS with a known concentration of IgD, and a commercial IgD standard (Meloy Labs., Springfield, Va.) to generate a standard curve to relate % inhibition to serum IgD concentration.

Cell Suspensions: Blood was obtained by cardiac puncture into heparinized tubes and the animals were sacrificed by cervical dislocation. Spleens were removed by laparotomy and trimmed of fat. Thymuses were removed by careful dissection to prevent removal of parathymic lobes. Thoracic and cervical lymph nodes were dissected from the submandibular region and separated from adipose tissue. Peyer's patches were excised from the small intestine. Isolated tissues were washed in cold RPMI 1640 without serum (GIBCO, Grand Island, N.Y.) and single cell suspensions were prepared in RPMI 1640. Tissues were finely minced and extruded through 100 gauge stainless steel mesh. The resulting suspensions were allowed to settle briefly to remove tissue debris and the single cell suspensions washed 2 times with 0.01 M phosphate buffered saline, pH 7.2 (PBS). All manipulations were done on ice.

Blood and splenic lymphocytes were isolated by centrifugation over a Hypaque-Ficoll gradient (24 parts of 9% aqueous (w/v) Ficoll (Sigma Chem. Co., St. Louis, Mo.) to 10 parts of 34% Hypaque (Winthrop Labs., New York, N.Y.)) at 400 g. for 25 min. at 25 C. Cells at the plasma-gradient interface were washed 3 times with cold PBS. Cell counts and viabilities were determined in 0.05% trypan blue in PBS.

Cell Surface Iodination: Cell suspensions were labelled by the lactoperoxidase technique of Marchalonis et al., (1971) as applied by Vitetta, Bauer and Uhr (1971). 3.0 - 20.0 X 10^7 cells were suspended in 0.3 ml. PBS containing 200 μg. lactoperoxidase (Sigma Chem. Co., St. Louis, Mo.) and 1.0 mCi carrier free, aqueous $Na^{125}I$ (New England Nuclear Corp., Boston, Mass.). 25 μl. of 8mM H_2O_2 (J.T. Baker Co., Phillipsburg, N.H.) was added and the reaction incubated at room temperature for 10 minutes, during which time 2 additional 25 μl. aliquots of 8mM H_2O_2 were added. The reaction was terminated by the addition of 5 ml. cold 5mM 1-cysteine HCl (Sigma Chem. Co., St. Louis, Mo.) in PBS. Labelled cells were washed 4 times with cold PBS. Washed, labelled cells were resuspended in 0.2 to 2.0 ml. 0.5% Nonidet P-40 (Shell Chem. Co., New York, N.Y.) in PBS and allowed to lyse at room temperature for 10 minutes. The nuclei and cellular debris were removed by centrifugation at 2,500 g. for 25 min. at 4 C.

Co-precipitation of Labelled Surface Ig: Labelled cell membrane proteins were "sandwich" precipitated by the addition of 12 μl of chicken anti-δ, rabbit anti-rat L chain or rabbit anti-μ antiserum to 0.2 ml. aliquots of each lysate. Following incubation for 1 hr. at 37 C the complexes were precipitated by the addition of excess RaCl or GaRGG, respectively. In the case of re-precipitation experiments, chicken anti-δ was precipitated with excess GaCl to avoid interaction of the second antibodies. Following the addition of second antibodies, incubation was continued at 4 C. overnight. The resulting precipitates were pelleted by centrifugation at 2,500 g. for 20 min. at 4 C. and washed 5 times with cold PBS. The supernatant from each wash was counted to ensure complete removal of unbound labelled proteins.

SDS - PAGE: SDS-PAGE was carried out on radioactive samples in 6.5 X 100 mm glass tubes using a modification of the method of Weber and Osborn (1969) employing 10% polyacrylamide gels. The samples were heated to 100 C. for 3 min. in 2% SDS (w/v) and 10% (v/v) 2-mercaptoethanol before electrophoresis, to facilitate reduction of the complexes. Following electrophoresis for 11 hr. at 25 C the gels were cut into 1 mm sections with a Savant Autogeldivider (Savant Inst., Hicksville, N.Y.). The ^{125}I labelled fractions were counted in a Beckman Biogamma well-type spectrometer (Beckman Inst., Fullerton, Calif.). The following external mole-

cular weight markers were used: phosphorylase a, (Sigma, St. Louis, Mo.; 94,000 daltons), bovine serum albumin, (Miles Labs., Kankakee, Ill.; 67,000 daltons), ovalbumin (Nutritional Biochemicals, Cleveland, Ohio; 45,000 daltons), pepsin (Worthington Biochemicals, Freehold, N.Y.; 35,000 daltons), and chymotrypsinogen a, (Worthington Biochemicals, Freehold, N.Y.; 25,000 daltons). After electrophoresis, marker gels were fixed and stained with a solution of 0.05% Coomassie brilliant blue (Sigma Chem. Co., St. Louis, Mo.) in methanol-acetic acid-water (5:1:4) for 3 hr. and destained in 7% acetic acid with 5% methanol.

In some studies, unreduced, ^{125}I-labelled surface Ig was electrophoresed for 3 hrs. at 8 ma/gel in 5 X 70 mm glass tubes containing 4.25% polyacrylamide gels. Immune precipitates were washed as before and dissolved by heating for 3 min. in 0.1 M phosphate buffer pH 7.6 with 2% SDS and 8 M urea. Following electrophoresis, samples were sliced as above and compared with the following stained external molecular weight markers: HuIgG (150,000 daltons); phosphorylase a (Sigma Chem. Co., St. Louis, Mo; 94,000 daltons); and BSA (Miles Labs., Kankakee, Ill; 67,000 daltons).

Preparation of Rabbit anti-rat Membrane IgD: 24 adult Sprague Dawley rats were sacrificed by exsanguination and the spleens and submaxillary lymph nodes were isolated. Single cell lymphocyte suspensions were prepared and pooled and subsequently lysed with

7.0 ml. of 0.5% NP40 in PBS with 1mM phenylmethylsulfonyl fluoride (PMSF) at 25 C. for 10 minutes. The lysate was centrifuged at 3,000 g. for 30 minutes at 4 C and the supernatant absorbed twice with Sepharose immobilized, pooled normal rabbit sera. The non-adherent material was absorbed with specifically purified chicken anti-δ covalently coupled to Sepharose 4B. The material eluting with 3M NaSCN was collected, dialyzed against PBS with 0.1 M EACA and concentrated to 2.0 ml. The resulting protein solution was centrifuged at 2500 g. for 15 minutes at 4 C and the supernatant (OD_{280} = 0.95) emulsified in an equal volume of CFA. The insoluble pellet was resuspended in 1.0 ml. of PBS and emulsified similarly. 5 Kg, New Zealand White rabbits were injected subcutaneously with 1.5 ml. of the pellet or supernatant emulsion and rested 2 weeks prior to boosting with an additional 0.5 ml. of the respective antigen. Animals were bled prior to each injection and eleven days following the booster injection. 5 mls. of each antiserum was absorbed twice each with Sepharose immobilized pooled normal rat sera and Sepharose immobilized rat IgM, and reconcentrated to 5.0 mls. 1.0 ml. of the absorbed antisera was further absorbed with 0.5 ml. of packed Sprague Dawley thymocytes at 25 C. for 1 hr. then at 4 C. for 2 hrs. The absorbed antisera was centrifuged at 1500 g. for 30 minutes at 4 C. and tested against a labelled Sprague Dawley splenocyte lysate by radioelectrophoresis.

In Vivo Administration of Anti-δ and Anti-μ: Normal Sprague Dawley pups less than 24 hrs. of age were injected with either rabbit anti-rat μ antisera or chicken anti-δ antibody, intraperitoneally. The concentration of anti-μ antibody in the rabbit antisera was assessed by reverse radial immunodiffusion against rat IgM and contained approximately 2.2 mg/ml anti-μ, as compared to standards of specifically purified rabbit anti-rat IgM of known OD_{280}. Eight animals of each group were injected with each antibody preparation. In addition, four controls received either normal rabbit serum, chicken IgY or PBS. Animals were injected according to the schedule in Table I. Control animals received a corresponding amount of control antigen or PBS on the listed days.

Two days after the last injection, animals were bled, the sera collected, and immunoglobulin levels determined by radial immunodiffusion. Two and nine days after the last injection, animals of each group were selected, sacrificed by exsanguination, the spleens, Peyer's patches, and submaxillary lymph nodes were removed, numbers of visible Peyer's patches determined, and representative pieces of tissue placed in 10 mls. of formalin (10% (v/v) formaldehyde in PBS, pH 7.4) for histological analysis. The remainder of the spleens were minced and the recovered lymphocytes analyzed for cell surface IgM and IgD by radioelectrophoresis.

TABLE I

ANTI-IG INJECTION SCHEDULE - ANTI-δ AND ANTI-μ IN VIVO ADMINISTRATION

Anti-δ		Anti-μ	
Age of animal (days)	Mg. antibody injected[1]	Age of animal (days)	Mg. antibody injected[1]
1	0.5	1	0.5
2	0.5	2	0.5
3	0.5	3	0.5
4	0.5	4	0.5
9	0.5	11	1.1
12	0.5	18	1.1
17	0.8	27	1.1
21	0.8	32	1.1
28	0.8	39	1.1
32	0.9		
36	0.9		
39	0.9		
42	1.8		

1) The amount of anti-δ antibody injected was determined from the OD_{280} using an extinction coefficient of 1.35. Anti-μ activity was quantitated by reversed radial immunodiffusion.

Preparation of Immunoadsorbents: Chicken anti-δ, IgD negative normal human serum, normal rat serum, normal rabbit serum, rat IgM, and rabbit anti-human IgD immunoadsorbants were prepared by the procedure of Cuatrecasus (1970). Briefly, Sepharose 4B was washed with distilled H_2O (~100 vol/vol seph). The washed, packed Sepharose (1.5 - 2 ml/4 mg. of protein to be conjugated) was resuspended in an equal volume of distilled water and 10 ml. of CNBr (1g in 10 ml. distilled H_2O) was added per 10 ml. of packed Sepharose. The pH was maintained at 11.0 with 4N NaOH until stable (8 - 10 min.). The activated Sepharose was washed with 1 L cold, distilled water and subsequently with 1 L cold 0.5M $NaHCO_3$, pH 9.0. The washed, packed, activated Sepharose was resuspended in an equal volume of cold 0.5M $NaHCO_3$, pH 9.0, the protein added, and stirred at 4C overnight. The immunoadsorbant was washed extensively with Sephadex buffer, 3M NaSCN and finally with Sephadex buffer a second time. Samples were directly applied to the immunoadsorbents in small glass columns and the columns washed until the effluents had 280 mμ readings less than 0.010. Columns were then eluted with 3M NaSCN until the absorption at 280 mμ decreased to less than 0.010 and finally extensively washed with Sephadex buffer to removed NaSCN.

Results

Chicken Anti-δ Antibody Specificity. Specifically purified chicken anti-δ was analyzed by double immunodiffusion and found to be unreactive against purified human IgG and IgM as well as rat IgG, IgM, normal rat serum, and rat saliva. Normal human sera with less than 10 μg/ml IgD, as determined by radial immunodiffusion against W.H.O. standardized IgD sera controls, also failed to react with chicken anti-δ. However, normal human sera containing 305 μg/ml IgD formed a single line against the chicken antibody in high salt agarose gels. To further assess the specificity of the chicken anti-δ, human IgE (obtained from Dr. K. Ishizaka). IgA, IgM and IgG myeloma sera were used to produce a pool with elevated Ig levels and thereby allow detection of IgE by Ouchterlony analysis. The pool contained 15.2 mg/ml IgG, 1.4 mg/ml IgM, 3.0 mg/ml IgA, 8438 IU/ml IgE, and 32 μg/ml IgD. Against this pool, chicken anti-δ formed a weak band which demonstrated non-identity when compared to bands produced by monospecific antisera to the other 4 classes of Ig, suggesting no anti-α,δ,ε, or μ chain activity. Immunoelectrophoresis against IgD bearing NHS produced a single band corresponding to IgD. To assess the binding capabilities of the reagent, a solid phase radiometric immunoassay was used. This assay is capable of detecting ~10 ng/ml human IgD in serum (Leslie & Teramura, 1976). Briefly, the assay detects binding of IgD to insolubilized chicken anti-δ and detection of

binding is facilitated by reaction of the bound IgD with radio-labeled chicken anti-δ. When NRS, or purified rat IgG or IgM at 0.1 or 10 mg/ml were used as ligands, no binding was detected indicating that chicken anti-δ was incapable of binding rat IgM, IgG or other NRS components at nanogram levels and furthermore suggested that there is less than 10 ng/ml of IgD in the serum of these animals. Binding was detectable, however, when human IgD was used as a ligand.

Immunodiffusion Studies

To determine if IgD was present in the sera of a variety of mammalian species, specific chicken anti-δ antiserum was reacted with the unconcentrated sera of various species in 1.5 M NaCl agarose gels by double immunodiffusion. Sera from amphibians (marine toad, leopard frog, bullfrog, snapping turtle); reptiles (alligator); birds (quail, chicken, pheasant,duck, goose); mammals (rat, dog, cow, horse, Guinea pig, mouse, rabbit); marsupials (oppossum); and primates (man, babboon, chimp, gorrilla, spider monkey) were tested. Precipitin bands were observed only with each of the primate sera tested.

The use of oversized antigen wells, multiple fillings of these wells, as well as 10 fold concentration of the globulin fraction of these sera (obtained by precipitation with half-saturated

ammonium sulfate at 4 C.) and incorporation of 2% (w/v) polyethylene glycol (PEG) into the gels failed to alter these findings. PEG may act as a hydrophilic agent which can increase the sensitivity of gel diffusion by competing for solvent within the gel structure and facilitate immune precipitation by reduction of the solubility of precipitates and thereby enhance precipitin line formation (Lundkvist & Ceska, 1972).

IgD Radioimmunoassay

Since immunodiffusion studies were negative for all readily manipulable and economic laboratory test species, selected sera (some of which had been previously analyzed by immunodiffusion) were assayed in a double antibody radioimmunoassay for IgD. This system, which detected inhibition of ^{125}I labelled, pooled human myeloma IgD-chicken anti-δ immune complex formation by unlabelled IgD was capable of detecting human IgD at concentrations as low as 20 ng/ml in whole sera. Since the volume of the complexes was minute, and since complexes mediated by chicken antibody do not form precipitates readily at physiologic salt concentrations (Goodman, Wolfe, & Stata, 1951), a second antibody (RaCL)was added to co-precipitate these complexes. Inhibition of label precipitation was indicative of IgD in the test sera. Inhibition was expressed as the relative percent inhibition compared to a control assay receiving PBS/BSA in lieu of test sera. The results obtained with 146 different sera are indicated in Table II.

TABLE II

RADIOIMMUNOASSAY FOR IGD IN THE SERA OF VARIOUS SPECIES.

Species	#tested	Relative % Inhib.[2] Mean	Range	Concentra. IgD range µg/ml
Guinea Pig				
(outbred Hartley)	28	6.2	1.9-21.1	< 0.1-1.0
(strain 13)	7	5.0	4.0-7.0	< 0.1-0.2
Rat				
(Sprague-Dawley)	20	2.6	0-12.6	0-0.5
Rabbit				
(New Zealand White)	4	3.5	0.4-7.1	0-0.2
Mouse				
(Balb/c)	5	4.5	0-9.3	0-0.3
Dog	9	10.9	0.1-34.2	0-4.0
Goat	3	6.2	5.0-8.4	0.1-0.25
Pigeon	2	2.3	0-4.7	0-<0.1
Chicken	3	75.8	(see note 1)	
Snapping Turtle	2	3.2	0.8-5.7	0-0.1
Human				
(Adult)	36	39.8	9.4-69.8	0.3-189
(cord blood)	27	22.0	8.2-61.0	0.25-56

Note: 1) Inhibition due to chicken serum is due to inhibition of second antibody reaction. Therefore inhibition does not reflect the presence of δ chain in chicken serum.

2) Relative % Inhibition = $\frac{\text{\% Precipitated CPM (Sample)}}{\text{\% Precipitated CPM (Control)}} \times 100$

The high average relative percent inhibition obtained with normal chicken serum is probably due to nonspecific inhibition of the secondary precipitation of the labelled immune complexes since the addition of NCS increases the amount of chicken IgY in the assay system. This would subsequently produce conditions of antigen excess in the secondary precipitation reaction between chicken anti-δ (IgY) and RaCL, resulting in decreased precipitate formation and thereby inhibit label precipitation. Relative percent inhibition values of less than 10% were considered to be insignificant. Some species, notably humans (both adult and neonatal cord sera) and dogs gave evidence of humoral IgD in this system, although certain individual Guinea pigs and rats showed levels of inhibition above 10%. The inhibition seen with these species corresponded to 4.0, 1.0, 0.5 μg/ml IgD, for dogs, Guinea pigs, and rats respectively, when compared to a standard curve generated with human IgD of known concentration. Direct conversion of these inhibition values to concentration must be made with reservation since the degree of cross-reactivity of C anti-δ for dog, rat, and Guinea pig IgD with human IgD was not determined.

Anti-L chain precipitation. To ascertain which classes of cell surface immunoglobulins were detectable on rat splenocytes by the radiometric technique, rabbit anti-rat L chain was used as a precipitating reagent and complexes of rat surface Ig and rabbit

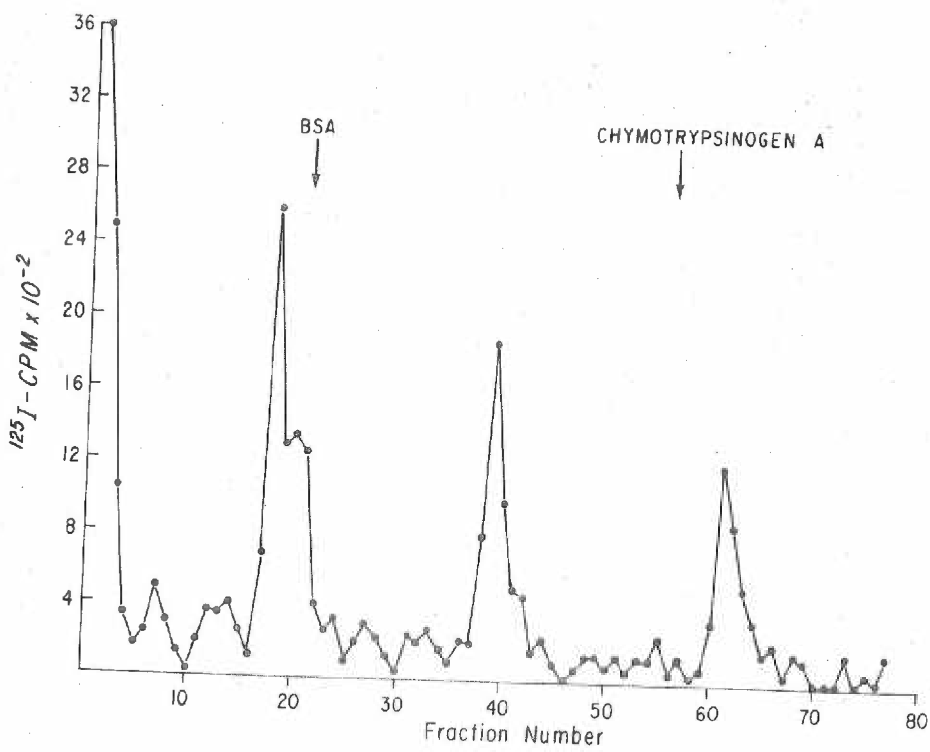
anti-rat L chain were precipitated with excess GaRGG. Anti-L chain precipitable material migrated as 3 distinct radiolabelled bands on 10% polyacrylamide-SDS gels, (Figure 1.) The radioactivity located in the heavy chain region of the gels (MW 50-75,000 daltons) migrated slower than γ and α chains and had an apparent molecular weight similar to μ chain (73,000 daltons). The small shoulder seen associated with peak 1 had a molecular weight of ~65,000 daltons. The H and L chain peak heights obtained with the anti-L chain reagent were always much higher than those obtained with anti-μ or anti-δ reagents alone, whereas the middle peak, which may represent an Fc receptor on rat lymphocytes, was only slightly higher under these circumstances.

Since others have reported the presence of IgG on the surface of murine lymphocytes (Mason, 1976), it was anticipated that γ chains would be observed between the μ chain and Fc receptor. However, no 50-60,000 dalton heavy chains were found on rat splenic lymphocytes.

Immunoprecipitation of rat cell surface IgD & IgM. The possibility that some species of animals do not bear an IgD-like molecule similar to that seen in the mouse has been raised by Finkelman et al. (1976), but admittedly their study was incomplete, although they did observe the co-migration of human δ and μ chains on SDS polyacrylamide gels. To assess the possibility that

Figure 1:

SDS-PAGE Electrophoretogram : Anti-L chain precipitated labelled rat cell surface Ig.


36
32
28
24
20
16
12
8
4
^{125}I-CPM x 10^{-2}
BSA
CHYMOTRYPSINOGEN A
10
20
30
40
50
60
70
80
Fraction Number

IgD in a non-primate species may act similarly, and more importantly, to determine if antigenic homology exists between δ chains from the rat and the human, attempts were made to precipitate IgD from a radiolabelled rat splenic lymphocyte lysate using primary antigenic considerations. The cells used were 90% lymphocytes and were 95% viable when surface labelled by the lactoperoxidase method. While different lot numbers of H_2O_2 or ^{125}I gave differing degrees of labelling, each cell surface component labelled with a characteristic efficiency relative to the other components. Therefore while absolute counts incorporated per molecule varied in separate experiments, the ratios of incorporated counts relative to a reference molecule (i.e. μ chain) were relatively consistent. Preliminary experiments showed NP-40 did not interfere with the precipitability of labelled components; thus NP-40 solubilized lysates were directly precipitated with the anti-immunoglobulin reagents.

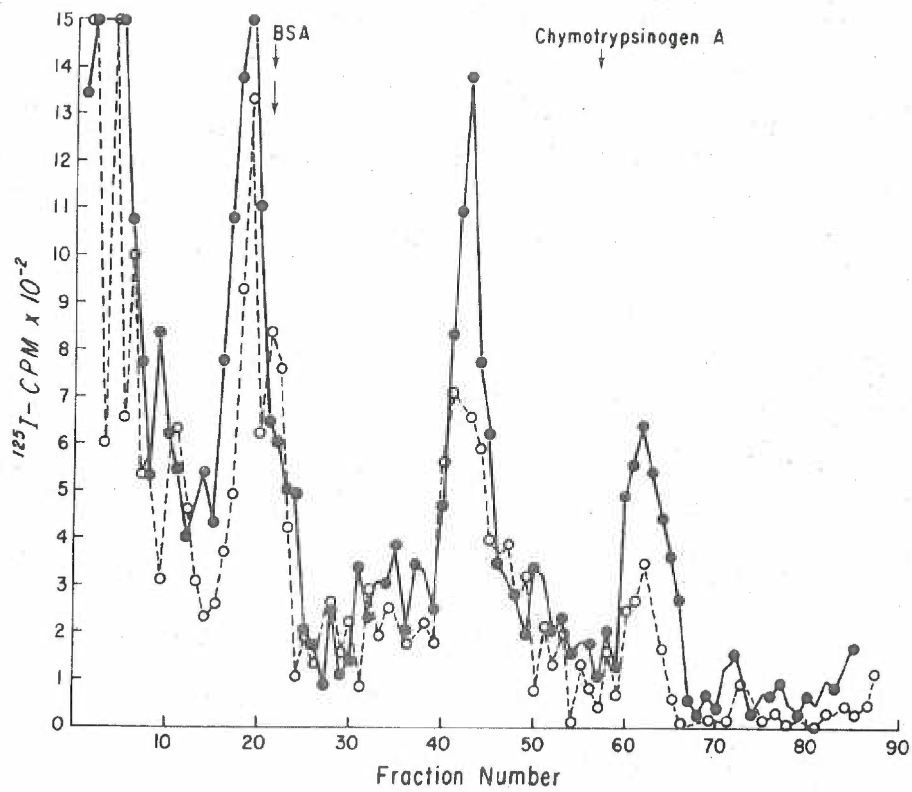
Figure 2 indicates the radioactive profile obtained following reduction and electrophoresis of a labelled rat splenic lymphocyte lysate precipitated with chicken anti-δ or rabbit anti-μ.
The rabbit anti-μ profile consists of 3 distinct peaks of labelled material corresponding to μ chain, Fc receptor and L chain. Rat μ and L chains migrated with apparent molecular weights of approximately 73,000 and 23,000 daltons,respectively. The intermediate peak, migrating at 30-35,000 daltons, probably

Figure 2:

SDS-PAGE Electrophoretogram : Anti-δ and anti-μ precipitated rat splenocyte surface Ig.

solid line = anti-μ

dotted line = anti-δ



BSA
Chymotrypsinogen A
^{125}I- CPM x 10^{-2}
Fraction Number

represents a rat Fc receptor with physicochemical properties similar to those seen in the mouse (Rask, et al., 1975). To further investigate this possibility, the lysate was reacted with non-immune chicken or rabbit sera plus the second antibody. The results in Figure 3 support this conclusion rather than the possibilty that these peaks represent an Ia-like antigen (Vitetta,Klein, & Uhr, 1974) since the 30-35,000 peak was precipitated with these "normal" reagents, as well as all other antisera used.

Chicken anti-δ precipitation resulted in the SDS-PAGE pattern seen in Figure 2. The δ chain peak coelectrophoresed with rat μ chain, although a small shoulder at approximately 65,000 daltons was also observed. Excluding relative peak heights, the Fc receptor and L chain peaks are superimposable with the respective peaks from the anti-μ precipitation. Therefore, apart from the appearance of the small δ chain shoulder, rat δ chain could not be readily distinguished from rat μ on the basis of electrophoretic mobility alone.

Specificity of chicken anti-δ precipitations.

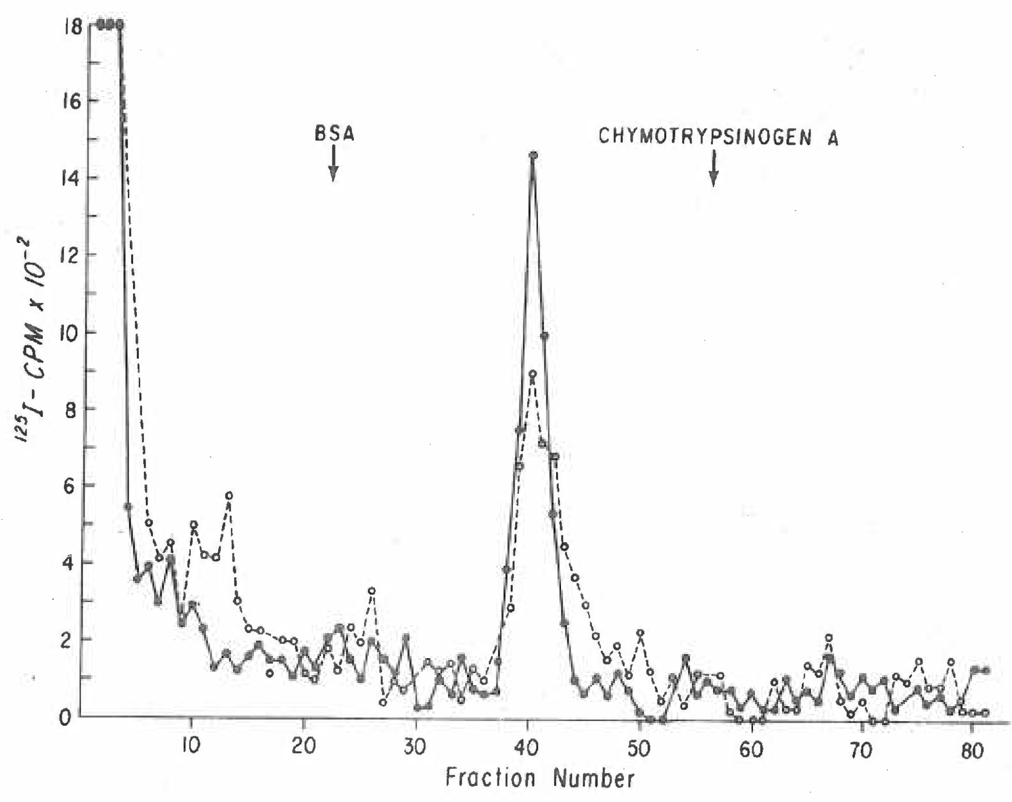
Absorption studies: Since both anti-μ and anti-δ antibodies precipitated 73,000 dalton proteins, the following absorptions were designed to ascertain if the anti-δ precipitable material was, in reality, cell surface IgM. Chicken anti-δ (0.5 mg/ml) was absorbed with 4 volumes of purified human IgD

Figure 3:

SDS-PAGE Electrophoretogram : Normal chicken serum and normal rabbit serum controls.

solid line = normal rabbit serum

dotted line = normal chicken serum.



BSA
CHYMOTRYPSINOGEN A
^{125}I - CPM x 10^{-2}
Fraction Number

(2.3 mg/ml) or purified rat IgM (9.6 mg/ml) for 18 hr. at 4 C. Following incubation, the absorbed antibody was centrifuged and the supernatant used to precipitate labelled surface immunoglobulin from adult Sprague Dawley splenocytes. The results, shown in Figure 4, confirm the ability of the anti-δ reagent to distinguish δ chain from μ chain determinants since absorption with human IgD blocked the anti-δ precipitation pattern whereas IgM failed to affect this reactivity. The small peak remaining after IgD absorption may indicate incomplete absorption of the antibody due, in part, to the presence of low affinity antibodies or the inability of some chicken antibody to form insoluble precipitates under these conditions (Goodman, et al., 1951). Sela, Wang, & Edelman (1975) have shown the presence of a naturally occurring antibody in the sera of normal chickens that has activity against the glycoprotein fetuin. To determine whether our antibody contained anti-fetuin activity, which could account for the precipitability of either the 35,000 dalton Fc receptor peak or the δ chain peaks, 4 volumes of a 10 mg/ml fetuin solution were used to absorb the chicken anti-δ antibody as previously described. As can be seen in Figure 5, the glycoprotein absorption failed to abrogate the precipitation of any peak in the anti-δ electropherogram.

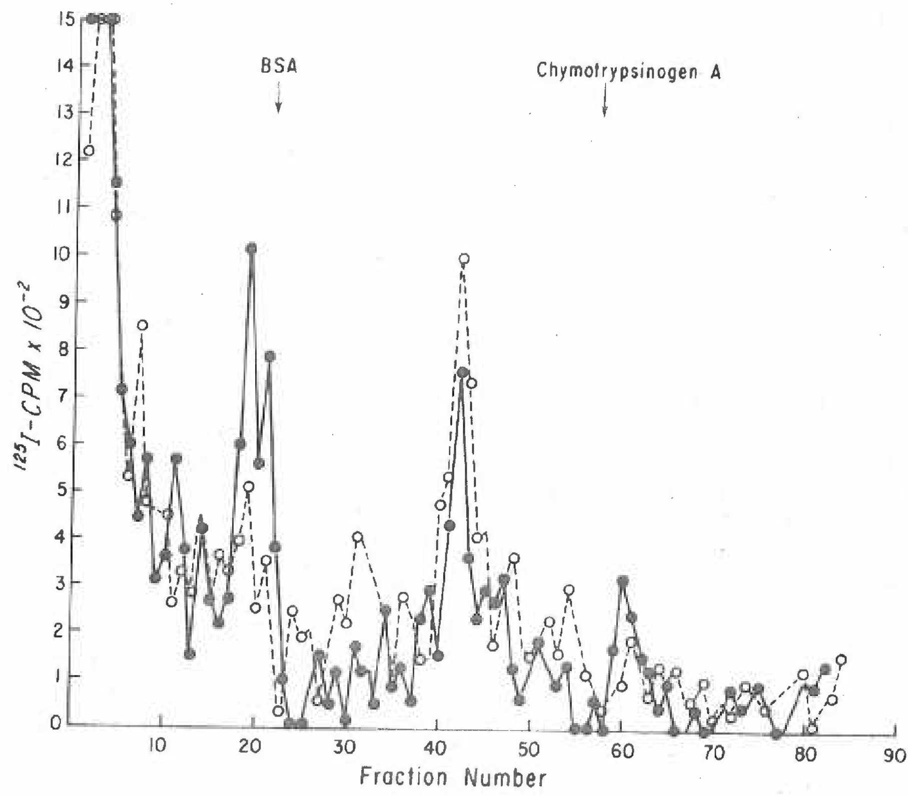
Reprecipitation Studies: To further evaluate the antigenic distinction between cell surface IgM and IgD in the rat, the presence of IgD in an IgM-depleted cell lysate was examined. This concept

Figure 4:

SDS-PAGE Electrophoretogram : Effects of absorption of chicken anti-δ with purified human IgD or rat IgM.

solid line = absorption with rat IgM

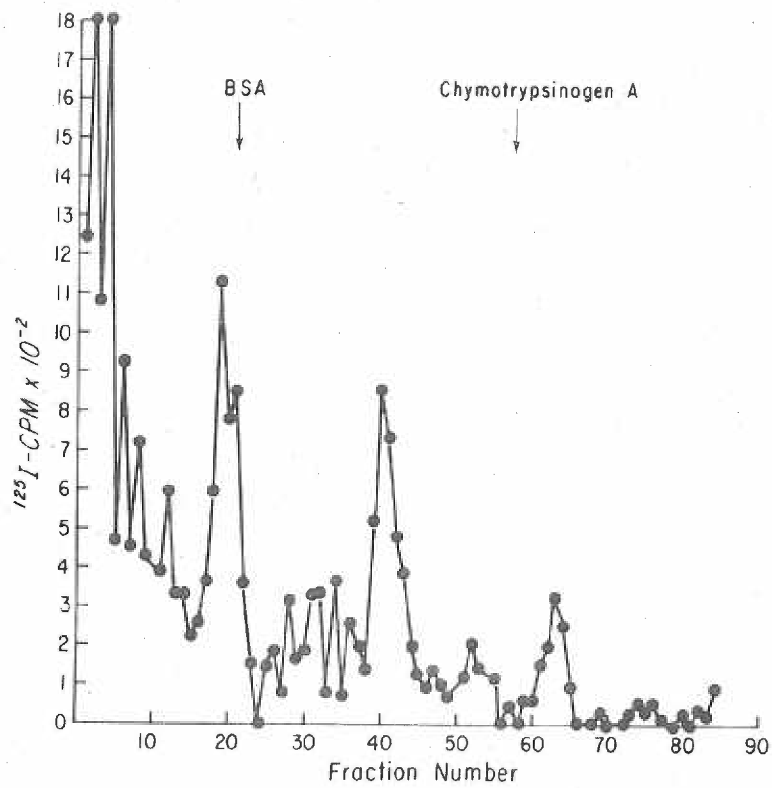
dotted line = absorption with human IgD



BSA
Chymotrypsinogen A
^{125}I-CPM x 10^{-2}
Fraction Number

Figure 5:

SDS-PAGE Electrophoretogram : Effects of absorption of chicken anti-δ with fetuin.


BSA
Chymotrypsinogen A
^{125}I-CPM x 10^{-2}
Fraction Number
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
10
20
30
40
50
60
70
80
90

is of interest not only from the viewpoint of establishing antigenic individuality between the 2 molecules but also in proving that, since the rabbit anti-μ antiserum used in this study gave no indication of having subclass or allotypic specificity, rat IgD is not merely an IgM subclass. Labelled splenic lysates were precipitated with either anti-δ or anti-μ and the dissolved precipitates electrophoresed. The supernatants from this first precipitation step were subsequently divided into 2 aliquots, 1 aliquot being reprecipitated with anti-δ, the other with anti-μ. To overcome reactivities between the GaRGG and RaCL reagents used to facilitate precipitation of the anti-Ig-surface Ig complexes, goat anti-chicken L chain was used in lieu of the rabbit antiserum. The results of the reprecipitations are compared with the first precipitation in Figure 6. Reprecipitation of the anti-μ depleted supernatant with the homologous reagent did not result in H or L chain peaks following electrophoresis. However, some material electrophoresing in the Fc receptor area remained. The heterologous precipitation with anti-δ, however, did provide evidence of both H and L chain peaks indicating the presence of an antigenically distinct δ chain in these lysates. The reversed experiment provided similar results, with virtual total removal of δ chains from the supernatant being effected following a single anti-δ precipitation while the precipitability of the μ chain in the same supernatant was unaffected. This experiment supports, therefore, the antigenic individuality of rat δ and μ chains.

Figure 6:

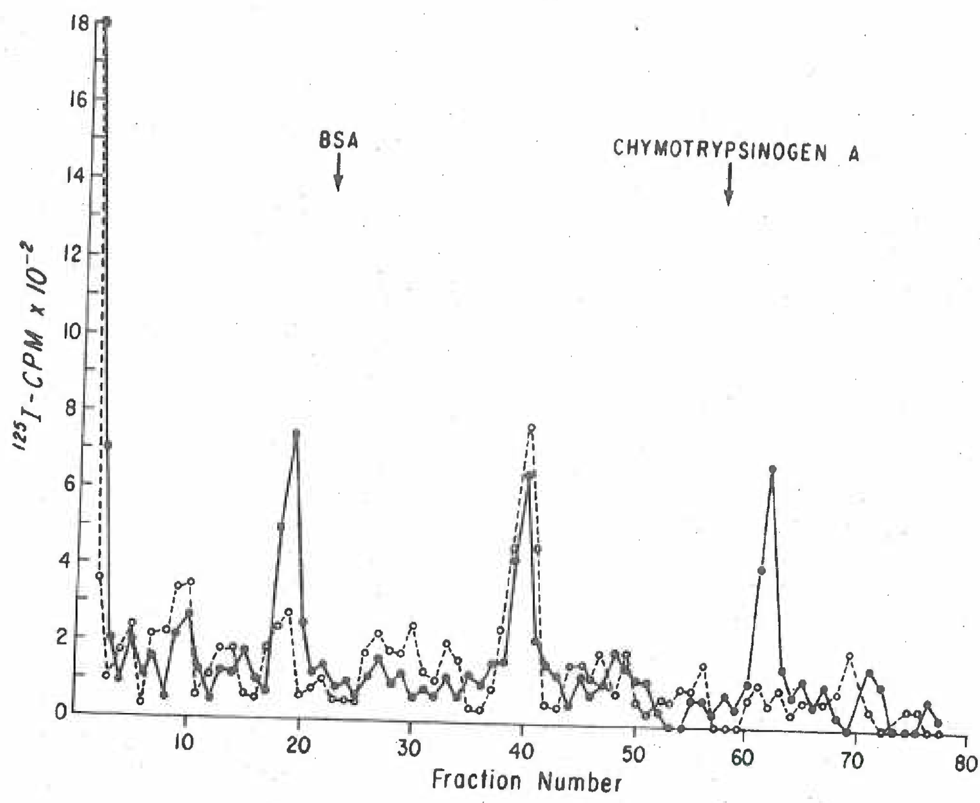
SDS-PAGE Electrophoretogram:

a) Anti-δ and anti-μ reprecipitation of an IgD depleted labelled rat splenocyte lysate.

b) Anti-δ and anti-μ reprecipitation of an IgM depleted labelled rat splenocyte lysate

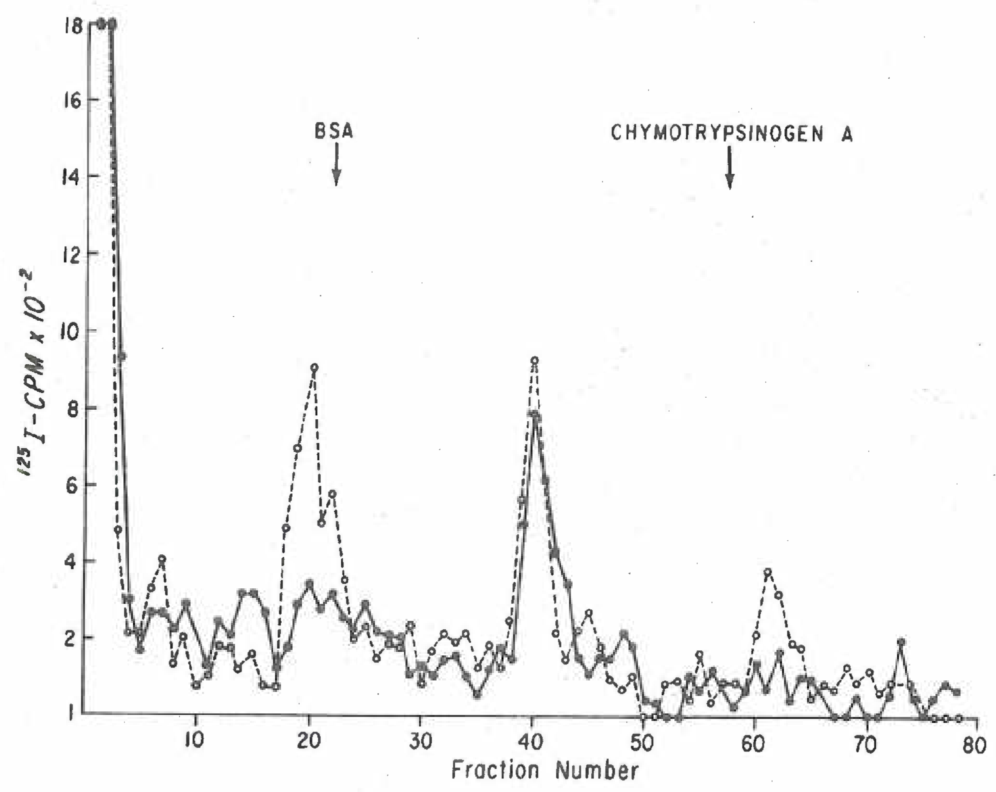
solid line = anti-μ precipitation

dotted line = anti-μ precipitation



6A↑

6B↓



Tissue Distribution of IgD Bearing Cells. To correlate the finding of IgD as a lymphocyte surface receptor with a particular class of lymphocytes, cells from various lymphoid tissues were examined for the presence of cell surface IgD and IgM. Labelled lymphocyte lysates from spleen, lymph nodes, thymus, Peyer's patches, and blood were analyzed for surface IgM and IgD. Equivolume aliquots were precipitated with each reagent and, following electrophoresis, the δ/μ peak height ratios were calculated. The results, summarized in Table III, suggest that IgD receptors occur only on the surface of B lymphocytes, since their absence is unique to thymus tissue, although their presence on post-thymic T cells is not excluded. IgD bearing cells are present in all the other lymphoid organs. Interestingly, the δ/μ ratio is not uniform. Organs considered to contain the most "mature" population of lymphocytes (i.e., Peyer's patches, lymph nodes and blood) exhibited higher δ/μ ratios than did splenocytes, with Peyer's patches, which may be antigen dependent for their development (Cooper et al., 1968; Pollard & Sharon, 1970), exhibiting the highest δ/μ ratio of all organs tested. Although thymocytes did not have demonstrable IgD or IgM, an Fc receptor-like peak could be detected with both the anti-δ and anti-μ reagents in accord with the finding of Stout and Herzenberg (1975) that certain subclasses of murine thymocytes bear an Fc receptor as a surface antigen.

TABLE III

TISSUE DISTRIBUTION OF IGD BEARING LYMPHOCYTES IN THE RAT

Tissue	IgD	IgM	δ/μ peak height ratio	
Spleen	+	+	0.53	(0.47-0.59)
Peripheral Blood	+	+	0.85	(0.74-0.96)
Lymph Node	+	+	1.50	(1.21-1.79)
Peyer's patch	+	+	2.15	(1.98-2.32)
Thymus	-	-		-

- = not detectable

+ = detectable

Ontogenic Emergence of IgD as a Cell Surface Ig. Since, ontogenically, Human IgD bearing cells appear after IgM cells are present (Gupta, et al. 1976), it was of interest to determine the developmental sequence of IgM and IgD in the rat. Rats 6,13, 25,42, and 180 days of age were sacrificed and the spleens removed and assayed for cell surface IgD and IgM. Electrophoretograms were scored on the basis of the ratio of the H-chain peak heights of the young animals to the heights of the corresponding peaks in a 6 mo. old control. Absence of a peak was indicated by a minus (-); peaks up to 1/3 of adult peak heights were termed (+); peaks of 1/3 to 2/3 of adult peak heights were termed (++); and adult levels were termed (+++). Table IV gives the results expressed in these terms. Spleen cells from animals of all ages tested had demonstrable IgM. Adult levels were reached by 1-2 weeks of age. IgD, however, was not demonstrable until ~3.5 weeks of age while adult levels were attained by 6 weeks of age. These data suggest an ontogeny of rat IgD similar to that of the human, with IgM appearing as the first detectable surface Ig.

Genetic Studies: Recent reports by Goding, et al. (1976), have suggested that an anti-allotype antisera raised in mice by allograft immunization could precipitate a molecule with mouse IgD-like molecule properties when the precipitate was reduced and electrophoresed similar to the technique employed in this study. Their data suggested that IgD in the mouse was not detectable in all strains with this antisera and therfore IgD in the mouse

TABLE IV

ONTOGENY OF CELL SURFACE IMMUNOGLOBULINS IN THE RAT

Age	IgM	IgD
6 days	+	-
13 days	+++	-
25 days	+++	+
42 days	+++	+++
6 months	+++	+++

- = not detectable

+ = slightly detectable

++ = moderately detectable

+++ = readily detectable at or near adult levels

may express allotypy. To determine if chicken anti-δ could detect an allotypic variation or, alternatively, a complete lack of IgD in certain strains, rats of varying histocompatability haplotypes (Ag-B loci) were assessed by radioelectrophoresis for the presence of IgD cell surface isotype. The results, presented in Table V suggest that no linkage of histocompatability haplotypes with IgD expression exists in the rat, since rats of Ag-B types 1-6 all revealed detectable δ chain on their surface. This data also implies, that should rat IgD express genetic polymorphism, the chicken anti-δ used here is class, rather than allotype specific, or, optionally, that all strains tested express the same allotype. Splenocytes used in this study were taken from animals at least 6 months of age and represented a pool of cells isolated from 3 animals of each strain. The cells were labelled and treated in a similar manner throughout the experiment. No significant differences were seen in the levels of IgM or IgD detectable on the surface of the inbred strains relative to outbred Sprague-Dawley splenocytes run in parallel.

Reelectrophoresis of δ chain peak. To determine the reproducibility of δ chain electrophoretic migration, as well as the integrity of the polypeptide chain, the δ chain peak, isolated by electrophoresis of the anti-δ precipitate of a labelled adult Sprague-Dawley splenocyte lysate, was reapplied to a second 10% polyacrylamide SDS gel and reelectrophoresed. The initial gel was sliced as in

TABLE V

CELL SURFACE IGD AND IGM : RELATIONSHIP TO STRAIN VARIATION AND AG-B LOCUS

Strain	Ag-B haplotype	SDS-PAGE Peaks Observed Fc(2)	μ	δ_1	δ_2	L
Sprague-Dawley	1,4,6(1)	+	+	+	+	+
Fischer (F344)	1	+	+	+	+	+
Wistar-Furth(W/fu)	2	+	+	+	+	+
Brown Norway	3	+	+	+	+	+
Copenhagem	4	+	+	+	+	+
August	5	+	+	+	+	+
M520	6	+	+	+	+	+

+ = detectable as a discrete ^{125}I labelled peak by SDS-PAGE

1) Sprague-Dawley rats may apparently be AgB 1,4, or 6

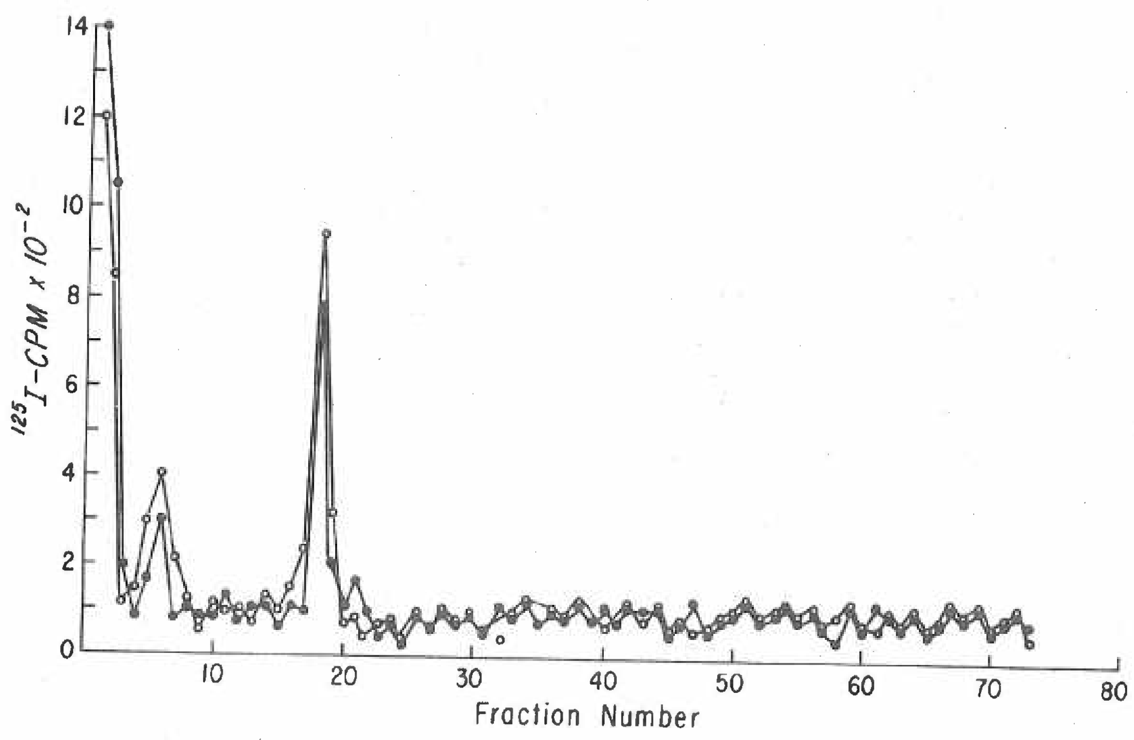
2) Fc = "Fc receptor-like" protein observed on SDS-PAGE as ~35,000 dalton peak.

previous experiments and the radiolabelled fractions collected in 1% SDS phosphate buffer (gel chamber buffer) with 1 mm phenylmethylsulfonyl fluoride (PMSF). The δ chain peak (fraction 18) was allowed to stand at 25 C. for 1 hr. to extract labelled material from the polyacrylamide gel. The polyacrylamide was then pelleted at 2500 at 4 C. for 15 minutes and the supernatant counted, mixed with tracking dye and glycerol, and applied to the second gel. The results seen in Figure 7 indicate that δ chain material remains a discrete, recoverable polypeptide chain during and after electrophoresis, and that the molecular weight, in the presence of 1 mM PMSF, does not appreciably change during this time. A parallel reelectrophoresis of rat μ chain gave identical results, and confirms the conclusion that rat μ and δ chains are discrete peptide chains with identical molecular weights. Since approximately 3000 CPMs of labelled μ chain and 2200 CPMs of labelled δ chain were applied to the secondary gels, approximately one third of the counts were recoverable as peaks in the second gels. The remainder of the counts may be accounted for as aggregated material in the first 5 fractions of the gels.

<u>Molecular Weight of Unreduced Cell Surface IgD</u>. Splenocytes from two 6 month old Sprague Dawley rats were labelled, lysed and precipitated with anti-δ or anti-μ as in the previous experiments. The washed precipitates were dissolved in 8M urea-SDS sample

Figure 7:

SDS-PAGE Electrophoretogram: Reelectrophoresis of rat splenic δ and μ chains.



^{125}I-CPM x 10^{-2}
14
12
10
8
6
4
2
0
10
20
30
40
50
60
70
80
Fraction Number

buffer (nonreducing sample buffer) and loaded on 4.25% polyacrylamide SDS gels. The results are expressed in Figure 8. Lysates precipitated with anti-μ antisera generated 2 peaks on SDS-PAGE corresponding to approximately 400,000 and 195,000 daltons while anti-δ precipitates electrophoresed as 2 labelled peaks at approximately 400,000 and 185,000 daltons. This first peak in both cases probably represents an aggregated (dimeric) form of the intact surface Ig based on its apparent molecular weight and on the observation that both bovine serum albumen and phosphorylase A marker proteins dimerized to some degree under these same circumstances on parallel gels. No major ^{125}I peaks were observed below 150,000 daltons in either case.

Precipitation of Cell Surface IgD with Rabbit anti-rat Membrane IgD. Absorbed rabbit anti-rat membrane IgD was tested for reactivity against labelled rat surface immunoglobulins by radioelectrophoresis. The lysates of radiolabelled Sprague-Dawley splenocytes were precipitated with rabbit anti-rat membrane IgD obtained following primary and secondary immunizations. 13 μl of the absorbed antisera were added to 0.2 ml. of the lysate and the complexes coprecipitated with excess GaRGG (200 μl). The peaks obtained with the rabbit anti-δ was compared with C anti-δ generated electrophoretic profiles obtained under identical conditions (Figure 9). The anti-rat δ chain antisera obtained from 2 of 3 immunized rabbits generated an electrophoretic profile

Figure 8:

SDS-PAGE Electrophoretogram: Intact, unreduced rat splenocyte surface IgM and IgD.

closed circles = IgM

open circles = IgD.

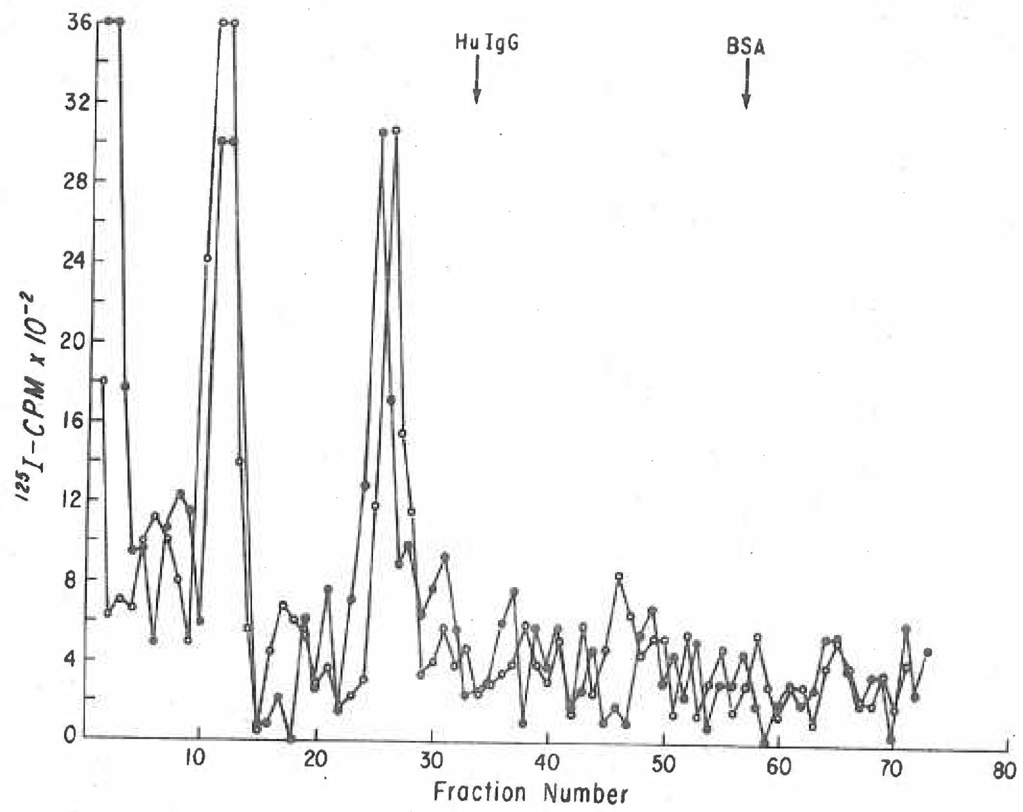
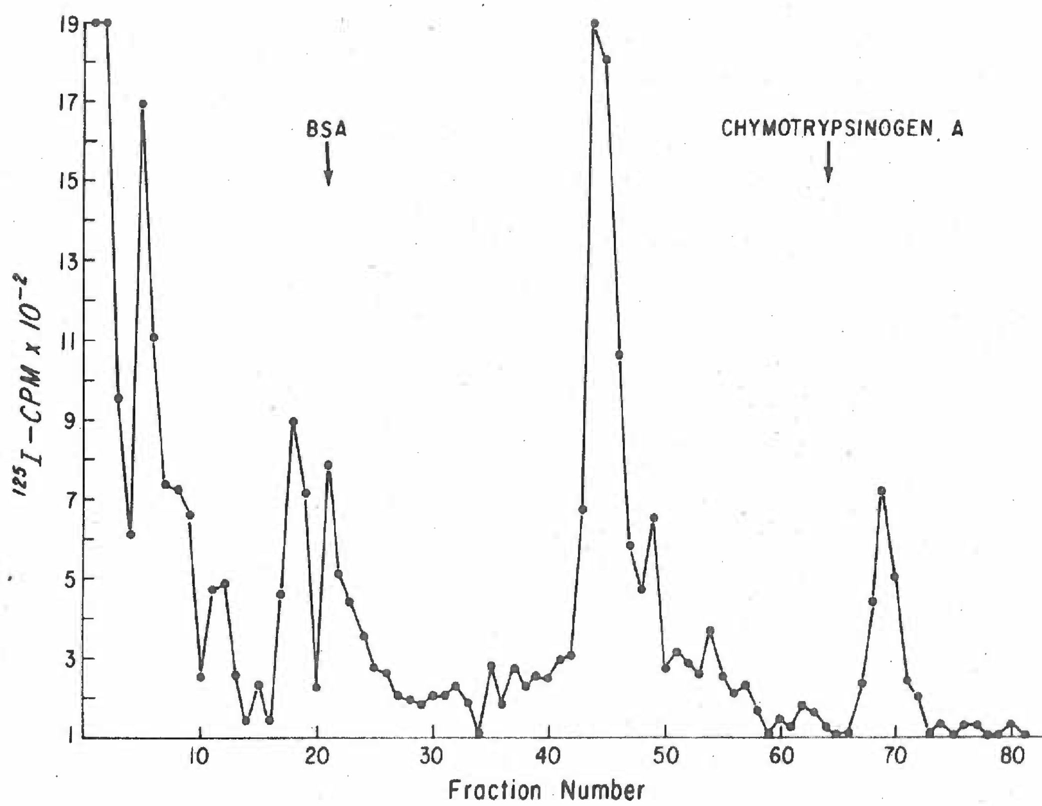

Hu IgG
BSA
^{125}I-CPM x 10^{-2}
Fraction Number
36
32
28
24
20
16
12
8
4
0
10
20
30
40
50
60
70
80

Figure 9:

SDS-PAGE Electrophoretogram : Precipitation of rat lymphocyte IgD with rabbit anti-rat membrane bound IgD.



BSA
CHYMOTRYPSINOGEN A
^{125}I-CPM x 10^{-2}
Fraction Number

virtually superimposable on the C anti-δ profile . Although all peaks were significantly lower than the C anti-δ peaks, only δ chain, Fc receptor, and L chain peaks were present and occurred in the same fraction as the corresponding peaks generated with C anti-δ. It is of interest that both the major δ chain peak and the δ chain shoulder appeared with all of the rabbit anti-rat IgD antisera in which H chain peaks were observed.

Anti-μ and Anti-δ In Vivo Administration. To determine if rat cell surface IgM and IgD may serve receptor functions and if these receptors generate heterogeneous signals to the cell bearing them which might result in different expressed functions of the same cell, anti-class specific immunoglobulin was injected, intraperitoneally, into neonatal rats. Since Lawton and Cooper, (1974), have given evidence for anti-μ chain mediated suppression in mice, it was of interest to see if such suppression could be extrapolated to the rat and to ascertain what effect, if any, anti-δ antibody would have on Ig production or lymphocyte development in this species.

To investigate this matter, animals were injected as described (see Materials and Methods, Table I). 6 of 8 rats survived the anti-μ treatment, 8 of 8 survived the anti-δ treatment, while all control animals survived. Sera from the 18 survivors were tested by radial immunodiffusion for quantitation of humoral immunoglobulins.

Of these 18 animals, 12 were selected for analysis of their cell surface Igs and 6 of these were selected for histological examinations. The results are indicated in Table VI. The serum immunoglobulin levels are expressed as mg/ml for IgM, IgG_2c and IgG_2a and as the diameter of the precipitin rings in mm for IgA. Detection of IgA by double immunodiffusion against anti-rat IgA is also noted while surface immunoglobulin peak heights are in counts per minute. δ_1 refers to the major δ chain peak whereas δ_2 refers to the secondary δ chain shoulder seen in gel fractions 20 - 21. μ represents the μ chain peak height from a parallel electrophoresis of IgM precipitated from an equal volume of the same lysate.

The anti-μ treated animals (nos. 4-8) had notably altered serum immunoglobulin levels as compared to the control group (#1-3). Animals #5 and 6, were virtually agammaglobulinemic with the exception of the low levels of IgG detected, which may be of maternal origin. Since the IgA ring diameters in these animals approach the diameter of the serum wells, this group demonstrates a significant reduction of IgA levels as compared to controls. The humoral Ig levels of the anti-δ treated group,however, did not differ significantly from the control levels. Concomittantly, cell surface immunoglobulins in the three groups showed significant variations, with all but one of the anti-μ treated group displaying decreased or undetectable levels of μ chain on their surfaces with

TABLE VI

SERUM IMMUNOGLOBULIN LEVELS AND CELL SURFACE IGD AND IGM LEVELS FOLLOWING IN VIVO ANTI-IG ADMINISTRATION

Animal #	Anti-Ig Treat.	Day(5) sacrif.	Humoral Ig (1)						Cell Surface Ig (2)					
			IgG2c	IgG2a	IgM	IgA	IgA	IgG	δ1	δ2	μ	Fc	L	δ/μ
1	NCS-IgY	ND (4)	0.46	2.30	1.15	4.5	+(3)	8.9	ND	ND	ND	ND	ND	-
2	NRabS	9	0.33	1.70	1.80	5.8	+	9.2	1858	1277	4065	1367	2111	0.46
3	PBS	2	0.66	3.00	2.50	5.3	+	9.0	2715	2533	5152	3575	1865	0.53
4	anti-μ	2	0.00	0.80	<0.10	3.2	-	5.8	1661	-	1376	2200	1300	1.21
5	"	2	0.00	0.13	<0.10	3.1	-	3.7	1994	1448	1260	2446	1201	1.58
6	"	2	0.00	0.19	<0.10	3.2	-	3.6	2119	-	1281	2181	1400	1.65
7	"	9	0.00	1.55	<0.10	3.0	-	5.5	1226	1080	880	2030	1095	1.39
8	"	9	0.12	2.30	1.5	3.5	trace	6.6	1520	-	3936	1610	1596	0.39
9	anti-δ	2	0.00	3.00	0.57	5.7	+	9.0	4881	2915	5412	4469	1924	0.90
10	"	2	0.35	2.05	0.30	5.0	+	6.5	4295	2385	4517	3206	2505	0.95
11	"	9	0.63	1.70	0.80	5.9	+	8.0	1623	1073	5824	1595	2346	0.28
12	"	9	0.23	3.40	0.70	5.2	+	8.4	1236	1108	5858	2194	2024	0.21
13	"	9	0.23	1.60	0.88	5.8	+	7.0	1531	1279	5398	2155	2518	0.28

NOTES:
1) Humoral Ig levels expressed as mg/ml for IgM, IgG2c, and IgG2a, and as radial immunodiffusion ring diameters (in mm.) for IgA and Ig.
2) Cell surface Ig levels expressed as ^{125}I CPMs of corresponding SDS-PAGE peak
3) - + not detectable; + = detectable by double immunodiffusion
4) ND = not done
5) Days between last anti-Ig injection and cell surface Ig analysis

All rats were approximately 40 days of age at the time of the last anti-Ig injection.

little decrease in the amounts of δ chain found concurrently on these cells. The anti-δ treated animals, however, had normal levels of IgM on their surfaces while the IgD receptors on these cells appeared to be increased soon after the termination of anti-δ treatment (#'s 9 and 10) but returning to near, or slightly lower than normal values within 9 days (#'5,11, 12 and 13).

The δ/μ peak height ratios have been calculated and represent the ratio of δ_1/μ . It can be seen that the ratio is approximately 0.50 for the control group regardless of the time interval between antiglobulin treatment and cell labelling whereas δ/μ ratios in anti-μ treated animals, with one exception, were considerably higher than controls. This anti-μ treated group showed δ/μ ratios of approximately 1.46 even as late as 9 days after the last injection. Anti-δ treated animals were notably different in respect to δ/μ ratios immediately after or following the 9 day rest. The earlier group showed an elevated ratio (0.93) whereas the latter group had a somewhat depressed δ/μ ratio (0.26) as compared to the control group. Histological examination of Peyer's patches revealed very little conclusive difference between normal, anti-μ treated or anti-δ treated animals. Splenic tissue however showed the differences observed in Figure 10. The anti-δ treated spleen, while only slightly larger[1] than control or anti-μ treated tissues, demonstrated large, well developed follicles, however these follicles lacked germinal centers, which were readily

[1]Determined by subjective visual inspection.

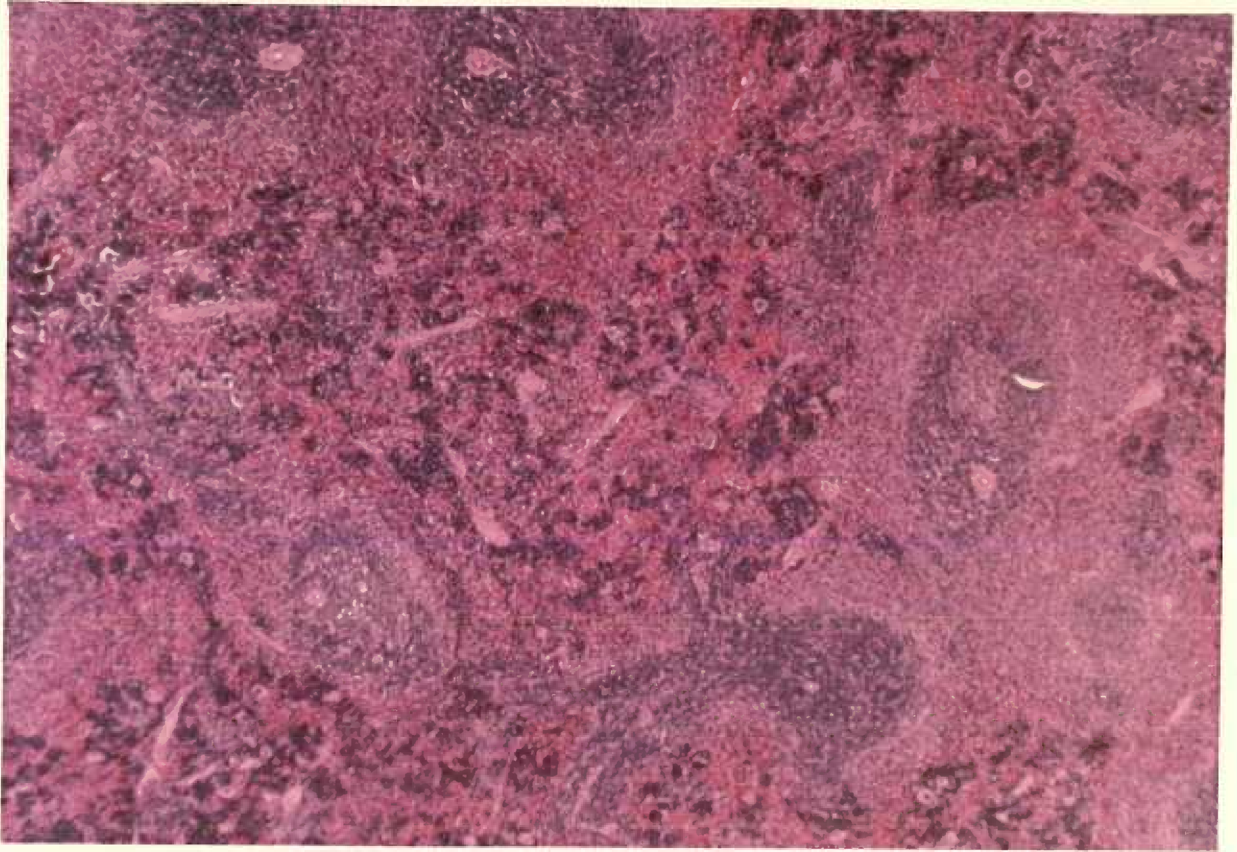
Figure 10 :

Histology of anti-δ treated, anti-μ treated, and control rat splenic tissues. (Hematoxylin and eosin, approx. 40 X)

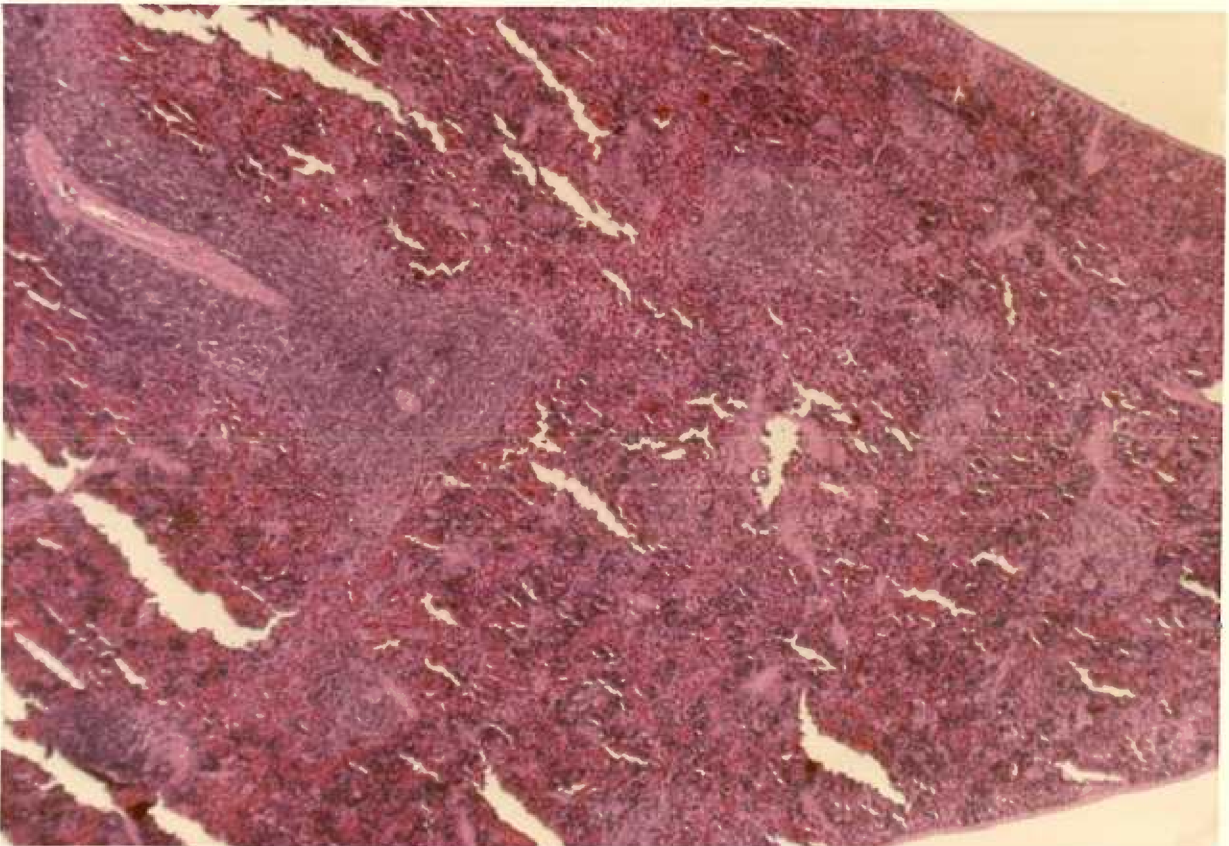
10a) control spleen

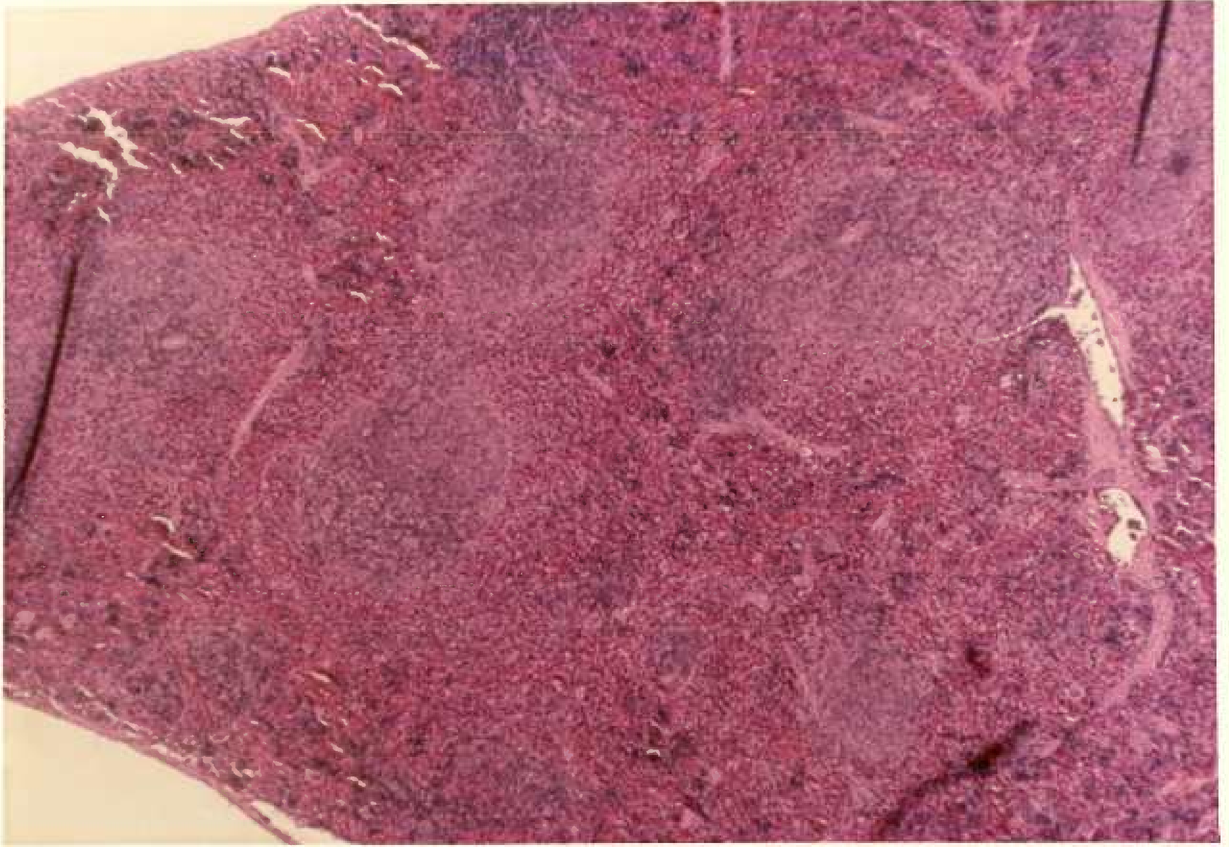
10b) anti-μ treated spleen

10c) anti-δ treated spleen



10A↑ 10B↑





10C

apparent in control animal tissue and should be readily seen following antigenic stimulation with chicken immunoglobulin. Periarteriolar lymphatic sheath regions were unaffected in all groups. Anti- treated animals, however, revealed splenic tissue in which few follicular structures could be observed and in which unorganized lymphatic tissue regions were considerably lymphopenic. Marginal zones (between organized and unorganized lymphatic regions) were considerably reduced in anti-μ treated animals. Lymph nodes from untreated or anti-μ treated rats were comparable, however anti-μ treated nodes displayed a general diminution of lymphatic elements suggestive of B cell depletion. Peyer's patches were counted on the entire length of the intestinal tract from the duodenum to the terminal ileum. The average number of Peyer's patches on the control group was 6.66 (range,6-7) while anti-μ and anti-δ treated animals averaged 3.66 (range, 1-6) and 21.50 (range 17-27), respectively. This increase in Peyer's patch number in anti-δ treated animals was not reflected in the histology of these lymphoid tissues, however, since Peyer's patches from treated or untreated animals had comparable size and lymphatic organization with follicular structures being readily apparent in the lamina propria of these sections.

Discussion

This research was designed to investigate the nature of lymphocyte surface Igs in the rat with special consideration to the possible existence of IgD as a major cell surface Ig in this species. The results described above indicate that the surface Ig pattern in the rat clearly resembles that of the human as described by Finkelman et al. (1976) and that the δ chain present on these lymphocytes is antigenically homologous with human δ chain. This is the first definition of IgD in a nonprimate species using primary antigenic criteria, and therefore confirms the possibility that IgD emerged phylogenetically prior to the appearance of hominids (Spiegelberg, 1975; Terhorst, et al. 1976). The approach used to detect cell surface IgD was based on the ability of lower vertebrate species (i.e., chickens) to detect antigenic determinants that are shared among various mammalian species, and may, therefore, be excluded from another mammal's antigenic recognition potential (Orlans & Feinstein, 1971). To augment this inherent cross reactivity, birds were hyperimmunized to stimulate antibody degeneracy and thereby accentuate cross reactivity (Eisen, et al., 1969; Little and Eisen, 1969). The salient points investigated in this theses include: 1) the detection and characterization of rat IgD, 2) the tissue distribution and ontogenic emergence of rat IgD and, 3) the effects of anti-δ and anti-μ administration, *in vivo*.

Specificity of Chicken Anti-δ. Although C anti-δ antisera was raised with all due consideration to preparing a highly cross reactive population of anti-δ antibodies, these antisera remained monospecific for δ chain following absorption and purification. The monospecific chicken anti-δ antibody did not however, react with NRS by radioimmunoassay suggesting that either humoral IgD does not exist, *per se*, in the rat, or does so only in very minute quantities or under rare circumstances. These data are in agreement with Neoh, et al., (1973), who were unable to detect IgD in the rat by immunodiffusion, and with Abney et al. (1976) who failed to detect humoral mouse IgD. This lack of reactivity with the sera of rats bearing IgD on their lymphocytes is noteworthy since humoral IgD was readily detectable in dogs and primates with this antiserum. It is interesting to speculate that the inverse relationship between human humoral IgD levels and the percentage of IgD bearing lymphocytes (Leslie, Lopez-Correa & Holmes, 1975; Kermani-Arab, Berger & Leslie, 1976), may exist not only at the level of the individual, but perhaps also at the species level.

Rat Cell Surface Ig. It has been claimed (Williams, 1975), that IgG is detectable on the surface of murine lymphocytes. The anti-L chain precipitation electrophoretogram presented here (Figure 1) suggests that, if IgG exists on rat lymphocytes it represents only a minor class of surface Ig in the spleen and is

undetectable by the techniques employed here. This conclusion was also reached by Parkhouse, Hunter and Abney (1976), who suggested that IgG was undetectable on murine splenocytes and by Melcher et al. (1974) who determined that only 7% and 10% of the counts incorporated into the anti-L chain precipitable ^{125}I labelled cell surface protein were precipitated with anti-α and anti-γ reagents, respectively. These counts could not be detected as discrete peaks on SDS-PAGE, however. Rat surface Ig is restricted to two H chain peaks implying that only 2 classes of Ig, both with H chain molecular weights greater than γ or α are present. It is possible that detection of γ and α bearing lymphocytes with fluorescien conjugated antiglobulin reagents can detect very low percentages of cells bearing a high density of γ or α whereas the radioelectrophoretic technique used here does not resolve the presence of isotypes that are rare in the total cell surface Ig population.

Rat Cell Surface IgD Precipitation of cell lysates with anti-δ produced a major peak comigrating with μ chain on SDS-PAGE as well as a lower molecular weight shoulder. This peak was detected on all inbred and outbred rats suggesting no linkage to histocompatibility loci, or anti-allotype activity in the C anti-δ used. Both the 73,000 dalton major peak and the 65,000 dalton shoulder could be specifically recovered from an IgM depleted cell lysate, suggesting that both peaks bear

characteristic IgD determinants. The antigenic distinction of both peaks from IgM is confirmed by the reverse experiment, namely recovery of IgM from an IgD depleted lysate. A similar reprecipitation pattern was seen by Finkelman, et al., (1976) in the human where μ and δ chains also comigrate. Advancing this concept is the absorption of anti-δ activity with unlabelled human IgD but not with rat IgM. Although the possible presence of an undetected contaminant present in the human IgD preparation but not in the rat IgM preparation is not excluded, since this contaminant is more likely to be present in human IgD and IgM than in rat IgM. The 65,000 dalton δ chain peak may result from either proteolytic degradation of the 73,000 dalton δ chain as suggested by Fu and Kunkel (1974) and Jensenius and Williams, (1974a), or by molecular rearrangement as suggested by Melcher and Uhr (1976). It is uncertain which of these mechanisms (or both) is creating the δ chain shoulder in the rat, although reelectrophoresis of the major δ chain peak did not result in the production of a biphasic peak in the second electrophoretogram. This suggests that the generation of the δ chain shoulder probably occurs during isolation from the membrane, and the proteolysis and/or rearrangement does not occur appreciably after this time. This type of molecular weight reduction upon isolation may be due to an additional homology region or peptide fragment on membrane bound Ig. The findings of Kennel and Lerner (1973) have demonstrated that human membrane bound IgM has a greater molecular weight on SDS-PAGE than 7S

IgM derived from serum IgM by partial reduction, perhaps due to an additional region embedded in the membrane, as suggested by Fu and Kunkel (1974). This additional fragment may be readily dissociable from surface IgM or, optionally, may have hydrophobic structures that account for a significant change of conformation resulting in a decreased apparent molecular weight. This may be the cause of the lower molecular weight of murine membrane IgM as opposed to serum IgM as noted by Jensenius and Williams (1974a). Results from this study however, suggest that these changes do not occur in intact, unreduced rat cell surface IgD or IgM, since the molecular weights of these molecules paralleled human serum IgD (Leslie, Clem, and Rowe, 1971), and rat serum IgMs (Jensenius and Williams, 1974a) at 185,000 and 195,000 daltons, respectively. The ratio of rat δ chain peak heights to the corresponding L chain peak heights warrants some discussion. Melcher and Uhr (1976) have shown that δ/L ratios in the mouse are approximately equal to μ/L or total H chain/total L chain ratios, implying that surface IgM and IgD have the same polypeptide chain structures as well as labelling efficiency. Finkelman, et al., (1976) have shown in the human, however, that the δ/L ratio is significantly higher than the μ/L or H/L peak height ratios. Both patterns had been noted in the course of this study, with the δ/L ratio, more often than not, being higher than the μ/L ratio. It must be concluded however that this decrease in associated L chain labelling is due to an alteration of the labelling efficiency

of this chain rather than an absence of δ associated L chain since anti-L chain electrophoretograms show that anti-L chain does precipitate rat IgD.

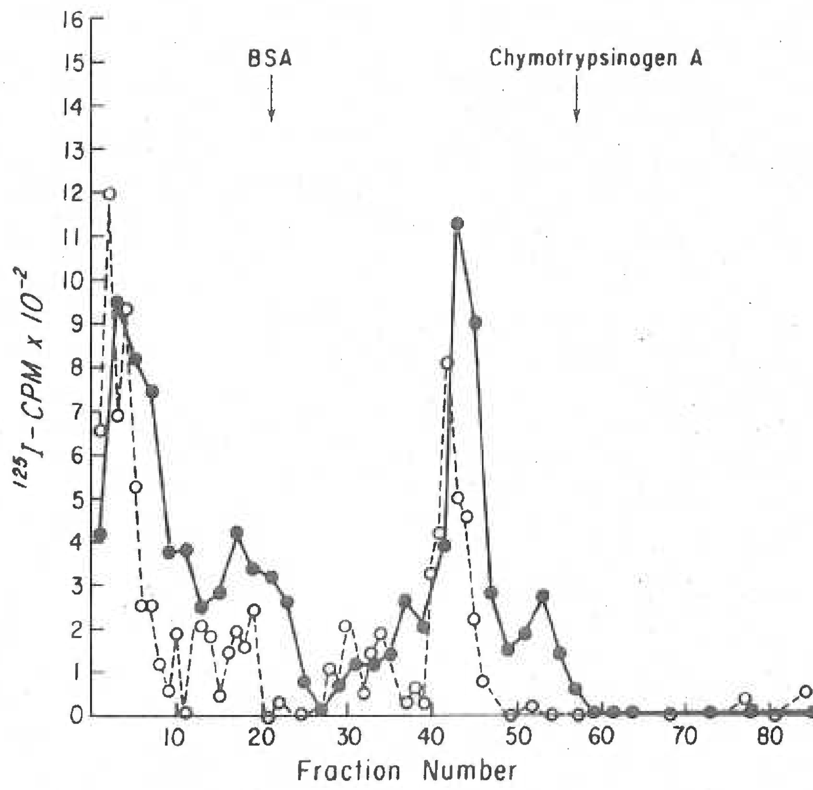
There are several other peaks on the 10% PAGE-SDS electrophoretogram that warrant discussion at this time. The first is a cluster of peaks of varying heights that precede the H chain region of the gels (fractions 1-15) and the second, the very pronounced peak around fraction 40. In our hands the material running before and after the δ and μ chain peaks could also be detected in non-immune precipitates (i.e. NRS, NCS, Figure 3). This high molecular weight material has been reported by Abney, Hunter and Parkhouse (1976) and Haustein, et al, (1974) and probably represents an aggregated form of surface Ig. The second peak (~fraction 40) was seen in all gels and may represent an Fc receptor in the rat. The apparent molecular weight of 30 - 35,000 daltons is in agreement with that of mouse Fc receptors (Rask, et al., 1975). NCS or NRS plus second antibody generated precipitates in which only this peak was apparent. A similar conclusion has been reached by Vitetta, Grundke -Iqbal, Holmes & Uhr (1974), and Anderson (1974). These studies also demonstrated that the Fc receptor was detectable on the surface of thymocytes and since Ig was not co-detectable, (Figure 11) suggested this finding was probably due to the presence of Fc receptor on T cells and not due to B cell contamination. The molecule that we have observed on T and B cells also appears quite

Figure 11:

SDS-PAGE Electrophoretogram: Anti-δ and anti-μ precipitation of a labelled rat thymocyte lysate.

solid lines = anti-μ

dotted lines = anti-δ



BSA
Chymotrypsinogen A
^{125}I-CPM x 10^{-2}
Fraction Number

similar to that described by Stout and Herzenberg (1975) and Santana and Turk, (1975) on murine T cells, but definition of this peak as a true Fc receptor is not possible at this time. Although the molecular weight of this putative Fc receptor is also in close agreement with that of murine Ia antigens (Vitetta, Klein, and Uhr, 1974), it is doubtful that a specific reaction between the anti-Ig reagents and a rat Ia antigen is taking place since 1) Ia antigens are present in serum and C anti-δ did not react with NRS by double immunodiffusion and 2) all anti-Ig reagents precipitated this peak suggesting that all the antisera used here would have to have anti-Ia activity.

To cast doubt on the possibility that C anti-δ contained a population of anti-carbohydrate antibodies that may cross react with IgM and/or other cell surface components (Merler, et al. 1974), the C anti-δ was absorbed with fetuin. This glycoprotein has been shown to react with a significant number of normal chicken sera (Sela, Wang and Edelman, 1975). This absorption suggests that these cross reactive anti-fetuin specificities do not exist in the C anti-δ antibody used in this study, although other anti-carbohydrate reactivities are not precluded by this absorption. To further assess the nature of rat IgD, an attempt was made to raise a rabbit anti-rat membrane IgD antiserum. As seen in Figure 10, the absorbed anti-rat δ was capable of precipitating δ_1,δ_2, and L chain peaks similar to C anti-δ. This antiserum not

only reacted in radioelectrophoresis with labelled rat splenocyte lysates but also reacted with normal and myeloma derived human IgD by immunoelectrophoresis and double immunodiffusion. No reactivity with rat IgM, IgG, or NRS was seen however. The implication of these results is that not only can rat IgD be distinguished from IgM experimentally, it can be differentiated _in vivo_ as an antigenically distinct moeity capable of eliciting an immune response in rabbits. This further implies that, should rabbits also have IgD, their IgD most probably bears determinents which are antigenically distinct from those seen in the rat. The ability of rabbits to respond to rat IgD poses an interesting insight into the antigenic relationship between rat δ chain and the hypothesized rabbit δ chain. While the rabbit can respond to both human and rat δ chains, rabbit anti-human δ chain fails to react with rat δ chain implying that the determinants that differ between rat and rabbit are distinct from those differing between human and rabbit, whereas some of the human determinants detected by the chicken are shared between both the rat and the human explaining the cross reactivity seen between rat and human IgD. Alternatively, certain membrane structures could be associated with rat IgD isolated from lymphocyte surfaces, providing an adjuvant or "carrier" effect capable of allowing a response to cryptic determinants on rat δ chains which are common to human δ chains, but not immunogenic in the absence of this carrier.

Tissue Distribution and Ontogeny of Rat IgD. Rat IgD was detected in all lymphoid tissues analyzed with the exception of thymus. These results are in good agreement with Vitetta, Melcher, McWilliams, Lamm, Phillips-Quagliata, & Uhr (1975), who concluded that murine IgD was not present on thymocytes or bone marrow cells, and with Santana, et al., (1976) and who concluded that a high molecular weight protein on thymocytes was not IgM or IgD. The absence of both IgM and IgD from T cells demands some qualification since others (Feldmann, et al., 1973; Jensenius & Williams, 1974,b; Haustein, et al., 1974) have detected IgM on these cells. This effect has been attributable to "hidden" immunoglobulins (Grey, Kubo & Cerottini, 1972) and the differences these authors noted with different extraction procedures may reflect the cause of the undetectability of T cell IgM noted by Vitetta, Bianco, Nussenzweig and Uhr (1972), and this study, or, optionally, the anti-IgM used in other studies may be reacting with a non-IgM carbohydrate (Merler, et al., 1974). It is of interest that IgD appears more frequently in tissues that are more distal to the spleen. This could be indicative of the maturity of the distal cells (Goodman, et al., 1975). These cells also seem to require a greater degree of T cell help for production of Ig, with the spleen (low δ/μ ratio) producing largely IgM whereas lymph nodes (intermediate δ/μ ratio) produce IgG as their major product (Parkhouse, 1973). Peyer's patches, with the highest observed δ/μ ratio, produce IgA primarily, (Jones and Cebra, 1974) sugges-

ting a possible relationship of δ/μ ratios to the class of Ig secreted.

In keeping with the concept of IgD denoting a mature population of cells, is the appearance of IgD following IgM ontogenically. This finding is in accord with human (Gupta, et al., 1976) and murine (Vitetta, Melcher, McWilliams, Lamm, Phillips-Quagliata & Uhr, 1975; and Abney and Parkhouse, 1974) developmental sequences. Since the presence of IgM and IgD on the same cell in murine lymphoid tissue has been shown (Vitetta and Uhr, 1976) and that the relative densities of these Igs on cells shifts from cells bearing only IgM to those bearing primarily IgD during normal development (Scher, Sharrow, Wistar, Asofsky & Paul, 1976) it can be suggested that the density of cell surface IgD relative to IgM may qualitatively determine the type of immune response of which these cells are capable.

In support of this concept is the finding of Bruyns, et al., (1976) that early murine B lymphocytes (up to 10 days of age) do not respond to anti-immunoglobulin or antigen stimulation. Furthermore, following exposure to these agents, the cells fail to reexpress surface immunoglobulin and can be specifically tolerized by antigens at this time. These cells, which should express mainly IgM, are similar to the cells of adult CBA/N mice. These mice have abnormally low amounts of IgD on their surface relative to IgM (Finkelman, et al., 1975) and are capable of producing a

T-dependent response to a variety of antigens,although they do not respond to pneumococcal (type III) polysaccharide, a T-independent antigen (Amsbaugh et al., 1972). Cambier, et al., (1976) have suggested that B cell tolerance is due to IgM receptor stimulation in neonates since adults are less easily tolerized while Vitetta, Forman & Kettman (1976) have suggested that IgD receptor stimulation results in proliferative responses.

These data, taken cumulatively, may indicate that IgD exists on mature lymphocytes, some of which carry immunologic memory (reviewed in Strober, 1975). Memory B cells occupy the same anatomical niches as do cells with high δ/μ ratios, although a direct correlation with this function has not yet been obtained. It should be noted here that while IgD has only been detected in mammals, and therefore appears to be mature by phylogenetic criteria, it has been suggested (Terhorst, et al., 1976; and Speigelberg, 1975) that IgD evolved prior to the emergence of mammals, although IgD probably evolved from IgM around 200 - 300 Myr. ago thereby maintaining its phylogenetically late emergence in comparison to IgM.

Although the simultaneous expression of 2 Ig classes on the same cell has implications in "switch" and V-C gene integration mechanisms, the genetics of this phenomenon are relatively unstudied. However, since both δ and μ are known in some cases to share the same V region (Salsano, et al., 1974) this could indicate the simultaneous

presence of 2 integrated genes (i.e. VH-Cδ & VH-Cμ). This concept is in agreement with the copy-choice mechanism of Williamson (1971), and based on a case of double paraprotein expression in multiple myeloma (TIL). This concept allows integration of VH genes into all possible CH gene "backbones" simultaneously. The receptor specificities would, therefore, remain identical, while phenotypic expression of 1 or more H chain isotypes with this specificity is possible depending on the environmental stimuli responsible for this "choice".

Anti-δ and Anti-μ Administration. The concept of signal heterogeneity elicited by either IgD or IgM receptor stimulation was examined by the *in vivo* administration of anti-δ or anti-μ. The results suggest that while anti-δ has no effect on humoral immunoglobulin synthesis, it may have a significant bearing on cell differentiation. It has been demonstrated in chickens (Kincade, et al., 1973; Leslie and Martin, 1973a) and in mice (reviewed in Lawton and Cooper, 1974) that neonatal anti-μ injections result in a suppression of Ig synthesis and that the nature and duration of this suppression is vitally dependent on the dose, route, and timing of anti-μ administration (Lawton et al., 1973). This research has provided data indicating similar suppressions in the rat suggesting that B cell differentiation in this species parallels the mouse. It is of interest that anti-μ suppressed mice reject allogeneic skin grafts as rapidly as do normal controls (Manning

and Jutila, 1972) indicating normal T cell function. Suppressed animals also have a profound decrease in Ig bearing splenocytes as detected by fluorescence (Lawton and Cooper, 1974) and these spleens have a mean weight about 1/2 that of control spleens. Of merit to this report is the extreme rarity of germinal centers and primary follicles in anti-μ suppressed spleens, (Lawton, et al., 1972) as well as a diminuition of Peyer's patch differentiation and numbers (Lawton and Cooper, 1974).

Prior to this study, no reports of in vivo anti-δ administration had been recorded, with the exception of the recent finding of Pernis (1975) that anti-human δ administration to monkeys resulted in a 5-10 fold increase of serum IgG. Adoption of the anti-μ protocol for anti-δ administration generated quite interesting histological and surface Ig pattern results not previously reported. The δ/μ ratio for untreated rats (Table VI, animals 1-3) was approximately 1/2 that of anti-δ treated rats immediately following anti-δ treatment(table VI, animals 9 & 10), implying an increase in splenic IgD receptors. This pattern reversed rapidly however, and after 9 days, a rapid decrease of δ chains had taken place resulting in a δ/μ ratio of almost 1/2 the control level (table VI, animals 11-13). This may be indicative of an anti-δ mediated proliferative response, the transient nature of this proliferation being anticipated due to clearance of anti-δ in vivo. The decrease in the δ/μ ratio after 9 days may reflect depletion of δ bearing cells through recruitment to other tissues, turnover, or compensatory IgM receptor proliferation. Anti-μ treatment

however caused a decrease in the amount of both δ and μ chains on the cells (table VI, animals 4-6) and generated a δ/μ ratio almost 3 times that of control animals. With the exception of 1 animal, which was clearly not suppressed humorally (table VI animal 8), this pattern remained unchanged after 9 days. Since anti-μ treatment decreased all surface Ig levels whereas anti-δ did not affect surface IgM appreciably, and while anti-μ concurrently decreased serum Ig levels while anti-δ did not, it is of interest to suggest that anti-μ treatment may suppress all surface Ig expression, and therefore may be considered tolerogenic, whereas anti-δ may result in IgD bearing cell proliferation of a transitory nature. This suggestion is consistant with Vitetta, Forman, & Kettman (1976) and Kermani-Arab, Leslie, & Burger, (1976) who showed that IgD receptor stimulation can lead to a proliferative event. It is not unreasonable to propose that this event may subsequently lead to Ig synthesis as suggested by Pernis (1975) although elevated Ig levels were not seen in the anti-δ treated animals in this study and *in vitro* suppression of Ig synthesis by anti-δ has been reported by Van Boxel, et al., (1976). In support of anti-δ mediated B cell proliferation is the finding of an increase in splenic follicles (Figure 10,c), as well as the number of visible Peyer's patches. Since these areas are B cell-dependent, their proliferation could be expected if anti-δ is indeed stimulatory to B cells. It is doubtful that this suggested, anti-δ mediated B cell proliferation is due to antigenic stimulation attributable to chicken immunoglobulin since histological examination

of anti-δ treated spleens revealed no germinal centers in this tissue, suggesting a lack of specific B cell clone stimulation. The lack of a similar effect in lymph node or Peyer's patch tissue may be due to relationships between these organs and the route of anti-globulin administration used in these experiments. Unfortunately, no studies were performed to assess T cell function in these animals, and the possibility exists that the lack of a histological response by these animals to chicken immunoglobulin may be due to a functional T cell destruction or recruitment to another anatomical site as suggested by Kermani-Arab, Leslie & Burger, (1976), or by a regulatory effect on T cell function mediated by anti-δ (Kermani-Arab, Burger & Leslie, 1976).

The mechanism of anti-Ig suppression or activiation is not fully understood (reviewed in Lawton & Cooper, 1974) although there is evidence that complement need not be involved since $F(ab')_2$ (of anti-μ) suppression of chicken Ig ontogeny did not appreciably differ from intact IgG anti-μ (Leslie and Martin, 1973a). This suggests that complement mediated cytotoxity is not the cause of the results observed in this study. An alternative proposal may involve the generation of suppressor T cells directed against membrane bound IgM (Grebenau, et al., 1976). In this theory, IgM is sequestered from recognition as self by anti-μ administration. Autoimmunization subsequently occurs with suppression being mediated by T cells. This type of suppression is very similar in nature

to that seen in infectious agammaglobulinemia of chickens as observed by Blaise, et al., (1974), in which suppression could be passed to untreated recipients via suppressed donor thymocytes. The third proposal involves receptor blockade, wherein cells are incapable of responding due to the steric masking of cell surface receptors by anti-μ. This hypothesis should lead to transient suppression if, in the absence of antiglobulin administration, regeneration of unblocked receptors occurs. However, the lack of IgM receptor reexpression in neonatal mice treated with anti-μ may explain long term tolerance (Bruyns, et al., 1976).

At this time, the second hypothesis is favored by this author since it allows permanent, dynamic suppression and can readily account for the results presented here although combinations of the above mechanisms can not be rejected. Anti-δ probably does not produce permanent T cell mediated suppression since its effect is stimulatory and receptors are re-expressed rapidly and thus are not totally hidden from tolerizing mechanisms. Furthermore, IgD receptors are not readily detectable until much later than IgM. Relevant to this concept is the finding by Mellby et al., (1975) that antibody to IgD exists in the sera of 8% of the normal adult humans tested. These individuals may represent a group who have failed to balance a fine line between tolerance to IgD and recognition of the receptor as non-self during B cell development. In this regard, it would be of interest to know if these individuals

demonstrate blast cells *in vivo*, have abnormal immunoglobulin levels or have an abnormal spectrum of Ig-bearing B cells.

Summary and Conclusions

An experimental model utilizing the rat was devised to study the biological role of IgD. Discovery of IgD in the rat, itself a novel finding, was furthered by the demonstration of antigenic cross reactivity of rat IgD with human IgD. Rat IgD was found to be associated with lymphocyte plasma membranes and was undetectable in rat serum. Characterization of rat IgD indicated it exists as a 185,000 dalton protein with both heavy and light chains in an H_2L_2 conformation. The δ chains coelectrophorese with μ chains on polyacrylamide-SDS gels and have a molecular weight of ~73,000 daltons. δ chain is relatively stable at this molecular weight, although partial degradation or rearrangement to a 65,000 dalton species does occur.

IgD was found on lymphocytes from all Ag-B haplotypes tested (1-6) and was found to appear, ontogenically, at 3.5 weeks of age. Not all lymphocytes bear δ chain equally. Cells obtained from peripheral lymphoid tissue (i.e. spleen, lymph nodes, blood) bore increasing amounts of δ chain as the tissue became progressively more distal to the spleen, with Peyer's patches exhibiting the most IgD of all tissues tested. Thymocytes did not demonstrate surface Ig by the techniques used. These results suggested that IgD bearing cells may represent a late stage in B lymphocyte differentiation.

Speculation:

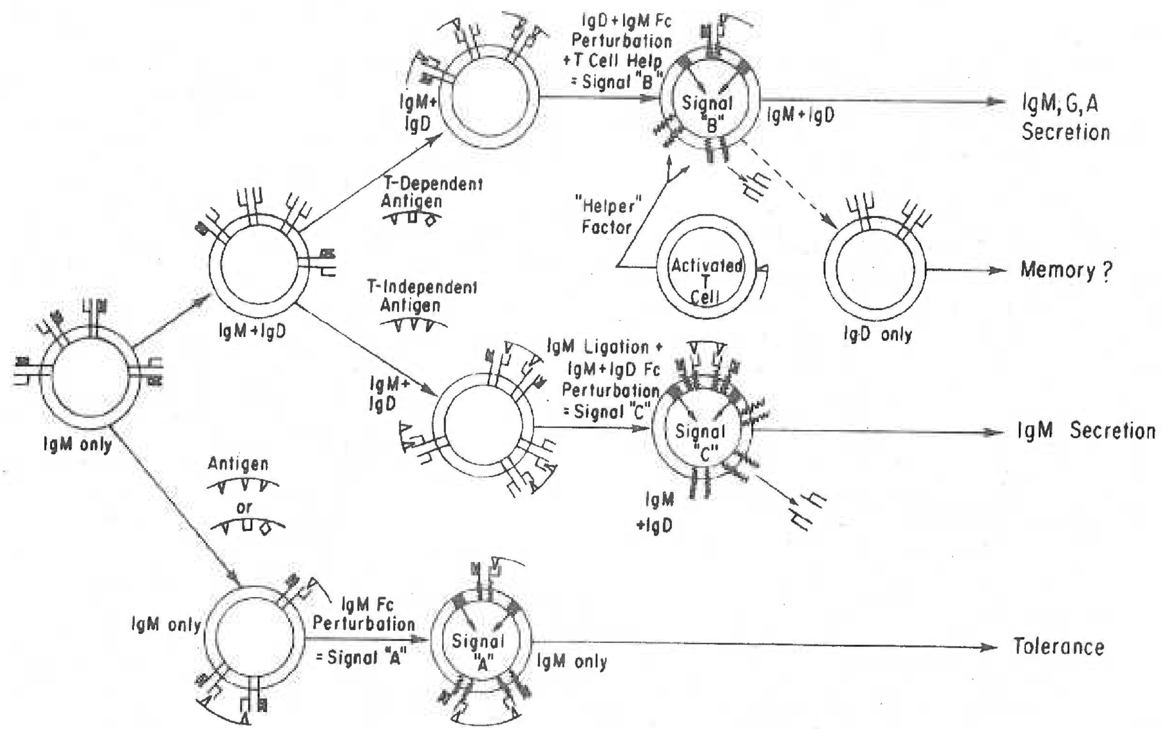
In the course of B lymphocyte development, a precursor B cell, although already committed to a B lymphocyte developmental sequence, but lacking detectable surface immunoglobulin, can be observed. This cell differentiates to a cell bearing only IgM prior to, or shortly after, parturition, with the actual time of IgM emergence varying between species. At this stage, it is of interest to consider the nature of IgM cell surface receptors and to speculate that, similar to humoral IgMs, they may be functionally monovalent. The resulting monogamous binding of antigen sets the stage for an explanation of the "IgM only" cells to antigen, i.e. tolerance.

Following confrontation of an IgM only cell with antigen, a series of events may be set in motion. Initially, epitope binding by cell surface IgM may cause a conformational distortion of the Fc region of this molecule, in a manner similar to that believed to facilitate complement binding in humoral antigen-antibody interactions. If, as has been demonstrated in numerous other receptor stimulation-cell response systems (i.e. peptide hormones), the structural rearrangement of these membrane imbedded regions mediates an alteration of the membrane lipid bilayer, then activation of the purine triphosphate cyclases through altered hydrophobic interactions with membrane lipids could mediate lymphocyte functional responses by

modulating the intercytoplasmic concentrations of cAMP and cGMP. It is easy to propose that antigen binding by "IgM only" cells may generate a specific membrane signal resulting in a precise level of membrane bound cyclase stimulation that may produce a tolerizing signal to that cell's synthetic and reproductive mechanisms, and which is termed signal "A" in figure 12. It should be noted that this signal may differ from other signals of a similar nature both in qualitative as well as quantitative features. Alternatively,then, the B cell bearing IgM may not encounter antigen until after it has differentiated to a more mature state and now bears, in addition to IgM, varying amounts of IgD. It is proposed that it is at this stage that T-dependent and T-independent antigenic responses occur. In the first case, the IgM and IgD bearing cell may be stimulated by T-dependent antigens in one of three different ways (uppermost sequence in Figure 12). First, IgM may bind antigen monogamously as in a tolerogenic response. Secondly, and possibly in addition to binding of antigen by IgM receptors, IgD may bind antigen either monogamously or divalently, since it is reasonable to suggest that cell surface IgD is functionally divalent as is humoral IgD. Since IgD appears to be very friable at or near its hinge region in the presence of plasmin, especially following interaction with antigen, cleavage of cell surface IgD may occur at this time. This cleavage could expose a site on the membrane which either binds, or otherwise interacts with, a T-cell "helper factor" elicited from activated T cells.

Figure 12:

Hypothetical working model for B cell differentiation and stimulation by antigen. The schematic drawing represents expansion of a monospecific clone of B lymphocytes with reactivity against the haptenic conformation indicated by the triangle. Monovalency of IgM molecules is indicated by an "X" in the Fab region of this receptor. Membrane bound cyclases are indicated by a dark intramembranous square.


IgD + IgM Fc
Perturbation
+T Cell Help
= Signal "B"
IgM+
IgD
Signal
"B"
IgM + IgD
IgM, G, A
Secretion
T-Dependent
Antigen
"Helper"
Factor
Activated
T
Cell
Memory ?
IgD only
IgM + IgD
T-Independent
Antigen
IgM Ligation +
IgM + IgD Fc
Perturbation
= Signal "C"
IgM+
IgD
Signal
"C"
IgM Secretion
IgM
+IgD
IgM only
Antigen
or
IgM only
IgM Fc
Perturbation
= Signal "A"
Signal
"A"
IgM only
Tolerance

The combination of IgM Fc conformational changes, IgD cleavage and T cell factor binding may produce a second type of signal (i.e. signal "B" in figure 12) by way of a membrane rearrangement-purine triphosphate cyclization sequence similar to that described for "IgM only" cell stimulation. It should be noted that in the absence of IgD receptor stimulation and/or T cell help, signal "A" is produced which results in tolerance or a tolerance-like signal to the more mature IgD bearing cell. It can be seen that variations in the nature or magnitude of signal "B" can be expected due to variations in the IgD/IgM ratio on the cell in question. Other factors such as epitope density, antigen concentration, receptor density, etc., may, of course, have specific roles, perhaps of a subtler nature. This hypothesis relates to the finding that high D/M ratio tissues produce more "mature" response such as IgA secretion, as opposed to IgG or IgM secretion for tissues with lower D/M ratios, thus accounting for the classical variations in the nature of the T-dependent response seen.

A third possible signal involves the binding of T-independent antigens by a D and M bearing cell and is presented in the middle sequence, figure 12. In this case, IgM receptors may be bridged causing IgM ligation over and above IgM Fc conformational distortions due to antigen binding. This additional signal (signal "C" in figure 12) may replace the T cell help required for other antigens

but may also prevent differentiation of the immune response of these cells to IgG or IgA secretion stages. This response appears to be very susceptible to epitope density differences and certain densities and antigen concentrations may produce tolerance even at this stage of B cell differentiation.

Finally, the existance of an "IgD" only cell has been postulated. In this author's opinion, such a cell may still bear minute amounts of IgM, but this fact notwithstanding, it is suggested here that these cells may contain the population of B cells bearing memory, and presumably are generated by T-dependent antigenic stimulation of D and M bearing cells.

REFERENCES

Abney, E.R., L.R. Hunter & R.M.E. Parkhouse, 1976. Preparation and Characterization of an Antiserum to the Mouse Candidate for Immunoglobulin D. Nature (London) 259 : 404.

Abney, E.R. and R.M.E. Parkhouse. 1974. Candidate for Immunoglobulin D Present on Murine B Lymphocytes. Nature (London) 252 : 600.

Amsbaugh, D.F., C.T. Hansen, B. Prescott, P.W. Staghak, D.R. Barthold & R.J. Baker. 1972. Genetic Control of the Antibody Response to Type III Pneumococcal Polysaccharide in Mice. Evidence That an X-linked Gene Plays a Decisive Rolê in Determining Responsiveness. J. Exp. Med. 236 : 931.

Anderson, J., L. LaFleur, & F. Melchers. 1974. IgM in Bone Marrow-derived Lymphocytes. Synthesis Surface Deposition Turnover & Carbohydrate Composition in Unstimulated Mouse B. Cells. Eur. J. Immunol. 4 : 170.

Binaghi, R.A., and E. Sarandon deMerlo. 1966. Characterization of Rat IgA and Its Non-identity With the Anaphylactic Antibody. Int. Arch. Allergy Appl. Immunol. 30 : 580.

Blaese, R.M., P.O. Weiden, I. Koski & N. Dooley. 1974. Infectious Agammaglobulinemia: Transmission of Immunodifficiency With Grafts of Agammaglobulinemic Cells. J. Exp. Med. 140 : 1097.

Bruyns, C., G. Urbain-Vansanten, C. Planard, C. DeVos-Cloetens, & J. Urbain. 1976. Ontogeny of Mouse B. Lymphocytes and Inactivation by Antigen of Early B. Lymphocytes. Proc. Nat. Acad. Sci. 73 : 2462.

Cambier, J.C., J.R. Kettman, E.S. Vitetta, & J.W. Uhr. 1976. Differential Susceptibility of Neonatal and Adult Murine Spleen Cells to In Vitro Induction of B. Cell Tolerance. J. Exp. Med. 144 : 293.

Ceska, M., and U. Lundkvist. 1972. A New and Simple Radioimmunoassay Method for the Determination of IgE. Immunochem. 9 : 1021.

Cooper, G.N., J.C. Thonard, R.L. Crosby, and M.H. Dalbow. 1969. Immunological Response in Rats Following Antigenic Stimulation of Peyer's Patches. II. Histological Changes in Germ-free Animals. Aust. J. Exp. Biol. Med. Sci. 46 : 407.

Cooper, M.D., J.F. Kearney, A.R. Lawton, E.R. Abney, R.M.E. Parkhouse, J.L. Preudhomme, & M. Seligmann. 1976. Generation of Immunoglobulin Class Diversity in B. Cells: A Discussion With Emphasis on IgD Development. Ann. Immunol. (Inst. Pasteur). 127 C : 573.

Cormane, R.H., S. Husz, & F. Hamerlinck. 1974. Immunoglobulin and Complement - bearing Lymphocytes in Allergic Contact Dermatitis and Atopic Dermatitis (Eczema). Brit. J. Derm. 90 : 597.

Cuatrecasas, P. 1970. Protein Purification by Affinity Chromatography. Derivatizations of Agarose and Polyacrylamide Beads. J. Biol. Chem. 245 : 3059.

Devey, M., C.H. Sanberson, D. Carter, and R.R.A. Coombs. 1970. IgD Antibody to Insulin. Lancet 2 : 1280.

Eisen, H.M., J.R. Little, L.A. Steiner, E.S. Simms, and W. Gray. 1969. Degeneracy in the Secondary Immune Response: Stimulation of Antibody Formation by Cross Reacting Antigens. Israel J. Med. Sci. 5 : 338.

Fahey, J.L., P.O. Corbone, D.S. Rose, and R. Bachmann. 1969. Plasma Cell Myeloma With D-myeloma Protein (IgD Myeloma). Am. J. Med. 45 : 373.

Feldmann, M., R.E. Cone, & J.J. Marchalonis. 1973. Cell Interactions in the Immune Response In Vitro. VI. Mediation by T. Cell Surface Monomeric IgM. Cellular Imm. 9 : 1.

Finkelman, F.D., J.A. Van Boxel, R. Asofsky, and W.E. Paul. 1976. Cell Membrane IgD; Demonstration of IgD on Human Lymphocytes by Enzyme-catalyzed Iodination and Comparison With Cell Surface Ig of Mouse, Guinea Pig and Rabbit. J. Immunol. 116 : 1173.

Finkelman, F.D., A.H. Smith, I. Scher, and W.E. Paul. 1975. Abnormal Ratio of Membrane Immunoglobulin Classes in Mice With an X-linked B-lymphocyte Defect. J. Exp. Med. 142 : 1316.

Fu, S.M., and H.G. Kunkel. 1974. Membrane Immunoglobulins of B Lymphocytes. Inability to Detect Certain Characteristic IgM and IgD Antigens. J. Exp. Med. 140 : 895.

Fu, S.M., R.J. Winchester, T. Feizi, P.D. Walzer, and H.G. Kunkel. 1974. Idiotypic Specificity of Surface Immunoglobulin and the Maturation of Leukemic Bone-marrow-derived Lymphocytes. Proc. Nat. Acad. Sci. 71 : 4487.

Fu, S.M., R.J. Winchester, and H.G. Kunkel. 1974. Occurrence of Surface IgM, IgD and Free Light Chains on Human Lymphocytes. J. Exp. Med. 139 : 451.

Gluick, G.J., R.C. Biegar, & R. Stankievic. 1969. Antigen Combining Activity Associated With Immunoglobulin D. Science (Wash. D.C.) 165 : 606.

Goding, J.W., G.W. Warr, & N.L. Warner. 1976. Genetic Polymorphism of IgD-like Cell Surface Immunoglobulin in the Mouse. Proc. Nat. Acad. Sci. 73 : 1305.

Goodman, S.A., E.S. Vitetta, U. Melcher, and J.W. Uhr. 1975. Cell Surface Immunoglobulin. XIII. Distribution of IgM and IgD-like Molecules on Small and Large Cells of Mouse Spleen. J. Immunol. 114 : 1646.

Goodman. M., H.R. Wolfe, and N. Stata. 1951. Precipitin Production in Chickens. VI. The Effect of Varying Concentrations of NaCl on Precipitate Formation. J. Immunol. 66 : 225.

Grebenau, M.D., S.P. Lerman, M.A. Palladino, & G.H. Thorbecke. 1976. Suppression of Adoptive Antibody Responses by Addition of Spleen Cells From Agammaglobulinemic Chickens "Immunized" With Histocompatible Bursa Cells. Nature (London) 260 : 46.

Grey, H.M., R.T. Kubo, and J.C. Cerottini. 1972. Thymus-derived (T-cell) Immunoglobulins. Presence of a Receptor Site for IgG and the Absence of Large Amounts of Buried Ig Determinants on T. Cells. J. Exp. Med. 136 : 1323.

Gupta, S., R. Pahwa, R. O'Reilly, R.A. Good, and F.P. Siegal. 1976. Ontogeny of Lymphocyte Subpopulations in Human Fetal Liver. Proc. Nat. Acad. Sci. 73 : 919.

Haustein, D., J.J. Marchalonis, and J.J. Crumpton. 1974. Immunoglobulin of T. Lymphoma Cells is an Integral Membrane Protein. Nature 252 : 602.

Heiner, D.C., and B. Rose. 1970. A Study of Antibody Responses by Radioimmunodiffusion With Demonstration of γ D Antigen-binding Activity in Four Sera. J. Immunol. 104 : 691.

Henney, C.S., H.D. Welscher, W.D. Terry, and D.S. Rowe. 1969. Studies on Human IgD. II. The Lack of Skin Sensitizing and Complement Fixing Activities of Immunoglobulin D. Immunochemistry 6 : 445.

Henson, P., H.J. Bell, and H.L. Spiegelberg. Unpublished Observations.

Jensenius, J.C., and A.F. Williams. 1974 a. Total Immunoglobulin of Rat Thymocytes and Thoracic Duct Lymphocytes. Eur. J. Immunol. 4 : 98.

Jensenius, J.C., and A.F. Williams. 1974 b. The Binding of Anti-immunoglobulin Antibodies to Rat Thymocytes and Thoracic Duct Lymphocytes. Eur. J. Immunol. 4 : 91.

Jones, P.P., and J.J. Cebra. 1974. Restriction of Gene Expression in B Lymphocytes and Their Progeny. III. Endogenous IgA and IgM on the Membranes of Different Plasma Cell Precursors. J. Exp. Med. 140 : 966.

Jones, P.P., S.W. Craig, J.J. Cebra, and L.H. Herzenberg. 1974. Restriction of Gene Expression in B. Lymphocytes and Their Progeny. II. Commitment to Immunoglobulin Heavy Chain Isotype. J. Exp. Med. 140 : 452.

Kearney, J.F., and A.R. Lawton. 1975 a. B-Lymphocyte Differentiation Induced by Lypopolysaccharide. I. Generation of Cells Synthesizing Four Major Immunoglobulin Classes. J. Immunol. 115 : 671.

Kearney, J.F., and A.R. Lawton. 1975 b. B-Lymphocyte Differentiation Induced by Lypopolysaccharide. II. Response of Fetal Lymphocytes. J. Immunol. 115 : 677.

Kennel, S.J., and R.A. Lerner. 1973. Isolation and Characterization of Plasma Membrane Associated Immunoglobulin From Cultured Human Diploid Lymphocytes. J. Mol. Biol. 76 : 485.

Kermani-Arab, V., G.A. Leslie, & D.R. Burger. 1976. Structures and Biological Function of Human IgD. IX. Anti-IgD Activation of Human Lymphocytes. Int. Arch. Allergy App. Immunol. in press.

Kermani-Arab, V., D.R. Burger, and G.A. Leslie. 1976. Structure and Biological Function of Human IgD. X. Enhancement of PHA Responsiveness by Anti-δ Activated Lymphocytes. J. Immunol. 117 : 467.

Kincade, P.W., K.S. Self, and M.D. Cooper. 1973. Survival and Function of Bursa-derived Cells in Bursectomized Chickens. Cell. Immunol. 8 : 93.

Knapp, W., R.L.H. Bolhuis, J.R Adl & W. Hijmans. 1973. Independent Movement of IgD and IgM Molecules on the Surface of Individual Lymphocytes. J. Immunol. 111 : 1295.

Konno, T., H. Hirai, & S. Inai. 1975. Studies in IgD-I. Complement Fixing Activities of IgD Myeloma Proteins. Immunochem. 12 : 773

Kubo, R.T., H.M. Grey, & B. Pirofsky. 1974. IgD : A Major Immunoglobulin on the Surface of Lymphocytes From Patients with Chronic Lymphocytic Leukemia. J. Immunol. 112 : 1952.

Lawton, A.R., R.M. Asofsky, J.M. Davie, & M.B. Hylton. 1973. Suppression of Immunoglobulin Synthesis in Mice: Age Dependence of Anti-μ Suppression and Effects of Anti-(γ1 & γ2), Anti-γ2, and Anti-α. Fed. Proc. 32 : 1012.

Lawton, A.R., & M.D. Cooper. 1974. Modification of B Lymphocyte Differentiation by Anti-immunoglobulins. In: Contemporary Topics in Immunobiology. Volume III. (M.D. Cooper & N.L. Warner, eds.) New York : Plenum Press. P. 193.

Lawton, A.R., R. Asofsky, M.B. Hylton, and M.D. Cooper. 1972. Suppression of Immunoglobulin Class Synthesis in Mice. I. Effects of Treatment with Antibody to μ Chain. J. Exp. Med. 135 : 277.

Lertora, J.J., F.J. Gomez, & G.A. Leslie. 1975. Structure & Biological Functions of Human IgD. V. Insulin Antibodies of the IgD Class in Sera From Some Diabetic Patients. Int. Arch. Allergy 49 : 596.

Leslie, G.A., and R.C. Armen. 1974. Structure and Biological Functions of Human IgD. III. Phylogenetic Studies of IgD. Int. Arch. Allergy Appl. Immunol. 46 : 9.

Leslie, G.A., and L.W. Clem. 1973. Chicken Antibodies to Group A Streptococcal Carbohydrate. J. Immunol. 110 : 191.

Leslie, G.A., L.W. Clem, and D. Rowe. 1971. The Molecular Weight of Human IgD Heavy Chains. Immunochemistry 8 : 565.

Leslie, G.A., and P. Hattier. 1974. Detection and Quantitation of Chicken Antibodies to Streptococcal Carbohydrate by High Salt Reverse Radial Immunodiffusion. J. Immunol. Methods. 4 : 53.

Leslie, G.A., R.H. Lopez-Correa, and J.N. Holmes. 1975. Structure and Biological Functions of Human IgD. IV. Ontogeny of Human Serum Immunoglobulin D (IgD) as Related To IgG, IgA and IgM. Int. Arch. Allergy. 49 : 350.

Leslie, G.A., and L.N. Martin. 1973 a. Suppression of Chicken Immunoglobulin Ontogeny by F $(ab')_2$ Fragments of Anti-μ Chain and by Anti-L Chain. Int. Arch. Allergy. 45 : 429.

Leslie, G.A., and L.N. Martin. 1973 b. Modulation of Immunoglobulin Ontogeny in the Chicken : Effect of Purified Antibody Specific for μ Chain on IgM, IgY and IgA Production. J. Immunol. 110 : 959.

Leslie, G.A., and G. Teramura. 1976. Manuscript in Preparation.

Little, J.R., and H.N. Eisen. 1969. Specificity of the Immune Response to the 2,4-dinitrophenyl and 2,4,6-trinitrophenyl Groups, Ligand Binding and Fluorescence Properties of Cross Reacting Antibodies. J. Exp. Med. 129 : 247.

Litwin, S.D. 1972. Allelic and Class Exclusion of Membrane-associated Immunoglobulins of Human Lymphocytes. J. Immunol. 108 : 1129.

Lundkvist, U, and M. Ceska. 1972. Effect of Non-ionic Polymers on the Formation of Immuno-precipitates in Single Radial Immunodiffusion Techniques. Immunology 23 : 413.

Luster, M.I., R.C. Armen, J.V. Hallum, & G.A. Leslie. 1976. Measles Virus-specific IgD Antibodies in Patients With Subacute Sclerosing Panencephalitis. Proc. Nat. Acad. Sci. 73 : 1297.

Malka, S., C.J. Oon, and J.R. Hobbs. 1974. Excess IgD Bearing Lymphocytes in Patients With Malignant Melanoma. Br. J. Cancer 30 : 370.

Manning, D.D., and J.W. Jutila. 1972. Effect of Anti-immunoglobulin Antisera on Homograft Rejection in Mice. Nature (New Biol.) 237 : 58.

Marchalonis, J.J., R.E. Cone, and V. Santer. 1971. Enzymatic Iodination. A Probe for Accessible Surface Protéins of Normal and Neoplastic Lymphocytes. Biochem.J. 124 : 921.

Martin, L.N., and G.A. Leslie. 1976. Surface Immunoglobulins D and M; Occurrence on Individual Lymphocytes in the Rhesus Monkey. Submitted for Publication.

Martin, L.N., G.A. Leslie, and R. Hindes. 1976. Lymphocyte Surface IgD and IgM in Non-human Primates. Int. Arch. Allergy Appl. Immunol. 51 : 320.

Mason, D.W. 1976. The Class of Surface Immunoglobulin on Cells Carrying IgG Memory in Rat Thoracic Duct Lymph : The Size of the Subpopulation Mediating IgG Memory. J. Exp. Med. 143 : 1122.

McConahey, P.J., and F.J. Dixon. 1966. A Method of Trace Iodination of Proteins for Immunologic Studies. Int. Arch. Allergy 29 : 185.

Melcher, V., E.S. Vitetta, M. McWilliams, M.C. Lamm, J.M. Phillips-Quagliata, and J.W. Uhr. 1974. Cell Surface Immunoglobulin. X. Identification of an IgD-like Molecule on the Surface of Murine Splenocytes. J. Exp. Med. 140 : 1427.

Melcher, U., and J.W. Uhr. 1976. Cell Surface Immunoglobulin. XVI. Polypeptide Chain Structure of Mouse IgM and IgD-Like Molecule. J. Immunol. 116 : 409.

Mellbye, O.J., H.M. Hoyeraal, T. Michaelsen, and J.B. Natvig. 1975. Human Anti-immunoglobulin Antibodies With Specificity for Native and Pepsin-digested IgD. Clin. Exp. Immunol. 20 : 479.

Merler, E., J. Gatien, and G. DeWilde. 1974. Significance of Immunofluorescent Staining of Lymphocytes With Antisera to IgM Immunoglobulins. Nature 251 : 652.

Neoh, S.H., D.M. Jahoda, D.S. Rowe, and A. Voller. 1973. Immunoglobulin Classes in Mammalian Species Identified by Cross-reactivity With Antisera to Human Immunoglobulin. Immunochem. 10 : 805.

Nerenberg, S.T., and R. Prasad. 1975. Radioimmunoassays for Ig Classes G, A, M, D, and E in Spinal Fluids : Normal Values of Different Age Groups. J. Lab. Clin. Med. 86 : 887.

Orlans, E., and A. Feinstein. 1971. Detection of Alpha, Kappa and Lambda Chains in Mammalian Immunoglobulins Using Fowl Antisera to Human IgA. Nature (New Biol.) 233 : 45.

Ovary, Z. 1969. Lack of Skin-sensitizing Property of γ D Myeloma Protein in Guinea Pigs. J. Immunol. 102 : 790.

Parkhouse, R.M.E. 1973. Assembly and Secretion of Immunoglobulin M (IgM) by Plasma Cells and Lymphocytes. Transplant. Rev. 14 : 131.

Parkhouse, R.M.E., I.R. Hunter, and E.R. Abney. 1976. Heterogeneity of Surface Immunoglobulin on Murine B Lymphocytes. Immunology 30 : 409.

Pernis, B. 1975. The Effect of Anti-IgD Serum on Antibody Production in Rhesus Monkeys. In: Membrane Receptors of Lymphocytes. (M Seligmann, J.L. Preudhomme, & F.M. Kourilsky, eds.) North Holland (New York, N.Y.) p. 25.

Pernis, B., J.C. Brouet, & M. Seligmann. 1974. IgD and IgM on the Membrane of Lymphoid Cells in Macroglobulinemia. Evidence for Identity of Membrane IgD and IgM Antibody Activity in a Case with Anti-IgG Receptors. Eur. J. Immunol. 4 : 776.

Perry, M.B., & C. Milstein. 1970. Interchain Bridges of Human IgD. Nature 228 : 934.

Peissens, W.F., P.H. Schur, W.C. Maloney, and W.H. Churchill. 1973. Lymphocyte Surface Immunoglobulins. Distribution and Frequency in Lymphoproliferative Diseases. N. Eng. J. Med. 288 : 176.

Preud'homme, J.L., & M. Seligmann. 1974. "Surface Immunoglobulins on Human Lymphoid Cells." In : Progress in Clinical Immunology. Volume II. (R.S. Schwartz, ed.) Grune & Stratton, New York N.Y. P. 121.

Pollard, M., and N. Sharon. 1970. Responses of Peyer's Patches in Germfree Mice to Antigenic Stimulation. Infect. Immun. 2 : 96.

Rask, L., L. Klareskag, L. Ostberg, and P.A. Peterson. 1975. Isolation and Properties of a Murine Spleen Cell Fc Receptor. Nature (London) 257 : 231.

Rowe, D.S., and J.L. Fahey. 1965 a. A New Class of Human Immunoglobulins. I. A unique Myeloma Protein. J. Exp. Med. 121 : 171.

Rowe, D.S., & J.L. Fahey, 1965 b. A New Class of Human Immunoglobulins. II. Normal Serum IgD. J. Exp. Med. 171 : 185.

Rowe, D.S., K. Hug, L. Forni & B. Pernis. 1973. Immunoglobulin D as a Lymphocyte Receptor. J. Exp. Med. 138 : 965.

Rowe, D.S., K. Hug, W.P. Faulk, J.N. McCormick, & H. Gerber. 1973. IgD on the Surface of Peripheral Blood Lymphocytes of Human Newborn. Nature New Biol. 242 : 155.

Saha, A., P. Chowdhurg, S. Samburg, Y. Behelak, D.C. Heiner, and B. Rose. 1970. Studies on Human IgD. II. Physicochemical Characterization of Human IgD. J. Immunol. 105 : 238.

Salsano, P., S.S. Froland, J.B. Natvig, & T.E. Michaelson. 1974. Same Idiotype of B-Lymphocyte Membrane IgD and IgM. Formal Evidence for Monoclonality of Chronic Lymphocytic Leukemia Cells. Scand. J. Immunol. 3 : 841.

Santana, V. N. Wedderburn, E.R. Abney, & R.M.E. Parkhouse. 1976. Identification of a High Molecular Weight Protein on the Surface of Murine Thymus and Thymus-dependent Cells. Eur. J. Immunol. 6 : 217.

Santana, V., and J.L. Turk, 1975. Binding of Aggregated Human Immunoglobulin to Murine Thymocytes and T Cells Through Receptors for the Fc Region. Immunology 28 : 1173.

Scher, I., S.O. Sharrow, R. Wistar, Jr., R. Asofsky, & W.E. Paul. 1976. B-Lymphocyte Heterogeneity : Ontogenic Development and Organ Distribution of B-Lymphocyte Populations Defined by Their Density of Surface Immunoglobulin. J. Exp. Med. 144 : 494.

Scher, I., S.O. Sharrow & W.E. Paul. 1976. X-linked B-Lymphocyte Defect in CBA/N Mice. III. Abnormal Development of B-Lymphocyte Populations Defined by Their Density of Surface Immunoglobulin. J. Exp. Med. 144 : 507.

Scher, I., A.D. Steinberg, A.K. Berning & W.E. Paul. 1975. X-linked B-Lymphocyte Defect in CBA/N Mice. II. Studies of the Mechanisms Underlying the Immune Defect. J. Exp. Med. 142 : 637.

Sela, B., J.L. Wang, and G.M. Edelman. 1975. Antibodies Reactive With Cell Surface Carbohydrates. Proc. Nat. Acad. Sci. 72 : 1127.

Sell, S., and P.G.H. Gell. 1965. Studies on Rabbit Lymphocytes In Vitro. IV. Blast Transformation of the Lymphocytes From Newborn Rabbits Induced by Anti-allotype Serum to a Paternal IgG Allotype Not Present in the Serum of the Lymphocyte Donor. J. Exp. Med. 122 : 923.

Spiegelberg, H.L. 1975. NH_2-terminal Amino Acid Sequence of the Fc Fragment of IgD Resembles IgE and IgG Sequences. Nature 254 : 723.

Spiegelberg, H.L. 1972. "γD Immunoglobulin". In : Contemporary Topics in Immunochemistry. Volume I. (F.P. Inman, Ed.). New York: Plenum Press. P. 165.

Spiegelberg, H.L. 1970. Structural Studies of Human γD Myeloma Protein. Biochemistry. 9 : 2115.

Stankus, R.P., and G.A. Leslie. 1976. Affinity-immunoadsorbent Fractionation of Rat Anti-streptococcal A Carbohydrate Antibodies of Restricted Heterogeneity. J. Immunol. Methods 10 : 307.

Stankus, R.P., and G.A. Leslie. 1974. Nonprecipitating and Electrophoretically Restricted Antibodies to Carbohydrate of Group A Streptococcus in the Rat. J. Infect. Dis. 130 : 169.

Stout, R.D., and L.A. Herzenberg. 1975. The Fc Receptor on Thymus-derived Lymphocytes. I. Detection of a Subpopulation of Murine T Lymphocytes Bearing the Fc Receptor. J. Exp. Med. 142 : 611.

Strober, S. 1975. Immune Function, Cell Surface Characteristics and Maturation of B Cell Subpopulations. Transplant. Rev. 24 : 84.

Terasaki, P. 1970. In : A Manual of Tissue Typing Techniques. U.S. Dept. Health, Education and Welfare. P. 42.

Terhorst, C., P. Parhan, D.L. Mann, and J.L. Strominger. 1976. Structure of HLA Antigens: Amino-acid and Carbohydrate Compositions and NH_2-terminal Sequences of Four Antigen Preparations. Proc. Nat. Acad. Sci. 73 : 910.

Van Boxel, J.H., W.E. Paul, W.D. Terry, and I. Green. 1972. IgD-bearing Human Lymphocytes. J. Immunol. 109 : 648.

Van Boxel., J.A., S. Broder, and T.A. Waldmann. 1976. Suppression of Immunoglobulin Synthesis In Vitro by Purified Antibody to IgD. In: Leukocyte Membrane Determinants Regulating Immune Reactivity. (V.P. Eijsvoogel, D. Roos, and W.P. Zeijlemaker, eds.) Academic Press (New York, N.Y.). P. 297.

Vitetta, E.S., S. Baur, and J.W. Uhr. 1971. Cell Surface Immunoglobulin. II. Isolation and Characterization of Immunoglobulin From Mouse Splenic Lymphocytes. J. Exp. Med. 134 : 242.

Vitetta, E.W., C. Bianco, V. Nussenzweig, & J.W. Uhr. 1972. Cell Surface Immunoglobulin. IV. Distribution Among Thymocytes, Bone Marrow Cells, and Their Derived Populations. J. Exp. Med. 136 : 81.

Vitetta, E.S., J. Forman, and J.R. Kettman. 1976. Cell Surface Immunoglobulin. XVIII. Functional Differences of B Lymphocytes Bearing Different Surface Immunoglobulin Isotypes. J. Exp. Med. 143 : 1055.

Vitetta, E.S., I. Grundke-Iqbal, K. Holmes, & J.W. Uhr. 1974. Cell Surface Immunoglobulin. VII. Synthesis, Shedding and Secretion of Immunoglobulin by Lymphoid Cells of Germ-free Mice. J. Exp. Med. 139 : 862.

Vitetta, E.S., J. Klein, and J.W. Uhr. 1974. Partial Characterization of Ia Antigens From Murine Lymphoid Cells. Immunogenetics. 1 : 82.

Vitetta, E.S., M. McWilliams, J.M. Phillips-Quagliata, M. Lamm, and J.W. Uhr. 1975. Cell Surface Immunoglobulin. XIV. Synthesis, Surface Expression and Secretion of Immunoglobulin by Peyer's Patch Cells in the Mouse. J. Immunol. 115 : 603.

Vitetta, E.S., U. Melcher, M. McWilliams, M.E. Lamm, J.M. Phillips-Quagliata, and J.W. Uhr. 1975. Cell Surface Immunoglobulin.. XI. The Appearance of an IgD-like Molecule on Murine Lymphoid Cells During Ontogeny. J. Exp. Med. 141 : 206.

Vitetta, E.S., and J.W. Uhr. 1976. Cell Surface Immunoglobulin. XV. The Presence of IgM and an IgD-like Molecule on the Same Cell in Murine Lymphoid Tissue. Eur. J. Immunol. 6 : 140.

Vitetta, E.S., and J.W. Uhr. 1975 a. Immunoglobulin Receptors Revisited. Science (Wash. D.C.) 189 : 964.

Vitetta, E.S., and J.W. Uhr. 1975 b. IgD on Murine Lymphocytes: Implications for Differentiation and Stimulation of B Lymphocytes. In: Membrane Receptors on Lymphocytes. Seligman, editor. North Holland Publishing Co., New York, New York. 27.

Weber, K., and M. Osborn. 1969. The Reliability of Molecular Weight Determinations by Dodecyl Sulfate-polyacrylamide Gel Electrophoresis. J. Biol. Chem. 244 : 4406.

Williams, A.F. 1975. IgG_2 and Other Ig Classes on the Cell Surface of Rat Lymphoid Cells. Eur. J. Immunol. 5 : 883.

Williamson, A.R. 1971. Biosynthesis of Antibodies. Nature (Lond.) 231 : 359.

Williams, C.A., and M.W. Chase. 1968. Methods in Immunology and Immunochemistry, Volume II. New York: Academic Press. p. 397.

Winchester, R.J., S.M. Fu, T. Hoffman, and H.G. Kunkel 1975. IgG on Lymphocyte Surfaces; Technical Problems and the Significance of a Third Cell Population. J. Immunol. 114 : 1210.