INDUCTION, ONTOGENY, AND ANTIGEN SPECIFICITY OF RAT IMMUNOGLOBULIN D

by

Michael Gillespie Steele

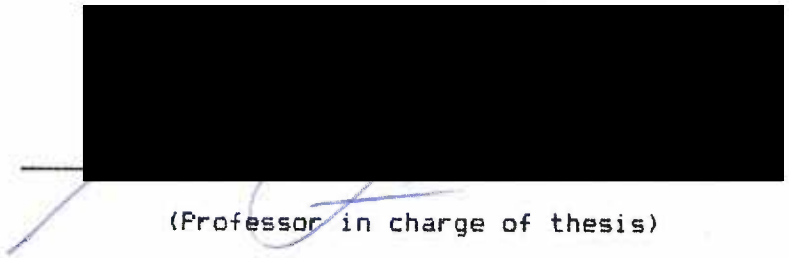
A THESIS

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

Doctor of Philosophy

June 1986

APPROVED:



(Professor in charge of thesis)

(Chairman, Graduate Council)

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

APPENDIX 2

Figure

APPENDIX 3

Figure

APPENDIX 4

Figure

1 IgA anti-DNP in milk and serum following intramammary and oral immunizations 218a

2 IgD anti-DNP in milk and serum resulting from DNP-OVA/DW immunization 218b

STATEMENT OF PROBLEM

Of the five classes of immunoglobulins, secreted IgD is unquestionably the least well understood. Reasons for this include: (1) its very low concentration in the normal serum of humans (Rowe and Fahey, 1965b; Rowe, Crabbe, and Turner, 1968; Leslie and Teramura, 1977) mice, (Bargellesi et al., 1979; Finkelman et al., 1979) and rats (Bazin et al., 1978; Steele and Leslie, 1985), which has tended to restrict quantitative studies of the immunoglobulin to simple measurement of total IgD (and even then in many instances only when it is abnormally high); (2) suitable animal models for the study of serum IgD were unavailable for a long time following its discovery in human serum which consequently restricted research to human clinical studies. Such studies, while certainly useful in developing hypotheses about IgD function, have rarely been definitive because of the limitations of human experimentation, the complicating factors of therapy, the usually low numbers of individuals tested, and the inherent complexity of disease etiologies. For these reasons it is desirable to develop an animal model.

The starting point for the investigations presented herein was the discovery by Olson and Leslie, (1982) of very high concentrations of IgD in rat milk. They reported that milk IgD in lactating rats was regularly present at concentrations between 80 and 300 µg/ml based on

radial immunodiffusion. However the investigation of the relationship between serum and milk IgD in the lactating mother and in the serum of the suckling pup required a more sensitive assay than radial immunodiffusion, therefore I specifically purified both rabbit and sheep antibodies to IgD, labeled them with alkaline phosphatase, and used this reagent to develop an ELISA capable of measuring IgD levels as low as 10 ng/ml in diluted samples.

The assay for total IgD allowed the investigation of the following questions concerning secreted IgD: (1) What is the relationship between IgD in milk and in serum, i.e., is its presence in milk the result of transudation from serum or the result of local production in the gland? (2) Does IgD in milk affect the serum IgD level found in the suckling rat? (3) What is the relationship between IgD in serum and saliva? (4) What influence does the athymic nude phenotype have on levels of serum IgD? (5) What is the ontogeny of IgD in rat serum? (6) What influence does age and sex have on serum IgD levels? (7) In what ways may IgD levels in serum be increased? (8) How may IgD be purified and what are its structural characteristics?

To fully address the question of the biological function of IgD it is necessary also to investigate its antigen specificity. However, the already quite low concentrations of IgD in serum compounds the problem of attempting to measure an antibody response. One way to overcome

this is to develop experimental systems in which the production of IgD is amplified, so that antibody responses are more readily measurable. In the course of my studies I found two situations where IgD levels might be sufficiently amplified to observe an antibody response. The first of these was the high IgD levels seen in rat milk. Using an intrammmary immunization protocol which had been shown previously to elicit both IgG and IgA antibodies to DNP in rat milk (Cox et al., 1980), I showed in several experiments that IgD anti-DNP antibodies could be regularly induced in milk and in subsequent experiments, I examined the efficacy of different methods of oral and intramammary immunization for inducing such a response (See appendix 4). I found a second instance of IgD amplification in the course of examining several pools of rat serum and ascitic fluid for their IgD levels. To my surprise a pool of rat ascitic fluid contained roughly 250 µg/ml of IgD. Subsequent tests showed that 18/18 pools of ascitic fluid from individual rats from experiments done by other workers in this lab had sufficient IgD to be detectable by Ouchterlony and by ELISA were found to have IgD concentrations ranging from 64 µg/ml to >800 µg/ml. Acting on these results I performed the experiments described in paper 3, which showed that IgD antibodies to DNP could be induced in rat ascitic fluid. Additional studies have been done to investigate the basic physical properties of IgD in rat ascitic fluid, milk, and normal rat serum.

REVIEW OF LITERATURE

I. Introduction

IgD was first identified by Rowe and Fahey (1965a) as an unusual paraprotein found in the blood of a myeloma patient. It had recognizable light chains of the lambda type, but the heavy chain did not react with antisera to any of the then known human isotypes. By raising an antiserum to the new protein, they were subseqently able to show that IgD was present in normal human serum, where it had a median concentration in 30 µg/ml (Rowe and Fahey, 1965b), thus normally making up less than 1% of the total serum immunoglobulin present. It was also a molecule which was unusually sensitive to proteolysis by papain (Rowe and Fahey, 1965b) as well as by plasmin and trypsin (Spiegelberg, Prahl, and Grey, 1970). In the two decades since its discovery, IgD or IgD-like molecules have been described on the surface of B lymphocytes in six mammalian species and one avian species. These include man (Rowe, Crabbe, and Turner, 1968), non-human primates (Leslie & Armen, 1974), rats (Ruddick and Leslie, 1977), rabbits (Sire, Colle, and Bourgois 1979; Wilder et al., 1979), mice (Abney and Parkhouse, 1974) swine (Zikan, Sima, and Tuckova, 1983) and chickens (Leslie, 1980; Chen et al., 1982). The evident conservation of membrane IgD in such diverse species (and hence through considerable evolutionary time) indicates that membrane IgD

must serve an important biological function or functions in each of these species. While unsuccessful early attempts to demonstrate IgD in non-primate species led some to the conclusion that serum IgD was a caprice of primate evolution (Fernis and Forni, 1976), IgD is now well established as a serum immunoglobulin present in a list of mammalian species which is of comparable diversity to that cited above. These include rats (Bazin et al., 1978), mice (Bargellesi et al., 1979; Finkelman et al., 1979), and swine (Zikan, Sima, and Tuckova, 1983). While admittedly, this is a shorter list, it is a sufficient indication of the likelihood that both serum and membrane IgD are regular components of the mammalian immune systems. Additional support for the dual nature of IgD as both a membrane and secreted immunoglobulin comes from studies of the human and murine delta gene, which has now been shown in both these species to contain provisions for both a secreted and membrane-binding form (Cheng et al, 1982; White et al., 1985).

In spite of the fact that for several years it has been clear that IgD may have a function both in a humoral and cellular context, until recently, apart from that of human clinical studies very little investigation of secreted IgD has been done. While an early study reported the absence of cytophilic activity for IgD on human lymphocytes, monocytes or neutrophils (Lawrence, Weigle and Spiegelberg, 1975), later studies have indicated that there may be IgD

Fc receptors on human T cells (Burger et al., 1982), both T cells and non-T cells (Sjoberg, 1980), or on B cells but not T cells (Rudders and Anderson, 1982). Meanwhile studies in mice have indicated that IgD Fc receptors are present on a population of Lyt 1+2- T cells which increase their numbers in the presence of IgD (Coico et al., 1985a). It has also been observed that mice injected with plasmacytoma IgD (TEPC 1017 or TEPC 1033) markedly enhanced (and in one case suppressed) PFC responses (Xue et al, 1984). Later it was shown that transfer of the IgD Fc receptor-bearing T cells could themselves enhance PFC responses in recipient animals (Coico et al, 1985b). These results indicate that a reevaluation of the biological significance of IgD is in order.

This review will discuss the recent literature on IgD in the following areas: (1) the concentration of IgD in serum and other body fluids with respect to its ontogeny, genetics, and alteration in disease, syndromes, and other physiological states, (2) the nature of antigen-specific IgD, (3) the histology of IgD-secreting cells, (4) the structure of IgD as it relates to its possible function and evolution, (5) IgD as a B lymphocyte receptor with respect to tissue distribution, ontogeny, tolerance induction and activation, (6) Fc receptors for IgD, and (7) the structure and function of the delta gene. Speculation on the possible biological function or functions of IgD will be discussed in the summary and conclusion section.

II. IgD Concentrations in Serum and Other Body Fluids

Normal IgD Concentrations. The concentration of IgD in normal serum has been examined in a number of studies. Using radial immunodiffusion, Rowe and Fahey (1965b) reported a median IgD concentration of 30 µg/ml with much individual variation in a sampling of 100 normal sera. Leslie and Teramura, using a more sensitive solid phase radioimmunoassay, were in essential agreement with this value for 114 individuals where they found a mean level of 34.9 µg/ml and a range from 5 to 240 µg/ml. Dunnette et al. 1977, also using a radioimmunoassay method, reported a geometric mean level of 13.0 IU/ml, a mean level of 30.1 IU/ml and a median level of 14.8 IU/ml in 112 adult sera.

The level of serum IgD in rats and mice is approximately 1/5 to 1/10 the level observed in humans. Bazin et al., (1978) using a radioimmunoassay, reported that normal rat serum IgD ranged between 5 ng/ml to 5 µg/ml, and, in another study, that six phenotypically normal rnu/+ rats had a mean level of 1.7 µg/ml (Bazin et al., 1980). Steele and Leslie (1985), using an ELISA found the concentration of IgD in 43 adult rats to be 5.4 $\pm$ 3.6 µg/ml. In mice, Bargellesi et al. (1979) reported a serum concentration range of between 0.2 and 4 µg/ml. The concentration of swine serum IgD was reported as 10 µg/ml (Zikan, Sima, and Tuckova, 1983). Thus a common characteristic of

serum IgD, where it has been examined, is its low serum concentration.

Using the relatively insensitive radial immunodiffusion assay, an initial report (Rowe, Crabbe, and Turner, 1968) did not find IgD in human saliva, colostrum, bile, urine, tears or cerebrospinal fluid (CSF). However, Leslie and Teramura (1977) by radioimmunoassay found IgD in 6/6 samples of saliva with a mean concentration of 0.07 µg/ml, while Sewell et al. (1979) detected IgD in 3/7 samples of saliva by an agglutination test, but did not quantify it. In rats, saliva IgD was determined to be 2.0 $\pm$ 1.7 µg/ml (n = 34), while saliva from five lactating rats had a mean IgD concentration of 0.9 $\pm$ 0.7 µg/ml (Steele and Leslie, 1985). Saliva and serum IgD were poorly correlated (r = 0.09) suggesting local rather than transudative production (Steele and Leslie, 1985). In cerebrospinal fluid, IgD has been shown to be present in very low concentrations by at least three different laboratories (Tavoloto and DiZanche, 1968; Nerenberg and Prasad, 1975; Leslie and Teramura, 1977), the latter two finding mean levels of 7 µg/ml and 0.09 µg/ml (12/13 samples) respectively. Low levels of IgD have also been reported in nasal secretions in at least two studies (Butcher et al., 1975; Dunnette et al., 1977), the latter also detecting IgD in low levels in bronchial washes.

IgD has been reported in human colostrum by two laboratories. Sewell

et al, (1979) found 1-20 µg/ml in 8/12 samples of colostrum. The findings of Bahna, Keller, and Heiner (1982) were in essential agreement, detecting between 0.02-20 µg/ml (n=39). In the latter study colostral and plasma IgD were only "moderately" correlated (r = 0.74) which the authors suggested was an indication that human colostral IgD was locally produced in the gland. In lactating rats, milk IgD concentrations are relatively high, ranging from 36 to 800 µg/ml. Nevertheless serum IgD in the rats was as low as in nonlactating rats, and correlated poorly with milk IgD levels (r = 0.13, n = 40). These data indicate that local synthesis rather than transudation is the likely mechanism of milk IgD production (Steele and Leslie, 1985). While similar studies of IgD in murine milk have not been published, a preliminary report found that IgD was detectable in mouse milk by Ouchterlony gel diffusion (Olson and Leslie, 1982).

Ontogeny of Serum IgD. Using radial immunodiffusion, Rowe, Crabbe, and Turner (1968) could not detect serum IgD during the first six months of life. Older children and adults had detectable levels within a wide range. By radioimmunoassay, subsequent studies determined a mean level of 0.6 µg/ml in neonatal cord blood, which increased to 2.2 µg/ml in children 1-6 months of age, and reaching adult levels at roughly 6-8 years (Leslie and Teramura, 1977). Serum IgG was at adult levels in cord blood, and dropped to 1/3 adult levels in the first six months of life, and only returned to adult levels at age 9-12 years.

In contrast, serum IgA and IgM were very low in cord blood, reaching adult levels respectively at 6-8 years and 7-12 months. In amniotic fluid IgD was found at 0.2 µg/ml, but the fetal or maternal origin of this IgD is not known (Leslie, 1975; Leslie and Teramura, 1977). Similarly, Cederqvist, Ewool and Litwin (1977) found an IgD concentration of 0.5 ±0.4 µg/ml. in 100 samples. By comparing the isotype levels in cord blood and that of maternal serum, this group concluded, as did Leslie and Swate (1972), that the IgD in cord blood was probably of fetal origin, and that maternal IgD does not cross the placenta. Recently, however, there was a report that IgD antibodies to rubella did cross the placenta (Salonen et al., 1985).

Unlike human neonates, serum IgD in newborn rats is at adult levels, and declines to 1/3 the level at birth by 7 days, where it remains to the end of the suckling period (Steele and Leslie, 1985). After day 30, the mean value of serum IgD begins to rise. The adult levels of serum IgD found following birth were not the result of ingestion of IgD-containing colostrum, since adult serum levels were seen in newborns and were not raised by 24 hours of suckling. In rats 30 and 66 days old, there was considerable individual variability in serum IgD and fluctuations in serum IgD with time. Transient elevations of serum IgD, which lasted from a few days to several weeks, were observed in several rats (Steele and Leslie, 1985). Unlike rat IgG2a, IgG2b, and IgG1, whose serum levels in suckling rats are

apparently augmented via milk (Leslie and Cuchens, 1982), IgD does not appear to enter rat serum since serum IgD remains low in spite of the relatively high IgD concentrations in the milk which the rat pups ingest (Steele and Leslie, 1985). At present it is not known whether the IgD in neonatal rats is of fetal or maternal origin.

Genetic Influence on IgD Levels. There are a number of studies which demonstrate a connection between heredity and human serum IgD levels. Walzer and Kunkel (1974) found an association between the IgG allotypes Gm (f+b+) and low serum IgD. This association was confirmed in a more recent study (Litwin et al., 1985). Dunnette, Gleich and Weinshilboum (1977) concluded from studies of 245 adult blood donors and follow up studies with 134 first-degree relatives of subjects with low serum IgD that low serum IgD was an autosomal recessive trait. In a study of 1549 blood bank donors, Fraser and Schur (1981) also found that low serum IgD was an autosomal recessive trait but only in some families. In others it appeared to be determined by multiple alleles. They also found an increased frequency of certain HLA antigens in persons with low serum IgD. Another study found intrapair variance among 16 monozygotic pairs of twins to be significantly lower than among 7 dizygotic pairs of twins ($p < 0.05$) (Lee et al., 1980). Among 284 Metis Indians in Saskatchewan, serum IgD was on the average 10 times higher than among 784 whites (Gerard et al., 1981). Whether the differences observed, however, were hereditary or caused by

socio-economic factors was not determined. At present there are no published studies on the influence of genetics on serum IgD levels in other species.

Alterations of serum IgD in clinical studies. The numerous conditions associated with changes in serum IgD maybe classified as lymphoproliferative diseases, viral, bacterial, and parasitic diseases, respiratory diseases, atopic disorders, immunodeficiency disorders, and autoimmunity, with many of the diseases occupying more than one category.

Apart from the obvious effect on serum IgD levels which IgD myelomas may cause, two other lymphoproliferative disorders, Hodgkin's disease and infectious mononucleosis are associated with elevated serum IgD. Corte et al., (1978) found that 65 of 100 patients with Hodgkin's disease had IgD levels 3 to 50x above normal, with older patients having lower serum IgD than younger ones, and patients with splenectomy having higher serum IgD than other patients. Elevated IgD levels were associated with greater disease severity. Therapy (unspecified) was found to depress serum IgD levels to a level significantly lower than that of untreated patients. It was speculated that the elevated serum IgD levels could either be the result of antigen stimulation by tumor antigens or the proliferation of IgD-secreting cells. Elevated serum IgD was also found in two

studies of infectious mononucleosis early after infection (Bachmann, 1967; Nordbring et al., 1972).

Among viral diseases, elevated serum IgD has been reported in influenza A2/Hong Kong (Wiedermann et al., 1974), infectious mononucleosis (as mentioned above), and most recently in acquired immune deficiency syndrome (Chess et al., 1983; Papadopoulos and Frieri, 1984), a disease characterized by loss of helper T cell function. Rostenberg and Penalosa (1978) reported significantly elevated serum IgD in acute hepatitis (type not specified). Other viral diseases have been associated with the detection of IgD antibodies (see below).

Among bacterial diseases, tuberculosis (Buckley and Trayer, 1972) and leprosy (Rowe et al., 1968), both mycobacteria, have been reported to result in elevated serum IgD levels. In experimental studies with rats, Freund's complete adjuvant containing killed *Mycobacterium tuberculosis* has been shown to result in significant elevations of serum IgD, though it is not yet known whether this is specific for the adjuvant or a general effect (Steele and Leslie, *submitted*). Another study reported elevated IgD in salmonellosis (Rostenberg and Penalosa, 1978).

In parasitic diseases, elevated serum IgD has been observed in some

patients with toxoplasmosis (Cederqvist et al., 1977), while low serum IgD was found to be associated with the most severe cases of giardiasis (Jokipii and Jokipii, 1982).

A number of respiratory diseases or states of respiratory compromise have been associated with changes in serum IgD. Bahna, Heiner, and Myhre (1983a) found that the geometric mean serum IgD in healthy smokers was about 2x that of nonsmokers or ex-smokers. The most elevated levels, interestingly, were found among light smokers. A similar relationship between smoking and serum IgE was also observed (Bahna, Heiner, and Myhre (1983b) while other serum immunoglobulins are usually suppressed by smoking, especially IgG (Bahna, Heiner, and Myrhe, 1983a). While elevated serum IgD has been reported in allergic bronchopulmonary aspergillosis (Luster, Leslie, and Bardana, 1976a), Stevens, de Backer, and Vermeire (1983) in a study of 902 patients with allergic or non-allergic respiratory diseases (which were not further broken down according to type) reported that IgA and IgD were lower in the serum of both groups than in normal serum.
The allergic diseases, atopic dermatitis and allergic bronchopulmonary aspergillosis, have been reported to result in elevated serum IgD levels (Kohler and Farr, 1967; Berg and Johannson, 1969; Butcher et al., 1975; Luster, Leslie, and Bardana; 1976). A number of more recent studies have found a link between certain atopic disorders and specific IgD antibodies (see section III).

While elevated serum IgD is found in some autoimmune diseases, it is not found in others. Juvenile rheumatoid arthritis patients had significantly elevated serum IgD (Geny et al., 1974), but patients with adult rheumatoid arthritis, Sjogren's syndrome, or autoimmune thyroiditis do not (Rowe et al., 1968, Marcolongo and Marsili, 1975). Scully, Yap, and Boyle (1983) found that among 80 patients with recurrent aphthous stomatitis, a disease of unknown etiology but with autoimmune features, both serum IgE and IgD were elevated over normal controls. Other studies have demonstrated specific IgD antibodies in autoimmune diseases (see section III.).

Buckley and Fiscus (1975) have presented the most thorough analysis of IgD concentrations in immunodeficiency diseases to date. They found among 165 patients with a variety of immunodeficiency diseases, that in patients which were hypogammaglobulinemic with respect to the three major immunoglobulin classes, levels of IgD and IgE were generally also low. In patients with hyperimmunoglobulinemia E however, IgD was also significantly elevated. Recurrent staphylococcal infections were found in many but not all patients with elevated IgE and IgD. Plebani et al., (1983) found that selective IgA deficency was associated with elevated serum and secretory IgM but not IgD. Rundle et al., (1971) found that IgD levels were significantly elevated in patients with Down's syndrome, as compared with non-Down's syndrome

mentally retarded subjects. A detailed discussion of IgD and immunodeficiency diseases can be found in Leslie and Martin (1977) and Preud'homme, Brouet, and Seligmann (1977).

Elevated serum IgD has been identified in a number of other diseases for which the etiology is yet unclear. Two such diseases are sarcoidosis, a systemic granulomatous disorder (Buckley and Trayer, 1972) and periodic fever, a syndrome characterized by occasional unexplained bouts of fever with lymphadenopathy present (Van der Meer et al., 1984; Reeves and Mitchell, 1984). In two patients with periodic fever Reeves and Mitchell (1984) found relatively large numbers of IgD-containing cells in biopsies of lymph node, lung, bone marrow, and rectal epithelium. Decreased numbers of T helper cells (OKT4+) and increased numbers of T suppressor cells (OKT8+) were also seen, which suggested to them that the disease was a subtle form of immunodeficiency.

Serum IgD levels become elevated late in pregnancy. Leslie (1973) found that while control nonpregnant women had 33 ± 46 µg/ml of serum IgD, women in labor had 89 ± 136 µg/ml. He also found that multiparity was assoiated with somewhat higher IgD levels. Oral contraceptive use is also associated with elevated serum IgD (Klapper and Mendenhall, 1971). It was suggested by Klapper and Mendenhall (1971) that the elevated serum IgD in pregnancy may reflect (1) an antibody response

peculiar to the feto-maternal relationship, (2) a change in IgD metabolism brought on by pregnancy, or (3) a change in IgD metabolism secondary to increased levels of sex steroids. These authors found no evidence for IgD crossing the placenta, and so concluded that the increase in serum IgD late in pregancy was probably not associated with its transfer to the fetus (Leslie and Swate, 1972; Cederqvist, Ewool, and Litwin, 1977).

IgD levels by themselves are of only limited usefulness in determining the biological significance of secreted IgD. Although serum IgD has been found to be elevated or depressed in a number of diseases, a definite connection between it and the etiology of a disease has usually not been established. It seems plausible that elevated or depressed IgD is more usually a consequence of disease than a cause. Neither greatly elevated nor low serum IgD is by itself necessarily associated with overt disease (Dunnette et al., 1977; Leslie and Teramura, 1977).

It is clear that in diseases in which there is a chronic exposure to antigen and/or in which there is an exaggerated or disordered immune response, IgD may become elevated. The antigen-specificity of IgD, however, is perhaps more illuminating of its biological significance.

III. Antigen Specific IgD

Clinical Studies. IgD antibodies to a variety of antigens have now been demonstrated in a number of clinical studies, and show that they may appear in serum or milk in response to probable respiratory, enteric, parenteral, intravenous, or autoantigen exposure.

Therapeutic substances such as penicillin, insulin, and diphtheria toxoid have been reported to sometimes stimulate IgD antibody production. An early study reported IgD antibodies to benzylpenicillin in several cases of penicillin allergy (Gleich, Bieger, and Stankievic, 1969). IgD antibodies to insulin in diabetic sera have been reported in two studies, (Devey et al., 1970; Lertora, Gomez-Perez, and Leslie, 1975). Another report has described IgD antibodies to diphtheria toxoid following immunization (Heiner and Rose, 1970).

IgD antibodies to bacterial, and viral antigens have been reported in several studies. Tamura, Fujinaga, and Kuruome (1982) found IgD antibodies to *Mycoplasma pneumoniae* antigens in 19 children with primary atypical pneumonia. They found that IgD antibodies, when compared to other isotypes, reached a peak at a relatively early stage in the infection, decreasing rapidly thereafter. In a study of

hepatitis B patients, while 30% of patients with acute hepatitis B had IgD antibodies to core antigen, 60% of patients with chronic hepatitis B and 66% of patients who were carriers of hepatitis B had such antibodies, indicating that chronic exposure was important in the development of detectable IgD antibodies (Brzozko et al., 1975). In a study of patients with subacute sclerosing panencephaplitis, a progressive degenerative neurologic disease which is thought to be a rare sequel to measles infections, Luster et al., (1976) found 5/5 patients had IgD anti-measles antibodies, while measles convalescent sera and multiple sclerosis patient serum did not. In patients with rubella, IgD and IgE increased rapidly after the onset of infection and remained high for as long as 2 months (Salonen et al., 1985). The authors suggested that assay of IgD anti-rubella antibodies were so reliably produced by the infection that they could be used to diagnose acute rubella. Sewell et al. (1978, 1979) have found natural IgD antibodies to *E. coli* in both serum and colostrum at very low levels in normal subjects. In one case of IgD myeloma, antibody activity of the paraprotein was reported to be against Streptolysin O (Swierczynska et al., 1976). Collectively, these studies indicate that IgD antibodies can be elicited to bacterial or viral antigens after respiratory, gut, or systemic exposure of a short or long duration.

A number of excellent studies have been done on antigen-specific IgD

in hypersensitivity syndromes. Bringel et al., (1982) found that of 177 atopic patients with sensitivity to *Lollium perenne* fraction C, a hay fever allergen, 23 had IgD antibodies to the antigen. In subsequent experiments, IgD antibodies were isolated on an anti-delta immunoadsorbant and shown to specifically block basophil degranulation and histamine release, and to specifically inhibit the RAST assay while control IgD myeloma protein did not. Weliky et al., (1979) in a study of 16 subjects, found a correlation between IgD but not IgE antibodies and hypersensitivity to tartrazine.

Another study of gluten-induced celiac disease (a food allergy to wheat), found that, while total IgD and IgE in serum were normal, IgD but not IgE antibodies specific for wheat antigens were high (Bahna, Tateno, and Heiner, 1980). This and the previous study suggest that IgD may play a more critical role than IgE in some hypersensitivities.

Several studies have reported IgD antibodies in milk. Keller et al., (1985) found evidence for specific IgD antibody production in human colostrum following likely enteric or respiratory exposure to a variety of antigens. They found that colostrum from 6 of 14 lactating mothers had IgD antibodies to bermuda grass and that the colostrum/plasma ratio for these antibodies was ten times that of the total IgD colostrum/plasma ratio. IgD antibodies to beta

lactoglobulin, BSA, and alpha gliadin were also found in some colostrum samples, suggesting that IgD antibodies in milk could be elicited following enteric and respiratory exposure to antigen.

IgD antibodies have also been reported in duodenal fluid and in serum as a result of exposure to bovine milk. One study has reported IgD and IgE antibodies to bovine milk antigens and soy protein were in the duodenal fluid of children suffering from a variety of intestinal disorders (not specified) (Freier, et al., 1983). Galant et al., (1983) found serum IgD antibodies to bovine milk antigens in a 2 year old child with Down's syndrome, pulmonary hemosiderosis, and cor pulmonale who was apparently aspirating the milk. Removal of bovine milk from the diet caused disappearance of symptoms and a drop in serum IgD antibodies, while a restoration of bovine milk caused a return of both symptoms and IgD antibodies to milk antigens in his serum. These studies indicate, again, a connection between gut or respiratory exposure and IgD antibodies in serum and secretions.

IgD antibodies to autoantigens have also been reported. IgD antibodies to nuclear antigens have been found in 50% of patients with systemic lupus erythematosus and 35% of patients with rheumatoid arthritis (Ritchie, 1968; Watson et al., 1969; Kantor et al., 1970; Schmidt and Mueller-Eckhardt, 1973; Luster, Leslie, and Bardana 1976b). IgD anti-nuclear antibodies have also been found in 50% of

patients with chronic liver disease and 33% of patients with scleroderma (Schmidt and Mueller-Eckhardt, 1973). Luster (1976) found between 20-30% of patients with either Raynaud's disease, scleroderma, and discoid lupus erythematosis had IgD antinuclear antibodies. IgD antibodies have been found against thyroid antigens in Hashimoto's thyroiditis (Kantor et al., 1970). Of course, autoantibodies of other isotypes have also been documented in autoimmune disease, therefore it is unclear whether IgD antibodies play a special role in disease etiology or are merely part of a generalized autoantibody induction process.

In summary, IgD antibodies appear to be produced under conditions of chronic or acute stimulation at either respiratory, systemic, or enteric sites. It seems quite possible that the reason IgD antibodies are detected in these clinical states has to do with the amplifying effects of such antigenic exposure on the IgD antibody response. In other states of antigenic stimulation less chronic or acute, IgD antibodies may be produced, but at levels that are not easily detected.

Animal Studies. Apart from the studies presented in this thesis, there is only one report of IgD antibodies induced through immunization of experimental animals (Pauwels et al., 1979). Their assay consisted of ovalbumin-coupled disks incubated with ovalbumen immune sera followed

by an incubation step with radioiodinated anti-IgD. Expressing results as per cent of background binding to disks, they found that intraperitoneal immunization with 5 µg. ovalbumen with *B pertussis* vaccine and aluminum hydroxide, or intraplantar immunization with 250 µg of ovalbumin with Freund's complete adjuvant resulted in peak median responses of 14-16% of background by day 14. A secondary immunization on day 21 appeared to boost and accelerate the response, reaching a second peak seven days later at 18% of background. Intraperitoneal immunization with 5 µg ovalbumin and aluminum hydroxide alone had no effect as did intramuscular immunization with the 1 mg of ovalbumen on day 0 or day 21. Comparison of total serum IgD responses with that of IgD antibody responses indicated that most of the IgD induced by immunization was not specific for the antigen, which necessarily must have been very small, as total serum IgD was between 3 and 10 µg/ml throughout the experiment.

Our studies have endeavored to take advantage of known amplifications of total serum IgD in order to induce more easily measurable IgD antibody responses. A substantial IgD anti-DNP response in rat milk, which was clearly dissociated from IgM and IgA anti-DNP responses, was regularly induced following intramammary immunization during pregnancy with 250 µg DNP-BGG in 1:1 FCA (Steele and Leslie, in press). Another study (see appendices) suggests that IgD antibody responses in milk can also be induced by oral immunization. In a third study, in which

total IgD was found to be elevated in ascitic fluid in rats given intraperitoneal complete Freund's adjuvant (CFA), we found that the IgD antibody response was similarly amplified. Although, as with the studies of Pauwels et al., (1979) the IgD antibodies specific for the immunizing antigen apparently represented only a small fraction of the total IgD produced, these results plus other evidence that the IgD ascitic fluid response could be further optimized, suggest that IgD antibodies to DNP could be specifically purified and their biological functions tested in *in vitro* or *in vivo* experiments.

IV. IgD-Containing Cells in Normal and Diseased Tissue

Pernis et al., (1966), in examining three human spleens found IgD-containing cells existed at a frequency of 0.13, 0.30 and 0.45 per 100 IgG-containing cells. This is in accordance with the estimate that IgD composed less than 1% of normal human serum immunoglobulins (Rowe and Fahey, 1965b). Rowe, Crabbe, and Turner (1968) found lymph nodes, spleen, and intestinal mucosa very poor in IgD-containing cells, with 2700 IgD cells per cubic millimeter, as compared with 350,000 IgA containing cells per cubic millimeter. Adenoids, however were found to be relatively rich in IgD-containing cells. Other studies have also found IgD-containing cells in tonsils and nasopharyngeal mucosa (Tada and Ishizaka, 1970; Ishikawa et al., 1972). In the latter study it was found that IgD-containing cells

were concentrated in the mantle zone surrounding the germinal centers, while IgM and IgG containing cells were scattered throughout the tissue, and IgA-containing cells were localized in the columnar epithelium. IgD-containing cells are also present in the bone marrow (Leslie and Martin, 1977; Van Nieuwkoop and Radl, 1985). In one study Brandtzaeg et al., (1979) had found virtually no IgD-producing cells in jejunal mucosa. In a subsequent study Brandtzaeg (1983) found a very low percentage of IgD-producing cells in colonic mucosa but a higher percentage in four other glandular tissues. The highest percentage of IgD-producing cells was in lacrimal gland tissue with 9.7%. Parotid and submandibular glands had 3.1% and 1.6% respectively, while two samples of mammary tissue had 2.1% and 0.7% IgD producing cells, the first taken from a woman who had been breast feeding regularly for eight months and the second from a woman in the eighth month of pregnancy, both having undergone surgery because of breast tumors. All specimens taken were said to be histologically normal.

The number of IgD-containing cells apparently may increase dramatically in certain diseases. In a case of ulcerative colitis Crabbe and Heremans (1966) reported the presence of a large number of IgD-containing cells in rectal mucosa. Fontaine et al., (1974) found that IgD and IgA-containing cells increased 40- and 3-fold respectively in lymph nodes invaded by non-lymphocytic tumor

metastases, in which the highest percentage was observed in a patient with Hodgkin's disease. This latter observation fits well with studies by Corte et al. (1977, 1978) indicating that IgD serum levels are elevated in some Hodgkin's disease patients (see section II). Similarly Reeves and Mitchell (1984) found unusually large numbers of IgD-containing cells in tissue samples from lung, lymph node, and rectal mucosa in two cases of periodic fever, which is characterized by lymphadenopathy, continual bouts of fever, and elevated serum IgD (see section II).

V. The Structure of Immunoglobulin D

Human IgD. Rowe and Fahey (1965a,b) identified IgD in humans by first observing that it was a protein which upon reduction and alkylation showed the characteristic heavy and light chains in a 3:1 mass ratio. The light chains were identifiable by antiserum to kappa or lambda determinants, but the heavy chains did not react with antisera to any of the then known immunoglobulin classes. The original protein isolated from myeloma patient S.J. was found to have a sedimentation coefficient of 7S, corresponding to an immunoglobulin monomer possessing two heavy and two light chains. The myeloma protein was rapidly digested by papain into Fc and Fab fragments. IgD was also found to be relatively sensitive to heat and acid denaturation (Heiner et al, 1968). Rowe, Dolder, and Welscher (1969) determined the

sedimentation coefficient for two IgD myeloma proteins to be 6.19 and 6.14$S_{20,w}$ respectively, estimating its molecular weight at about 184 kDa. Spiegelberg, Prahl, and Grey (1970), using the same method, found the molecular weight of human IgD to be 172 kDa, while by adding up the molecular weights of component heavy and light chains they arrived at a figure of 166 kDa. They found that there was only one heavy-heavy disulfide link, and one disulfide link between each heavy and light chain. The protein had 11.3% carbohydrate, which was associated with the delta chain. When this carbohydrate was taken into account, the delta chain was found to be identical in size with gamma chains. Plasmin and trypsin cleaved the heavy chain on the C-terminal side of the disulfide link in the hinge region, while papain cleaved the molecule on the N-terminal side. Wolcott et al., (1975) found that when IgD was digested with trypsin at 56°C, the Fc fragment was resistant to further degradation, whereas the Fab portion of the molecule was rapidly degraded. Another study confirmed these results indicating that trypsin cleaved the Fab fragment near the junction of the constant and variable region in both the delta Fd and lambda light chain (Kocher and Spiegelberg, 1979). Jefferis and Matthews (1977) also confirmed these results finding that IgD was rapidly degraded by trypsin to Fab and Fc fragments, with the Fab fragment undergoing further degradation of both the heavy and light chain peptides. As with the intact molecule, the Fc fragment was susceptible to acid and heat denaturation, as detected by changes in

circular dichroism (Jefferis, Matthews, and Bayley, 1978).

Goyert, Hugli, and Spiegelberg (1977), in examining ten different IgD myeloma proteins, found that they consistently showed multiple heavy chain bands when analyzed by polyacrylamide gel electrophoresis, with an average molecular weight of 63.9 kDa and 60.5 kDa. They explained the heterogeneity to the putative action of a "spontaneous" trypsin-like degradation of the carboxy terminal part of the delta chain, since the tryptic Fc, but not the Fab or Fd fragments showed a similar multiple banding on electrophoresis. A similar heterogeneity was found by Corte et al., (1979a,b) but a different conclusion about it was drawn. When IgD from the serum of Hodgkin's disease patients or from normal human serum was isolated and analyzed by SDS-polyacrylamide gel electrophoresis, they found a slow and faster-migrating form of the delta peptide, which they "fingerprinted" by a two-dimensional chromatography and electrophoresis technique. Their results indicated that the faster-migrating delta peptide had three extra peptide fragments. Corte et al. termed the slow and fast forms IgD1 and IgD2 respectively. They subsequently found that membrane-bound IgD had a mobility similar to IgD1, while plasma cells produced both IgD1 and IgD2. They asserted that these were two different forms of IgD, based on the observation that (1) the conversion of one type to the other was never observed, and (2) myeloma patients existed who produced exclusively the IgD1 form (Corte

et al., 1979b). They suggested that the two forms were either different IgD subclasses or glycosylation variants.

The complete amino acid sequence of an IgD myeloma protein (WAH) was determined by Putnam et al., (1982). The delta chain consisted of one variable and three heavy chain domains with an unusually long hinge region between the the first and second constant heavy chain domains, four times as long as that of gamma-1 or gamma-2 chains. The hinge region was subdivided into two regions, an N-terminal region which is resistant to proteolysis and O-glycosylated at four or five sites with GalN glycans, the other region highly susceptible to proteolysis and unglycosylated. N-linked GlcN glycans are attached at three other sites in the Fc region.

Mouse and human IgD show the greatest sequence homology in the third constant domain (53%), while the first constant domain possesses only 25% homology (Putnam et al., 1982). The extended hinge region found in both species also had relatively high homology (about 50%). Putnam et al. speculate that the 64-residue human delta hinge arose from tandem duplication of a 32-residue hinge. The 32-residue hinge found in mouse IgD may be the result of a deletion of one of two hinge exons along with the second constant domain (Tucker et al., 1982). The greater sequence homology in the third constant delta domain and the hinge region suggests that they are of greater importance to the

putative biological function or functions of the molecule.

Using the sequence information for the five immunoglobulin classes, Lin and Putnam (1981) proposed two hypothetical pathways leading to the evolution of the human heavy chain genes. In both pathways the genes evolved through successive tandem duplications of a primordial four-domain complex. In the first pathway the mu-delta-alpha precursor line diverged from the gamma-epsilon precursor line followed successively by divergence of the mu from the delta-alpha and finally divergence of the latter into the delta and alpha genes. The second model suggests that, through a series of duplications ancestral mu-alpha, delta, and gamma-epsilon precursor lines (5'-3') evolved, followed by the duplication of the three genes into six, producing (5'-3') mu, delta, gamma, alpha, a delta-derived gene (subsequently either deleted or undiscovered) and an epsilon gene. However subsequent work by Flanagan and Rabbits (1982) does not support either of these models. They found that the gamma, epsilon, and alpha genes are arranged (5'-3') as gamma 3, gamma 1, epsilon 1 (pseudogene), alpha 1, gamma 2, gamma 4, epsilon, alpha 2. This would suggest that the mu-delta precursor diverged from a gamma-epsilon-alpha precursor line which became gamma, gamma, epsilon, alpha. This latter four-gene complex would then undergo duplication to form an eight gene complex with the 5' epsilon copy becoming a pseudogene.

Mouse IgD. Melcher et al., (1974) identified mouse IgD on mouse splenocytes by its appearance as a cell surface immunoglobulin whose heavy chains were not immunoprecipitable with anti-mu, anti-gamma, or anti-alpha, which migrated faster than mu chains on polyacrylamide electrophoresis, and which was sensitive to proteolysis. Similar observations were made by Abney and Parkhouse (1974). Golding et al, (1979) showed that there was cross-reactivity between mouse, rat and human IgD, allowing their formal identification as IgD. The heavy chain was smaller than the mu chain with an estimated molecular weight of 65.8 kDa, while the intact molecule was between 160-170 kDa. Other estimates of the membrane delta chain molecular weight have been 69 kDa (Radl, Van den Berg, Van der Zidje, 1980) and 68 kDa (Eidels, 1979).

As with human IgD a substantial proportion the heavy chain is composed of carbohydrate. Goding and Herzenberg (1980) in investigating the synthesis of membrane IgD in B lymphocytes found that an intracellular 59 kDa delta chain was produced shortly after a pulse of sulfur-35 labeled methionine, while several hours passed before the 63-72 kDa forms were produced. The 59 kDa delta chain could be reduced to 50 kDa by treatment with endoglycosidase H, indicating that partial translation with "high mannose" type oligosaccharide moieties had taken place shortly after glycosylation, while galactose and sialic acid moieties are added shortly before appearance on the surface of

the cell. Finkelman et al., (1981) showed that, while the secreted IgD heavy chain of IgD plasmacytomas TEPC 1017 and TEPC 1033 had a molecular weight of 63 kDa, TEPC 1017 delta chain mRNA in a cell-free system produced delta chains which were 43 and 44 kDa. Intracytoplasmic synthesis in the presence of tunicamycin, an inhibitor of N-glycosylation, produced a delta chain which was 54 kDa in size. They concluded that, as with membrane IgD, secreted IgD was glycosylated shortly after translation and again shortly before secretion. Similar results were found by Neuberger and Rajewsky (1981) with surface and secreted forms of IgD hybridoma B1-8 delta-1 being 64 kDa and 61 kDa respectively, while the cytoplasmic delta chains were 57 and 56 kDa. Treatment of cells with tunicamycin reduced the molecular weight of both secreted and cytoplasmic forms, with cytoplasmic delta chains reduced to a molecular weight of 44 kDa.

IgD isolated from B lymphocytes, was much more sensitive to trypsin digestion than IgM, producing Fc and Fab fragments within 10 minutes at 25°C (Bourgois et al., 1977). Similar results were observed by two other laboratories, showing that mouse IgD had an inter-chain disulfide link in the Fc region, C terminal to the trypsin cleavage site. (Kessler et al., 1979; Goding, 1980). Goding, (1980) found that trypsin cleaves the Fc fragment within its intra-chain disulfide loop as well. Trypsin treatment of cell-bound IgD removes the Fab but not

the Fc portion of the molecule (Kessler et al., 1979). A secreted IgD anti-NIP hybridoma protein, B1-8.delta-1, was readily degraded by trypsin while the the IgM secreted from parent hybridoma cell line (B1-8) was not (Neuberger and Rajewsky, 1981).

Eidels (1979) found that lymphocyte membrane IgD existed as a monomer and as a half molecule, showing that the half molecules he observed were not likely the result of proteolysis, disulfide interchange, or sulfhydryl-catalyzed reduction. Pollock and Mescher (1980) also reported monomer and "half-mer" forms for membrane IgD, which they designated as IgDI and IgDII respectively, but with somewhat lower estimates for their molecular weights. While Eidels estimated the IgD monomer and half molecule to be 185 kDa and 105 kDa respectively, Pollock and Mescher found them to be 150 kDa and 94 kDa. Pollock and Mescher ascribed their differences in molecular weight estimates to the SDS-polyacrylamide gel electrophoresis system employed, though it was admitted that their values were less in agreement with other estimates based on summation of the estimated molecular weights of individual light and heavy chains. Subsequently this group found that this heterogeneity was correlated with IgD allotype, with mice bearing Igh-5e having a low IgDI/IgDII ratio, while mice bearing Igh-5a and Igh-5b having a high IgDI/IgDII ratio (Pollock, Dorf, and Mescher, 1980). IgDI and IgDII were shown to appear simultaneously during ontogeny (Pollock and Mescher, 1982).

Size heterogeneity has also been observed in secreted IgD. Although secreted IgD from the mouse plasmacytomas TEPC 1017 and TEPC 1033 was reported to have a dimeric structure with a disulfide link between monomeric units (Finkelman et al., 1981), secreted IgD from the mouse hybridoma B1-8 delta-1 was reported to be a monomer (Neuberger and Rajewsky, 1981).

In addition to the heterogeneity in the number of heavy and light chains in IgD, size heterogeneity has also been observed for individual delta chains. Pearson et al., (1977) found delta chains to exist which were apparently 68 kDa and 70 kDa. Mescher and Pollock (1979) confirmed these results and found differences in their sensitivity to proteases and the degree to which they were glycosylated. It was suggested that the two forms may have different biological functions. Vasilov and Ploegh (1982) found that the N-glycosylation of IgD in the B1-8 delta-1 hybridoma was responsible for the heterogeneity observed in this protein. In light of delta chain size heterogeneity it is of interest that Mushinski et al. (1982) found five forms of delta mRNA, two of which appear to be associated with secreted mouse delta chains two associated with membrane forms, and one of unknown function.

Allotypic specificity was first described for mouse IgD by Goding,

Warr, and Warner (1976) At least five IgD allotypic specificities have now been found (Stall and Loken, 1984). Woods et al., (1980 a,b), in surveying the IgD allotypes of 27 strains of mice found no additional ones. In attempting to determine the site of allotypic determinants on the IgD molecule, Kessler et al., (1979) showed that the determinants for allotypic specificities were present both on the trypsin-derived Fab and Fc fragments. Stall and Loken (1984) also found allotypic determinants both in the tryptic Fab and Fc regions of murine IgD.

The primary structure of mouse IgD has been determined indirectly through the sequencing of the mouse IgD gene complex (Tucker et al, 1980; Cheng et al., 1982) which consists of seven exons. The structure and function of the gene complex will be discussed in more detail in section VIII of this review, however the gene structure shows several things about the structure of mouse IgD. First, alternative secretory and membrane exons coding for the secreted and membrane IgD are present, as has also recently been shown for human IgD (White et al., 1985). This indicates that the delta gene, like that of other isotypes, has a provision for secreted and membrane forms. Secondly, the mouse delta hinge is relatively large, though only half as large as that of human IgD (Putnam et al., 1982). This may indicate that the hinge region is relatively important to the putative function of IgD. Thirdly, the domain which would correspond

to the second human constant domain is missing. This indicates that a deletion event occurred late in rodent evolution but before rat and mouse lines diverged. It also suggests that neither the second constant domain nor the absolute length of the delta chain is especially important to a putative biological function.

Rat IgD. As with mouse IgD, rat IgD was first identified as an immunoglobulin on B lymphocytes. Using chicken anti-human IgD, Ruddick and Leslie (1977) found an immunoglobulin which could be immunoprecipitated from radioiodinated surface proteins isolated from B lymphocytes. Among other findings they observed size heterogeneity in the delta chain, with a major species of 73 kDa which co-migrated with rat mu chain and a minor one at 65 kDa. It was speculated that the minor species was a partially degraded delta chain. A similar heterogeneity has also been found in mouse IgD but has been attributed to non-degradative processes (see above). Alcaraz et al. (1981), however, did not find two forms of delta chain in rat B lymphocyte membrane. Their estimate of the molecular weight of the mu chain was higher (80 kDa) and that of the delta chain was lower (60 kDa). The estimated molecular weight of the membrane IgD molecule was 165 kDa.

Bazin et al., (1978) described a transplantable IgD-secreting plasmacytoma derived from Lou/C/Wsl rats, IR-731. Its product

possessed kappa chains but did not react with antiserum to rat IgA, IgM, IgE, or IgG. Its heavy and light chains were 60 kDa and 20 kDa, thus indicating a monomeric structure of 160 kDa. Gel filtration showed it to elute consistently at an apparent molecular weight greater than 570 kDa, which they speculated was a noncovalent aggregate, as the apparent molecular weight on a non-reducing 6% polyacrylamide gel was 140 kDa. Since this was somewhat lower than that of human IgD, they speculated that this was perhaps a submonomeric fragment. Antiserum to the plasmacytoma protein reacted with 30% of spleen or lymph node cells, but not with thymus cells. The determinants recognized appeared late in ontogeny and cross-reacted with mouse lymphocytes in a similar ontogenic pattern. These observations confirmed previous studies of rat B lymphocyte IgD (Ruddick and Leslie, 1977), and based on these observations they identified the IR-731 protein as rat IgD.

A more detailed structural study of the protein was undertaken by Alcaraz et al., (1980). By gel-filtration they found the protein to have a molecular weight of approximately 300 kDa, which suggested that rat IgD, like mouse IgD, might exist as a dimer in its native form. Nonreducing SDS-polyacrylamide electrophoresis, however, yielded a molecular weight of 150 kDa. Their estimate of the size of the rat delta chain was somewhat lower than given by Bazin et al., (1978), 50 kDa. In a subsequent study by Sire et al.(1981), a mutated

nonsecreting form of IR-731 had a cell surface delta chain with a molecular weight of 48 kDa, and was present in the form of covalent dimers without light chains. If rat IgD is similar to that of mouse IgD (see above), one would predict that membrane delta chains are somewhat larger than secretory forms. However this membrane form of the IR-731 delta chain was already less than the previously reported 50 kDa secreted IR-731 delta chain. Since an N-terminal deletion of the delta chain has been observed in mutated forms of the mouse IgD plasmacytoma TEPC 1017 (Thiele et al., 1985) it may be that a similar mutation event has taken place with IR-731.

As with human and mouse IgD, a relatively large amount of carbohydrate is associated with the rat delta chain most or all of which is present in the Fc region and the hinge region of the molecule (Alcaraz et al., 1980). The peptide mass was found to account for 37 kDa of the 50 kDa heavy chains. Sire et al., (1981) confirmed this conclusion by examining the cell-free translation product of IR-731 delta mRNA, which was a 37 kDa delta peptide, indicating that carbohydrate accounted for the additional mass.

The behavior of the IR-731 plasmacytoma protein on exposure to trypsin is quite similar to that of human IgD (Alcaraz et al., 1980). Digestion produced Fc and Fab fragments within 1 minute at 0°C, while in five minutes the Fab fragment but not the Fc fragment showed

evidence of further fragmentation. In a subsequent report they found the tryptic Fab fragment to be 50 kDa, and the Fc fragment 36 kDa. The Fab fragment, on reduction produced 30 kDa and 23 kDa fragments, while the Fc fragment produced a unique 19 kDa fragment. Plasmin degradation of IgD was much slower, taking several hours to produce Fab and Fc fragments. An intermediate 100 kDa fragment was produced which had a delta chain, a light chain, and a 19 kDa peptide (Alcaraz et al., 1981). The presence of lysyl and arginyl residues in the delta hinge region is apparently responsible for the extreme trypsin sensitivity of rat IgD (Sire et al., 1982).

The apparent shortness of the IR-731 delta chain relative to the human delta chain can be accounted for by a deletion of the second constant domain, as has been suggested by amino acid sequencing and sequencing of delta mRNA (Alcaraz et al., 1980; Sire et al., 1981, 1982), which is identical to the region deleted in mice (see above). Homology with the mouse delta chain was highest in the third constant domain and in the DC region (85-90%) and lower in the first constant domain and the hinge region. The size of the hinge region is quite similar to that of mouse delta chains, being half the size of the corresponding region of human delta chains (see above) (Sire et al., 1981, 1982). The higher degree of homology found in the Fc portion of IgD delta chains (as compared to other parts of the molecule) in the rat, mouse, and human indicate that this region of the molecule is

perhaps of greatest functional significance, since its structure has been more strictly conserved through evolution.

VI. IgD as a B Lymphocyte Receptor

IgD is well-established as a major B lymphocyte antigen receptor in several species (see section I). The volume of research which had been devoted to it is perhaps greater than in all other areas of IgD research combined. This considerable body of work has largely addressed questions of (1) its tissue distribution, (2) ontogeny, (3) activation characteristics, (4) role in tolerance and tolerance resistance, (5) functional differences between sIgD and sIgM, (6) functional differences of B lymphocyte subpopulations as defined by surface immunoglobulin phenotype, and (7) the regulation of antibody responses by T lymphocytes. Many experimental approaches have been used to address these questions and much controversy has been generated over the years. Because of the size of the literature and multiplicity of issues it leads into, this review will of necessity only cover what the author considers the major questions and the more recent work bearing directly on IgD.

Tissue distribution and Ontogeny of IgD-bearing B lymphocytes. IgD appears on the surface of a portion of human peripheral blood lymphocytes, where it is usually co-expressed with IgM (Winchester et

al., 1975; Rowe et al., 1973). Similarly, IgD and IgM are coexpressed on the majority of tonsil lymphocytes (Ferrarini et al., 1976). In mice, roughly 36% of spleen cells bear both IgM and IgD, 7% IgM only and 6% IgD only. In Peyer's patches 38% of cells coexpress IgM and IgD, 2-3% are IgM-only, and about 5% are IgD-only. Lymph node cells have about 18% IgM- and IgD-bearing cells, 2-3% bearing IgD or IgM singly. Bone marrow, however, has only 2-3% IgM and IgD bearing cells, about 5% IgM-only cells, and a very small percentage of delta-only cells (about 1%) (Goding, 1978). In rats, Ruddick and Leslie (1977) found IgM and IgD on lymphocytes from the spleen, peripheral blood, lymph nodes, and Peyer's patches, but not the thymus. The ratio of IgD/IgM in membranes from these cells ranged from 0.5 in spleen cells to 2.2 in Peyer's patches. Leslie and Cuchens, (1982) showed that 80% of B lymphocytes coexpressed IgM and IgD.

In human B lymphocyte ontogeny, IgM precedes the appearance of IgD (Gupta et al., 1976). In rats, while IgM was detectable on rat lymphocytes by day 6, IgD did not appear until day 25 (Ruddick and Leslie, 1977). Leslie and Cuchens (1982) later found that fetal rats (day 13) had about 5% IgM+ cells in the liver and < 1% IgD+ cells. Similarly, in mice, IgM+ B lymphocytes are present in day 13 fetuses, (Rosenberg and Parish, 1977), while IgD is first detectable by 3 to 5 days after birth (Kearney et al., 1977).

Several models have been proposed for the ontogeny of B lymphocytes. An early model proposed by Vitetta and Uhr (1975) suggested that the ontogenic sequence was IgM+, IgM+IgD+, IgD+, giving rise to cells responsible for tolerance, primary antibody responses, and secondary antibody responses respectively. Pernis (1977) proposed a somewhat different ontogenic sequence for IgG secreting cells: IgM+, IgM+IgD+, IgM+IgG+, IgG+. IgM+ cells were immature cells which were easily tolerized, which then become mature IgM+IgD+ cells, resistant to tolerization. Differentiation beyond this stage was antigen-driven, giving rise to IgM+IgG+ bearing memory cells and IgG+ antibody secreting cells. Another model proposed by Preud'homme et al. (1977), drew from data from immunodeficiency and immunoproliferative disorders. In it they proposed that the pivotal cell was an IgM+IgD+ cell which, on differentiating to IgG or IgA-secreting plasma cells, gives rise to triple isotype-bearing cells, in the process sequentially losing sIgD and sIgM. Precursors of IgD-secreting cells lose their sIgM, while precursors of IgM-secreting cells may or may not lose sIgD. Cells may in some cases switch to other isotype lineages beyond the pivotal cell stage.

Leslie and Martin (1978) proposed a model for B lymphocyte differentiation which also made the pivotal cell IgM+IgD+. In their model immature IgM+ B cells arise from pre-B cells,and these cells in turn become the mature IgM+IgD+ B cell. In the case of IgG, IgA, and

IgE-secreting cell lineages, antigenic stimulation causes the IgM+IgD+ pivotal cell to acquire a third sIg isotype identical to that it ultimately secretes, which then, during clonal B cell expansion, sequentially loses its sIgM, sIgD and finally all sIg as it becomes a plasma cell. In IgD and IgM-secreting cell lineages, the pivotal cell loses the alternate surface immunoglobulin isotype on terminal differentiation. The decision as to what class of isotype-secreting cell the pivotal cell becomes is determined by the nature of the antigen and by influences of accessory cells. The model was developed from observations that antiserum to IgD and IgM given early enough in ontogeny could eliminate partially or completely certain serum immunoglobulin isotypes. Similar to the Preud'homme model, it was suggested that in some cases cells may be "forced" into another differentiation pathway. An example given of this is the observation that in spite of evidence that most IgG secreting cells have as precursors IgM+IgD+ cells, chronic anti-delta treatment from birth does not eliminate IgG secretion in rats (Leslie and Cuchens, 1982).

A somewhat different model of B lymphocyte differentiation was proposed by Parkhouse and Cooper (1977) and by Cooper et al., (1982). In this model the pivotal cell is the "immature" IgM+ B cell. Isotype-committed sublines diverge from this cell first acquiring the sIg of the isotype ultimately secreted in addition to sIgM. Only afterwards does the subline acquire sIgD. These steps are non-antigen

driven and T cell-independent since they take place in germ free and athymic nude mice. Following antigen or mitogen stimulation these treble-isotype bearing cells, either sequentially lose sIgM and sIgD or just lose sIgD as they differentiate to plasma cells. Memory B cells, developing from the treble-isotype cells may or may not retain these isotype determinants. Parkhouse and Cooper based this model on their observation of the appearance of IgG and IgA on immature IgM+ B cells during ontogeny (Parkhouse and Cooper, 1977; Abney et al., 1978). Such experiments however do not exclude the possible traffic of maternal B lymphocytes into fetal circulation (Vitetta, 1982). Also the sequence of immunoglobulin appearance on B cells is difficult to reconcile with currently accepted theories of the function of the mouse heavy chain gene (see section VIII).

It is fairly well established by now that B lymphocytes do not obligately pass through an IgD+ stage on their way to becoming plasma cells. Bazin et al., (1982) found that anti-delta treatment of rats from birth resulted in a lymphoid B cell population which was exclusively IgM+IgD-, apparently not different from IgM+IgD- cells in untreated control animals. Apart from the not-too-surprising absence of serum IgD after chronic anti-delta treatment, such rats lacked serum IgE and IgG2a, while IgM, IgG1, IgG2b were unaffected, and levels of IgG2c and IgA were elevated. This would suggest that IgM+IgD+ and IgM+IgD- cell lineages give rise to some classes or

subclasses in common but that others are produced preferentially or completely by one or the other lineage. The presence of this pattern in both athymic nude rats and phenotypically normal littermates indicated that T cells were not involved in the phenomenon. Skelly et al., (1983) found that treatment of mice with anti-delta from birth resulted in a population of IgM+IgD- B cells which were phenotypically mature, since they possessed the Lyb-5 marker. Similarly Finkelman, Mond, and Metcalf (1983) found that, while considerable depletion of B cells in murine lymphoid tissues occurred following similar anti-delta treatment, the remaining B cells were a stable population of IgM+IgD-Ia+ cells. Such cells nevertheless could respond to a variety of antigens *in vivo* or *in vitro* (Metcalf, Mond, and Finkelman, 1983)

It also seems likely that sIgD appears on more than one functional type of B cell. Neonatal IgM+IgD- cells do not appear to be functionally identical to adult IgM+IgD- cells. sIa, a determinant on B cells which participates in a number of immunologically important interactions, appears earlier in ontogeny than sIgD (Kearney et al., 1977). Black et al., (1980) identified some memory B cells as IgD+. Kincade et al., (1980) found that anti-delta treatment inhibited the proliferation of some but not all IgD+ cells in soft agar. Roth, Tonda, and Pernis (1982) found that only 2/3 of IgD+ cells internalized anti-delta following binding. Layton and Howard (1978)

found that IgM+IgD+ cells in adult mice show considerable size heterogeneity, and Hardy et al., (1982) using a dual-laser cell sorter defined three IgM+IgD+ B lymphocyte populations. One population made up the majority of B cells in lymph nodes and spleen but is absent in CBA/N mice. The other two populations both stained more strongly with IgM but showed bright and dull IgD staining respectively. From these studies it is apparent that it may be an oversimplification merely to consider the sIg phenotype of a cell and nothing else.

Functional differences between sIgD and sIgM. The existence of IgD and IgM as the major B lymphocyte surface immunoglobulins has led to various attempts to discover a functional difference between the two isotypes. It is well established that individual B lymphocytes show allelic exclusion, a single light chain phenotype, and a single antigen and idiotype specificity. Therefore the functional difference between sIgD and sIgM must reside elsewhere. Structural differences include IgD's possession of a hinge region making it a much more flexible molecule, and its much greater sensitivity to proteolysis (see section V). At least in the mouse, since the intracytoplasmic C-terminal structure is identical in both sIgM and sIgD, these do not offer a structurally-based explanation for a putative functional difference.

An early hypothesis was that sIgD served as a triggering receptor for

the mature cell, while sIgM served as a tolerizing receptor (Vitetta and Uhr, 1975). This was based on the observation that neonatal B cells, which were IgM+IgD- were susceptible to tolerization, while adult IgM+IgD+ B cells were resistant. In support of this hypothesis Vitetta et al. (1977) found that removal of the sIgD from mouse splenic B cells using anti-delta increased the susceptibility of the cells to tolerance induction by thymus dependent (TD) antigens, while removing sIgM from mouse splenic cells decreased susceptibility to tolerization by both TD and thymus independent (TI) antigens. Similarly Cambier et al., (1977) showed that papain treatment of B cells, which selectively removes the sIgD, increased the tolerization susceptibility. Likewise were the findings of Dosch et al., (1979) in examining human peripheral blood lymphocytes. They found that papain removal of sIgD caused cells to become highly sensitive to antigen inactivation and to become less able to cooperate with T-helper cells or their humoral products.

Other studies however do not support sIgM as a tolerizing receptor. Metcalf and Klinman (1976) found that the decline of tolerance susceptibility preceded the appearance of sIgD in ontogeny. Layton et al. (1979) found that IgD+ and IgD- cells separated by cell sorting were equally susceptible to tolerance induction, while, in using a splenic focus assay, they found that IgD+ cells yielded the same number of anti-DNP precursors as did IgD- cells. It is also doubtful

whether neonatal IgM+ cells are identical with adult IgM+ cells or IgM+ cells produced in adult mice following chronic anti-delta treatment from birth. The finding that the latter are capable of responding to immunization by a variety of antigens (Metcalf, Mond, and Finkelman, 1983) and possess Lyb-5 determinants indicates that they are not identical. The observation that adult IgM+IgD- cells (as produced in that experimental system) could respond to antigen indicates that the sIgM does not necessarily mediate tolerance.

There are a number of conflicting reports of the degree to which IgD participates in TI and TD antigen responses. Zitron et al., (1977) reported that anti-delta treatment blocked *in vitro* responses to TI-2 but not TI-1 antigens. Similarly, Cambier et al., (1978) found that *in vitro* responses to the TI-1 antigen TNP-BA were not blocked by anti-delta, while *in vitro* responses to the TD antigen TNP-SRBC were. Buck, Yuan, and Vitetta (1979), however, found that while anti-mu completely blocked *in vitro* or adoptive responses to the TI-1 antigens TNP-BA and TNP-LPS, anti-delta reduced these responses by 70%, and McFadden and Vitetta (1984) found that by negatively enriching splenocytes from 5-12 day old mice for IgD- cells, virtually all of the cells able to respond to TNP-BA had been removed. However, Layton et al.,(1979) found that *in vitro*, sorted sIgD+ and sIgD- splenic B cells from 2 1/2 week old mice both responded to TI and TD antigens, though the sIgD+ cells appeared to respond better. Using the Klinman

splenic focus assay, the same group found that both sIgD+ and sIgD- cells had equal anti-DNP precursor frequencies, although IgM-secreting clones were more frequently derived from sIgD- cells and IgG-secreting clones more frequently derived from IgD+ cells. Marshall-Clarke, Keeler, and Parkhouse, (1983), using a cell sorter to separate IgD+ and IgD- mouse splenic B lymphocytes, found that both populations could respond *in vitro* to TD and TI antigens, but that, among IgD+ cells, responses to TD antigens apparently required sIgD, since these responses could be blocked by anti-delta. TI responses, however, were not blocked by anti-delta among IgD+ cells, which suggested that sIgD did not participate in TI responses. Similarly, experiments in which mice were anti-delta suppressed so as to leave a population of phenotypically mature IgM+IgD-Ia+ cells indicate that both TI and TD responses can be elicited from IgD- cells (Finkelman, Mond, and Metcalf, 1983; Metcalf, Mond, and Finkelman, 1983; Skelly et al., 1983).

In an interesting series of experiments by Pure and Vitetta (1980) it was shown that the differing requirements for sIgD and sIgM in responses to TD and TI antigens may reflect the different functional valence of sIgM and sIgD and the different epitope density of TD and TI antigens. TNP was coupled to polyacrylamide beads at high and low epitope density and used as the stimulating antigen in spleen cell cultures. It was found that, while the low epitope density responses

could be completely abolished by pre-treatment of the cells with anti-Thy 1.2 plus complement, only 50% of the higher epitope density antigen responses were similarly abolished. Likewise anti-delta pretreatment of cells blocked stimulation with low epitope antigen but less so with high epitope antigen. Vitetta (1982) proposed that, because of the greater flexibility of sIgD and the more rigid structure of sIgM, sIgD may be considered divalent and sIgM univalent. TI antigens, because of their high epitope density, do not need the added binding capacity of sIgD for optimal cross-linking, while TD antigens, because of their lower epitope density, require both sIgD and sIgM. She went on to suggest that TI responses in the absence of sIgD may occur because of the ability of the antigens to stimulate or induce the expression of B cell differentiation factor (BCDF) receptors. Alternatively, IgD-binding lymphokines from T cells generate a signal for a specific switch sequence.

IgD and B Lymphocyte Activation. Antibodies to IgD and IgM have been used extensively as probes for the investigation of how B lymphocytes become activated. Sell and Gell (1965a,b) were the first to use anti-sIg in this way, demonstrating *in vitro* blast transformation in rabbit B lymphocytes. Since then, others have shown similar effects with anti-mu, anti-gamma, anti-alpha, anti-f(ab) , and anti-L (reviewed in Kermani-Arab, Leslie, and Burger, 1977). Using anti-delta a variety of results have been reported, ranging from cell death,

suppression, activation, proliferation, differentiation, and migration.

Several studies have examined the relationship between anti-delta treatment and the mitogen responsiveness of cells. Kermani-Arab, Burger, and Leslie (1976) found that treatment of human peripheral blood lymphocytes with anti-delta 1-12 hours prior to stimulation with PHA resulted in an enhancement of PHA-stimulated proliferation. Anti-mu pretreatment did not produce a similar enhancement nor did anti-delta treatment after PHA stimulation. This would suggest that anti-delta caused T lymphocytes to become more amenable to PHA stimulation, perhaps via an efferent stimulus from IgD+ B lymphocytes following IgD binding or, as suggested by Burger et al., (1982) perhaps by reaction of the anti-delta with Fc receptor-bound IgD on T lymphocytes. A subsequent investigation found that anti-delta stimulation of peripheral blood lymphocytes *in vitro* was inversely related both to the ability of the cells to be stimulated by PHA and to serum IgD levels (Kermani-Arab, Leslie, and Burger, 1977). Since the *in vitro* cultures were presumably free of serum IgD, the effect was likely not simply the result of blocking of anti-delta by secreted IgD.

Kermani-Arab, Leslie, and Burger (1977) found that anti-delta *in vitro* resulted in an increase in tritiated thymidine incorporation by

the peripheral blood lymphocytes of patients with chronic lymphocytic leukemia. While patients with 5-52% IgD-bearing lymphocytes had peak stimulation by 3 days, a patient with 1% IgD-bearing lymphocytes showed peak stimulation by day 6. When Martin and Leslie (1979) injected rhesus monkeys with goat anti-delta and then tested peripheral blood lymphocytes for blastogenesis 0, 7, 14, and 21 days post-injection, they found increased tritiated thymidine incorporation in B lymphocytes between days 7 and 21. In another experiment using rabbit anti-delta in rats, Cuchens, Martin, and Leslie, (1978) found that IgM+IgD+ lymphocytes in peripheral blood disappeared from circulation within 24 hours after treatment with anti-delta. Peripheral blood IgM+IgD+ cells recovered to previous levels by day 4 and then peaked at 2 times their pre-treatment level at day 8, which was similar to the pattern observed in rhesus monkeys (Martin and Leslie, 1979). By day 8 hypergammaglobulinemia developed, which was accompanied by increased DNA synthesis in lymphoid organs. Subsequently Cuchens et al., (1979) found that the disappearance of IgM+IgD+ B lymphocytes from peripheral blood was associated with an increase in B lymphocytes in peripheral lymphoid organs, which, because they were not metabolically active, were thought to be the result of homing to these tissues by peripheral blood lymphocytes.

A number of studies have shown B lymphocyte proliferation by *in vitro* treatment with anti-delta in mice (Parker, 1980; Sieckmann, 1980;

Isakson *et al.*, 1980; Pure and Vitetta, 1980; Zitron and Clevinger, 1980), however anti-delta/IgD binding alone cannot cause B cells to terminally differentiate into Ig-secreting cells. Finkelman *et al.* (1982a,b) found that treatment of splenic B lymphocytes with heterologous anti-delta *in vivo* had a similar effect as that seen *in vitro*, however in this case, the cells apparently went on to polyclonal immunoglobulin secretion. Finkelman et al. found that the *in vivo* effect of anti-delta treatment could be dissected into two stages: (1) an early, T cell-independent, carrier-independent stage between 1 and 5 days after injection, and (2) a later T cell-dependent, carrier-dependent stage after day 6, leading to polyclonal secretion of immunoglobulin. The immunoglobulin secretion seen after day 6 was thought to be polyclonal rather than specific for the heterologous antiserum, since very few cells producing immunoglobulin appeared to be producing immunoglobulin to goat IgG, based on binding of FITC-goat IgG, and little IgG1 was removed from the serum by goat IgG immunoadsorbants. From these results they postulated that *in vivo* treatment with goat anti-delta resulted in a direct activation of B lymphocytes, an indirect activation of T lymphocytes which recognized the heterologous antibody as foreign, and a polyclonal stimulation of B cells by T cells or other accessory cells causing them to terminally differentiate and switch to IgG secretion. Muul, Mond, and Finkelman (1983) found that the second TD stage of anti-delta polyclonal activation could be induced in the

spleen cells of 6-8 day old mice, in spite of the fact that the first TI stage of activation apparently did not occur. This indicated to them that at least some of the early phenomena observed in the TI stage were not necessary for the TD stage to occur. In a study of the effect on germinal centers, Flotte, Finkelman, and Thorbecke (1984) showed that during the TI stage, mantle layer B cells undergo blastogenesis, while germinal center proliferation occurs during the TD stage. According to another study by Finkelman et al., (1984), the requirement for anti-delta lasts about 3 days. If goat anti-delta was neutralized by mouse IgD from TEPC 1017 *in vivo* before 3 days, the TD stage was interfered with, while after 3 days neutralization has little effect *provided* the goat anti-delta was replaced by goat IgG. Goat IgG by itself could not induce the two stages of polyclonal activation, but was sufficient to induce T cell help after three days anti-delta exposure. Therefore the T cell help must be non-antigen specific.

In a more recent study Finkelman et al. (1985) found that the dose of anti-delta used was critical to the effect one observed. At low doses (12.5 μg) the antibody failed to activate splenic B cells, but instead had the effect of modulating the IgD from their surface. A somewhat higher dose (50 μg) was found to be able to increase the sIa expression on the cells, while a still higher dose was required to induce DNA synthesis to occur (> 200 μg), which became optimal at 800

μg. The TD stage of anti-delta activation required the higher doses of anti-delta. Thus at least some of the varying effects observed with anti-delta treatment may be a function of the effective concentration of anti-delta used in the experiment. However since administration of a normally suboptimal amount of goat anti-delta (50 μg) along with 750 μg of goat IgG enabled splenic B cells to undergo the TD stage-associated development of IgG1 secreting B cells, the more optimal response of higher doses of goat anti-delta were due to the need for goat IgG and not for more anti-delta. Since neither non-immunogenic anti-delta (Finkelman et al, 1980) nor goat IgG alone could bring about the TD stage of polyclonal B cell activation, the TD stage clearly requires both anti-delta-IgD interaction and the heterologous IgG (Finkelman et al., 1982ab; 1985).

As may be suspected from the results of the studies discussed above, mouse monoclonal anti-delta can also serve as an adjuvant. Finkelman et al. (1980) showed that goat anti-delta, when injected into mice 24 hours before immunization with either TNP-Ficoll (i.p) or TNP-SRBC (i.v), resulted in a twofold enhancement of IgM antibodies and a 3 to 10-fold increase in IgG antibodies. When suboptimal amounts of antigen were used the enhancement was less dramatic. In rats, Cuchens et al. (1981) showed that anti-delta given 3 days before immunization with either DNP-Ficoll or SRBC (both s.c.) resulted in maximal enhancement of antibody responses. An enhancement was observed when

doses of antigen were either suboptimal or optimal. Anti-delta treatment also had the effect of accelerating immune responses and, at least with the TD antigen, SRBC, shifting them from IgG to IgM earlier than normal. The authors speculated that anti-delta may have an adjuvant effect by expanding the pool of antigen-reactive clones through expansion of the B cell population as a whole. However in a subsequent study in which anti-delta treatment was shown to increase lymph node and spleen B cells by between 66 and 100%, PFC against SRBC were greater than would be expected from such an increase, which indicated that there had been either a clonal expansion of antigen-specific B cells or a more extensive recruitment of antigen-specific B cells already present (Cuchens et al., 1982).

Another laboratory found that the route of immunization was a factor in the effect observed. Jacobson et al., (1981) reported that mice treated with monoclonal anti-mouse delta (anti-allotype) from birth followed by i.v. immunization had an enhanced response to antigen, while other studies using s.c. or i.p. immunization found no such enhancement (Baine et al., 1982; Layton et al., 1978). Recently Jacobson et al., (1985) reported that monoclonal mouse anti-delta (anti-allotype) resulted in *decreased* mouse PFC responses to TNP-ficoll or TNP-BA (i.v.) when given a day before immunization. However if the anti-delta was given along with an IL-2 containing supernatant (SN), an enhancement was observed. If the anti-delta

allotype was given without SN in experiments with heterozygous mice responses of the opposite allele were enhanced, while those of the allele recognized by the anti-delta allotype were suppressed. When the anti-delta allotype was given with SN, however, responses from *both* alleles were enhanced. From this the authors concluded that anti-delta allotype had a suppressive effect on IgD+ B cells, but that through its reaction with the B cells it exerted an indirect augmenting effect on B cells of the opposite allotype.

Summary. The function of IgD on B lymphocytes has been difficult to pin down experimentally. In spite of its presence on most mature B lymphocytes, its presence is not mandatory for the development of immune responses. While its appearance in ontogeny is relatively late, its association with the maturation of B cells and the resistance to tolerance induction may only be coincidental. The precise sequence through which sIg is gained and lost in ontogeny and its functional significance is still a matter of controversy. Activation of B cells through anti-delta treatment may lead to the expression of receptors for T cell help, the production of lymphokines which act on T cells to produce help, or it may act directly on T cells via IgD bound to Fc receptors. The exact regulatory circuit is at present largely undetermined. To date there is very little data which relates the possible participation of secreted IgD in the regulatory circuit, nor much consideration of the potential

consequences of the reaction of anti-delta reagents *in vivo* with secreted IgD. Considering the recent reports that IgD in the form of TEPC 1017 and TEPC 1033 can have suppressive or enhancing effects on PFC responses in mice (Xue et al., 1984), and that IgD can induce delta Fc receptor-bearing T cells (Coico et al., 1985a), which themselves have an enhancing effect, it seems increasingly likely that secreted IgD may indeed play a role in the regulation of antibody responses. Whether the function of secreted IgD is one which complements that of membrane IgD or is completely separate remains to be determined.

VII. IgD Fc Receptors

The presence of IgD-specific Fc receptors on lymphoid cells has major implications in assigning to IgD a possible immunoregulatory function. An early survey of cytophilic activity by human Ig isotypes found no cytophilic activity between IgD and human lymphocytes, monocytes, or neutrophils (Lawrence, Weigle, and Spiegelberg, 1975). However Sjoberg (1980), using latex particles coated with IgD, found rosetting among T cell and non-T cell populations which was specifically inhibitable by IgD. Between 0 and 6.5% of peripheral blood lymphocytes (PBL) had IgD receptors, which were mostly associated with the non-T cell population. Nevertheless some of the Fc receptors

appeared to be present on a small percentage of T cells based on staining of rosetting cells. Using aggregated IgD, Rudders and Anderson (1982) found Fc receptors on 2-3.7% of PBL, but found none on human T cells nor on four T leukemia cell lines. Burger et al., (1982) presented indirect evidence for IgD Fc receptors by showing that E-rosetting mononuclear cells from IgD-rich peripheral blood had 2.7% IgD+ cells. When such cells were cultured for five days in the presence of PHA and anti-IgD, the per cent IgD+ cells increased to 3.9%.

In mice, Xue et al., (1984) found that plasmacytoma-derived IgD (TEPC 1017 and TEPC 1033) could enhance antibody responses in euthymic but not athymic nude mice, an effect which could be transferred by T cells from the mice. In a follow-up study Coico et al., (1985a), using a rosetting assay with IgD-coated erythrocytes, found that IgD Fc receptor-bearing T cells (which were Lyt 1+2-) could be induced *in vivo* or *in vitro* by exposure to the plasmacytoma IgD. Subsequently Coico et al., (1985b) found that immune responses to TNP-KLH could be augmented by adoptive transfer of these cells into syngeneic mice. Thus it appears that secreted IgD induces a population of T cells which in turn secrete non-antigen-specific helper factors to enhance antibody responses. In view of the fact that treatment of T cell populations with IgE also induces an analogous increase in Lyt 1+2- IgE Fc receptor-bearing T cells which in turn secrete IgE binding

factors regulating IgE antibody responses (Ishizaka, 1984), and that IgG and IgA-binding factors have also been identified which may have similar properties (Fridman, Guimezanes, and Gisler, 1977; Yodoi et al., 1983); it seems plausible to suspect that the IgD Fc receptor-bearing T cells described by Coico (1985ab) may confer their augmentation through the secretion of an IgD-binding factor. If this is true, then one may expect that IgD+ B cells but not IgD- B cell responses would be enhanced by such factors.

VIII. Structure and Function of the Delta Gene

The structure of the delta gene has now been determined for both mice (Tucker et al., 1980) and humans (White et al., 1985). The coding exons of the murine delta gene are the following (5' to 3'): first constant domain exon, hinge region exon, third constant domain exon followed by a large intervening sequence, the secreted IgD C-terminus exon, an exon resembling the secreted C-terminus of undetermined function, and finally two exons coding for the membrane IgD C-terminus. The human delta gene (5' to 3') consists of the first constant domain exon, two exons coding for the hinge region, the second and third constant domain exons, the secreted IgD C-terminus exon, and two exons coding for the membrane C-terminus. Thus major differences in the human and mouse gene superstructure are (1) the lack of a second hinge exon and the second constant region domain in

mouse IgD, (2) the presence of only three C-terminus coding exons rather than four as in the mouse, (3) the different spacing of the exons in the gene complex and the greater distance from the mu gene of the human delta gene. Major similarities are (1) the location of the delta gene immediately 3' from the mu gene, (2) the fact that both the secreted and membrane C-terminal exons are separated from the body of the delta gene by an intervening sequence, and (3) the fact that both genes occupy a relatively large area (10 kbp). The amino acid sequence of the human delta chain which was deduced from the gene sequence confirmed the findings previously determined by direct amino acid sequencing with only minor exceptions (Putnam et al., 1982).

The coexpression of IgD and IgM and of other isotypes on B lymphocytes presents a theoretical problem concerning the production of transcripts from the heavy chain genes. Studies of the gene organization of murine plasmacytomas has shown that under these circumstances the expression of a given isotype is accompanied by the rearrangement of the V region 5' of the corresponding constant region gene (reviewed by Blattner and Tucker, 1984). However experiments in which IgD and IgM are both removed from B lymphocytes have shown that both isotypes reappear, indicating that both are synthesized at the same time. From this it is conceivable that either mu chain mRNA persists following a rearrangement of the V region 5' to the delta gene, or that the mu and delta mRNA are products of the analogous gene

on different homologous chromosomes. However using cell hybridization studies Wabl et al., (1980) showed that IgM and IgD may be simultaneously expressed by a single chromosome containing the mouse heavy chain, and found no evidence for long-lived mRNA to account for dual expression. Simultaneous production of functional H chain mRNA from both homologous chromosomes seems unlikely in view of the generally accepted concept of allelic exclusion and the absence of evidence to the contrary. Other studies have shown that IgM+IgD+ B cells possess a V region gene rearranged 5' of the mu gene in the expressed allele (Knapp et al., 1979; Moore et al., 1981; Maki et al. 1981). Therefore the explanation for the coexpression of IgD with IgM requires that delta mRNA be the result of splicing of a transcript running the length of the mu-delta locus. Experimental evidence in support of this hypothesis has come from studies of transcription in IgM+IgD+ B lymphomas (Mather et al., 1984).

The phenotypic expression of sIgD and sIgM does not appear to reflect the relative transcriptional rate of the mu vs the delta gene. In spite of the fact that IgD is present on the majority of murine B lymphocytes at a ratio to sIgM of 10:1 (Havran, DiGiusto, and Cambier, 1984), the transcriptional rate of mu/delta is present in such cells at a ~3:1 ratio (Yuan and Tucker, 1984). Yuan and Tucker also found that the ~3:1 ratio was maintained in neonatal mouse B cells which express much more sIgM than sIgD, and that although IgD quickly

disappears from cells stimulated by LPS, the transcription ratio is still relatively low at 8:1. The authors conclude that the differential expression of IgM and IgD is regulated by a complex mechanism operating at several levels. Yuan (1984) reported that the turnover rate for resting B lymphocyte sIgM was higher than for sIgD, which indicates one way in which this regulation may be achieved and how sIgD can so predominate on a cell producing 3 times more mu chain mRNA.

The simultaneous production of delta and mu mRNA implies a mechanism whereby a variety of transcripts are produced from the same mu/delta locus. Tucker (1985) suggests that RNA "cleavage events" determine the nature of subsequent processing. Thus a transcript which is cleaved 3' of the body of the mu gene but 5' of the membrane mu C-terminal exons is processed to become secreted mu mRNA, while a transcript which terminates after the membrane delta C-terminal exons is processed to become membrane delta mRNA. Termination at other points result in mRNA's for the other products of the mu/delta locus. Two models have been proposed to explain how a resting B cell can produce both sIgD and sIgM while an IgM-secreting plasma cell produces primarily secreted IgM. One model proposed by Mather et al., (1984) is that there are small number of regulatory factors which control the cleavage events of the immunoglobulin genes and those of other analogously functioning genes. The other model, referred to as the

"gauntlet model", proposed by Blattner and Tucker (1984), has the regulation of transcript cleavage controlled by the regulation of levels of an endonuclease or "endase". Thus in a resting B cell, in which the endase is at low concentrations, a greater number of intact transcripts reach the end of the delta gene and are processed to membrane delta mRNA. In contrast, in an IgM-secreting cell, in which the endase is at higher concentrations, fewer intact transcripts will reach the end of the delta gene and most will be cleaved before transcription reaches the mu C-terminus exons, thus resulting in mostly secreted mu mRNA.

The simplest explanation for gene arrangement in IgD-secreting plasma cells would be the deletion of the mu gene and the rearrangement of the V region 5' to the delta gene. This appears to have taken place with the TEPC 1017 IgD plasmacytoma (Gilliam et al., 1984) and in the rat IR-731 IgD plasmacytoma (Moore et al., 1981). In the mouse system at least, although isotype switching is thought to be mediated by "switch sites" for most isotypes, no sequences identifiable as switch sites have been found between the murine delta and mu gene. Gilliam et al., (1984) concluded from studies of the TEPC 1017 IgD plasmacytoma that deletion of the mu gene had taken place through illegitimate recombination and suggested that this may explain the low frequency of IgD-secreting cells in mouse tissues. However Blattner and Tucker (1984) believe that such "non-switch rearrangement" was an

artifact of transformation, and that normally, IgD secretion is regulated transcriptionally. Whether switching of the V region 5' to the IgD gene is a product of illegitimate recombination in the rat and human systems and what the arrangement of exons is in normal non-neoplastic IgD-secreting cells remains to be determined.

References

Abney, E.R., Cooper, M.D., Kearney, J.F., Lawton, A.R., and Parkhouse, R.M.E. (1978). Sequential expression of immunoglobulin on developing mouse B lymphocytes: A systematic survey that suggests a model for the generation of immunoglobulin isotype diversity. *J. Immunol.* 120: 2041.

Abney, E.R., and Parkhouse R.M.E. (1974). Candidate for immunoglobuin D present on murine B lymphocytes. *Nature (Lond.)* 252: 600.

Alcaraz, G., Bourgois, A., Moulin, A., Bazin, H., and Fougereau, M. (1980). Partial structure of a rat IgD molecule with a deletion in the heavy chain. *Ann. Immunol. (Inst. Pasteur)* 131C: 363.

Alcaraz, G., Colle, A., Boned, A., Kahn-Perles, B., Sire, J., Bazin, H., and Bourgois, A. (1981). Tryptic and plasmic cleavage of a rat myeloma IgD. *Mol. Immunol.* 18: 249.

Bachmann, R. (1967). Serum gamma-D globulin in conditions with pathological proteins (M-components) and in mononucleosis. In *Gamma Globulins* (J. Killander, ed.) *Nobel Symposium* Vol. 3. Almquist and Wiksell, Uppsala. p. 605.

Bahna, S.I., Heiner, D.C., and Myhre, B.A. (1983a). Changes in serum IgD in cigarette smokers. *Clin. exp. Immunol.* 51: 624.

Bahna, S.I., Heiner, D.C., and Myhre, B.A. (1983b) Immunoglobulin E pattern in cigarette smokers. *Allergy* 38: 57.

Bahna, S.I., Keller, M.A., and Heiner, D.C. (1982). IgE and IgD in human colostrum and plasma. *Pediatr. Res.* 16: 604.

Bahna, S.I., Tateno, K., and Heiner D.C. (1980). Elevated IgD antibodies to wheat in celiac disease. *Ann. Allergy* 44: 146.

Baine, Y., Chen, Y., Jacobson, E.B., Pernis, B., Siskind, G.W., and Thorbecke, G.J. (1982). Physiology of IgD: II. lack of humoral immune responsiveness in lymph nodes of mice treated with anti-IgD from birth. *Eur. J. Immunol.* 12: 882.

Bargellesi, A., Corte, G., Corsulich, G., and Ferrarini, M. (1979). Presence of serum IgD and IgD-containing cells in the mouse. *Eur. J. Immunol.* 9: 490.

Bazin, H., Beckers, A., Urbain-Vansanten, G., Pauwels, R., Tilkin, A.F., Platteau, B., and Urbain, J. (1978). Transplantable IgD immunoglobulin-secreting tumors in rat. *J. Immunol.* 121: 2077.

Bazin, H., Gray, D., Platteau, B., and MacLennon, I.C.M. (1982). Distinct delta + and delta- B- lymphocyte lineages in the rat. In *Immunoglobulin D: Structure and Function*, (G.J. Thorbecke and G.A. Leslie, eds.). *Ann. N.Y. Acad. Sci.* 399: 157.

Bazin, H., Platteau, B., Pauwels, R., and Capron, A. (1980). Immunoglobulin production in nude rats with special attention to the IgE isotype. *Ann. Immunol. (Inst. Pasteur).* 131C: 31.

Berg, T., and Johansson, S.G.O. (1969). Immunoglobulin levels during childhood with special regard to IgE. *Acta Paediatr. Scand.* 58: 513.

Black, S.J., Tokuhisa, T., Herzenberg, L.A., and Herzenberg, L.A. (1980). Memory B cells at successive stages of differentiation: expression of surface IgD and capacity for self-renewal. *Eur. J. Immunol.* 10: 846.

Blattner, F.R., and Tucker, P.W. (1984). The molecular biology of immunoglobulin D. *Nature* 307: 417.

Bourgois, A., Abney, E.R., and Parkhouse, R.M.E. (1977). Mouse immunoglobulin receptors on lymphocytes: identification of IgM and IgD molecules by tryptic cleavage and a postulated role for cell surface

IgD. *Eur. J. Immunol.* 7: 210.

Brandtzaeg, P., Gjeruldsen, S.T., Korsrud, F., Baklien, K., Berdal, P., and Ek, J. (1979). The human secretory immune system shows striking heterogeneity with regard to involvement of J-chain positive IgD immunocytes. *J. Immunol.* 122: 503.

Brandtzaeg, P. (1983). The secretory immune system of lactating human mammary glads compared with other exocrine organs. In *The Secretory Immune System* (J.R. McGhee and J. Mestecky, eds.) *Ann. N.Y. Acad. Sci.* 409: 353.

Bringel., H., Vela, C., Urena, V., Gurbindo, D., Garcia, R., and Lahoz, C. (1982). IgD antibodies in vitro blocking activity of IgE mediated reactions. *Clinical Allergy* 12: 37.

Brzozko, W.J., Mikulska, B., Cianciara, J., and Babiuch, L. (1975). Immunoglobulin classes of antibody to hepatitis B core antigen. *J. Inf. Dis.* 132: 1.

Buck, L.B., Yuan, D., and Vitetta, E.S. (1979). A dichotomy between the expression of IgD on B cells and its requirement for triggering such cells with two T-independent antigens. *J. exp. Med.* 149: 987.

Buckley, R.H. and Fiscus, S.A. (1975). Serum IgD and IgE concentrations in immunodeficiency diseases. *J. Clin. Invest.* 55: 157.

Buckley, R.H. and Trayer, H.R. (1972). Serum IgD in sarcoidosis and tuberculosis. *Clin. exp. Immunol.* 10: 257.

Burger, D.R., Regan, D, Williams, K., and Leslie, G.A. (1982). Human T-cell activation is augmented by monoclonal antibody to IgD. In *Immunoglobulin D: Structure and Function*, (G.J. Thorbecke and G.A. Leslie, eds.). *Ann. N.Y. Acad. Sci.* 399: 227.

Butcher, B.T., Salvaggio, J.E., and Leslie, G.A. (1975). Secretory and humoral immunologic response of atopic and non-atopic individuals to intranasally administered antigen. *Clin. Allergy* 1: 33.

Cambier, J.C., Ligler, F.S., Uhr, J.W., Kettman, J.R., and Vitetta, E.S. (1978). Blocking of primary in vitro antibody responses to thymus-independent and thymus dependent antigens with antiserum specific for IgM or IgD. *Proc. Nat. Acad. Sci.* 75: 432.

Cambier, J.C., Vitetta, E.S., Kettman, J.R., Wetzel, G.M., and Uhr, J.W. (1977). B cell tolerance: III. Effect of papain-mediated cleavage of cell surface IgD on tolerance susceptibility of murine B

cells. *J. exp. Med.* 146: 107.

Cederqvist, L.L., Kimball, A.C., Ewool, L.C., and Litwin, S.D. (1977).Fetal immune response following congenital toxoplasmosis. *Obstet. Gynecol.* 50: 200.

Cederqvist, L.L., Ewool, L.C., and Litwin, S.D. (1977). IgD and the fetal immune response. *Scand. J. Immunol.* 6: 821.

Chen, C.H., Lehymeyer, J.E., and Cooper, M.D. (1982). Evidence for an IgD homologue on chicken lymphocytes. *J. Immunol.* 6: 2580.

Cheng, H., Blattner, F.R., Fitzmaurice, L., Mushinksi, J.F., and Tucker, P.W. (1982). Structure of genes for membrane and secreted murine IgD heavy chains. *Nature* 296: 410.

Chess, Q., Daniels, J., North, C., Lerner, C., Cheskin, L., and Macris, N.T. (1983). Elevation of serum immunoglobulin D (IgD) in patients with the acquired immuno-deficiency syndrome (AIDS). *Fed. Proc.* 42: 1335.

Coico, R.F., Xue, B., Wallace, D., Pernis, B., Siskind, G.W., and Thorbecke, G.J. (1985). T cells with receptors for IgD. *Nature* 316: 744.

Coico, R.F., Xue, B., Wallace, D., Siskind, G.W., and Thorbecke, G.J. (1985). Physiology of IgD: VI. Transfer of the immunoaugmenting effect of IgD with T-delta containing helper cell populations. *J. exp. Med.* 162: 1852.

Colwell, E.J., Bernier, G.M., and Fife, E.H., (1971). Serum immunoglobulin D and malria antibodies in South Vietnamese. *Trans. R. Soc. Trop. Med. Hyg.* 65: 310.

Cooper, M.D., Kuritani, T., Chen, C., Lehmeyer, J.E. and Gathings, W.E. (1982). Expression of IgD as a function of B-cell differentiation. In *Immunoglobulin D: Structure and Function*, (G.J. Thorbecke and G.A. Leslie, eds.). *Ann. N.Y. Acad. Sci.* 399: 146.

Corte, G., Ferrarini, M., Tonda, P., and Bargellesi, A. (1977). Increased serum IgD concentrations in patients with Hodgkin's disease. *Clin. exp. Immunol.* 28: 359.

Corte, G., Ferraris, A.M., Rees, J.K.H., and Bargellesi, A. (1978). Correlation of serum IgD level with clinical and histologic parameters in Hodgkin disease. *Blood* 52: 905.

Corte, G., Tonda, P., Corsulich, E., Milstein, C.P., Bargellesi, A.,

and Ferrarini M. (1979). Characterization of IgD: I. Isolation of two molecular forms from serum. *Scand. J. Immunol.* 9: 141.

Corte, G., Viale, G., Corsulich, E., Bargellesi, A., and Ferrarini, M. (1979). Characterization of IgD: II. Molecular forms of IgD in human B cells. *Scand. J. Immunol.* 10: 275.

Cox, D.S., Taubman, M.A., Ebersole, J.L., and Smith D.J. (1980). Secretory Antibody response to local injection of soluble or particulate antigens in rats. *Mol. Immunol.* 17: 1105.

Crabbe, P.A., and Heremans, J.F. (1966). Presence of large numbers of plasma cells containing IgD in the rectal mucosa of a patient with ulcerative colitis. *Acta Clin. Belg.* 21: 73.

Cuchens, M.A., Bost, K.L., Hoover, M.L., and Leslie, G.A. (1981). Anti-IgD enhancement of primary antibody responses in rats. *Cell. Immunol.* 63: 293.

Cuchens, M.A., Hoover, M.L., Bost, K.L., and Leslie, G.A. (1982). The enhancing effects of anti-IgD on B lymphocyte functions in rats. In *Immunoglobulin D: Structure and Function*, (G.J. Thorbecke and G.A. Leslie, eds.). *Ann. N.Y. Acad. Sci.* 399: 329.

Cuchens, M.A., Martin, L.N., and Leslie, G.A. (1978). The effect of anti-IgD on serum immunoglobulins, antibody production, and immunoglobulin-bearing cells in adult rats. *J. Immunol.* 6: 2257.

Devey, M., Sanderson, C.J., Carter, D., and Coombs, R.R.A. (1970). IgD antibody to insulin. *Lancet* 2: 1280.

Dosch, H.M., Kwong, S., Tsui, F., Zimmerman, B., and Gelfand, E.W. (1979). Role of surface IgM and IgD in the functional differentiation of human and B lymphocytes: effect of papain treatment. *J. Immunol.* 123: 557.

Dunnette, S.L., Gleich, G.J., Miller, R.D., and Kyle, R.A. (1977). Measurement of IgD by a double antibody radioimmunoassay: Demonstration of an apparent trimodal distribution of IgD levels in normal human serum. *J. Immunol.* 119: 1727.

Dunnette, S.L., Gleich, G.J., and Weinshilboum, M. (1978). Inheritance of low serum immunoglobulin D. *J. Clin. Invest.* 62: 248.

Eidels, L. (1979). IgD is present on the cell surface of murine lymphocytes in two forms: delta L and delta-L. *J. Immunol.* 123: 896.

Ferrarini, M., Corte, G., Viale, G., Durante, M.L., and Bargellesi, A. (1976). Membrane Ig on human lymphocytes: rate of turnover of IgD and IgM on the surface of human tonsil cells. *Eur. J. Immunol.* 6: 372.

Finkelman, F.D., Kessler, S.W., Mushinski, J.F., and Potter, M. (1981). IgD-secreting murine plasmacytomas: Identification and partial characterization of two IgD myeloma proteins. *J. Immunol.* 126: 680.

Finkelman, F.D., Mond, J.J., and Metcalf, E.S. (1983). Effect of neonatal anti-delta antibody treatment on the murine immune system. I. Suppression of development of surface IgD+ B cells and expansion of a surface IgM+IgD- B lymphocyte population. *J. Immunol.* 131: 593.

Finkelman, F.D., Scher, I., Mond, J.J., Kessler, S., Kung, J.T., and Metcalf, E.S. (1982). Polyclonal activation of the murine immune system by an antibody to IgD: II. Generation of polyclonal antibody production and cells with surface IgG. *J. Immunol.* 129: 638.

Finkelman, F.D., Scher, I., Mond, J.J., Kung, J.T., and Metcalf, E.S. (1982). Polyclonal activation of the murine immune system by an antibody to IgD. I. Increase in cell size and DNA synthesis. *J. Immunol.* 129: 629.

Finkelman, F.D., Smith, J., Villacreses, N., and Metcalf, E.S. (1985). Polyclonal activation of the murine immune system by an antibody to IgD. VI. Influence of doses of goat anti-mouse delta chain and normal goat IgG on B lymphocyte proliferation and differentiation. *Eur. J. Immunol.* 15: 315.

Finkelman, F.D., Smith, J., Villacreses, N., and Metcalf, E.S. (1984). Polyclonal activation of the murine immune system by an antibody to IgD. VII. Demonstration of the role of nonantigen specific T help in in vivo B cell activation. *J. Immunol.* 133: 550.

Finkelman, F.D., Woods, V.L., Berning, A., and Scher, I. (1979). Demonstration of mouse serum IgD. *J. Immunol.* 123: 1253.

Flanagan, J.G., and Rabbitts, T.H. (1982). Arrangement of human immunoglobulin heavy chain constant region genes implies evolutionary duplication of a segment containing gamma, epsilon and alpha gene. *Nature* 300: 709.

Flotte, T.J., Finkelman, F.D., and Thorbecke, G.J. (1984). Polyclonal activation of the murine immune system by an antibody to IgD: V. Effect on germinal center. *Eur. J. Immunol.* 14: 725.

Fontaine, M., Mercier, P., and Mongin, M. (1974). Report of IgA, IgD,

IgG and IgM secreting plasma cells in cancerous lymph nodes. *Biomedicine* 21: 168.

Fraser, P.A., and Schur, P.H. (1981). Hypoimmunoglobulinemia D: Frequency, family studies, and association with HLA. *Clin. Imm. Immunopathol.* 19: 67.

Freier, S., Lebenthal, S., Freier, M., Shah, P.C., Park, B.H., and Lee, P.C. (1983). IgE and IgD antibodies to cow milk and soy protein in duodenal fluid: effects of pancreozymin and secretin. *Immunol.* 49: 69.

Fridman, W.H., Guimezanes, A., and Gisler, R.H. (1977). Characterization of immunoglobulin binding factors (IBF): I. Production of IBF by a theta-positive lymphoma (L 5178 Y). *J. Immunol.* 119: 1266.

Galant, S., Nussbaum, E., Wittner, R., DeWeck, A.L., and Heiner, D.C. (1983). Increased IgD milk antibody responses in a patient with Down's syndrome, pulmonary hemosiderosis, and cor pulmonale. *Ann. Allergy* 51: 446.

Geny, B., Rodary, C., Griscelli, C., and Mozziconacci, P. (1974). Immunoglobulin D (IgD) in childhood I. Maturation of serum IgD: study

of a population of healthy children. *Biomedicine* 20: 40.

Gerard, J.W., Heiner, D.C., Ko, C.G., Mink, J.T., and Myers, A. (1981). IgD levels in white and Metis communities in Saskatchewan. *Ann. Allergy* 46: 321.

Gilliam, A.C., Shen, A., Richards, J.E., Blattner, F.R., Mushinski, J.F., and Tucker, P.W. (1984). Illegitimate recombination generates a class-switch from C-mu to C-delta in an IgD-secreting plasmacytoma. *Proc. Nat. Acad. Sci.* 81: 4164.

Gleich, G.J., Bieger, R.C., and Stankevic, R. (1969). Antigen combining activity associated with immunoglobulin D. *Science* 165: 606.

Goding, J.W. (1978). Allotypes of IgM and IgD receptors in the mouse: A probe for lymphocyte differentiation. *Cont. Top. Immunobiol.* 8: 203.

Goding, J.W. (1980). Structural studies of murine lymphocyte surface IgD. *J. Immunol.* 124: 2082.

Goding, J.W., and Herzenberg, L.A. (1980). Biosynthesis of lymphocyte surface IgD in the mouse. *J. Immunol.* 124: 2540.

Goding, J.W., Warr, G.W., and Warner, N.L. (1976). Genetic polymorphism of IgD-like cell surface immunoglobulin in the mouse. *Proc. Nat. Acad. Sci.* 73: 1305.

Golding, H., Cuchens, M., Leslie, G.A., and Rittenberg, M.B. (1979). Cross-rectivity of rat, mouse and human IgD. *J. Immunol.* 123: 2751.

Goyert, S.M., Hugli, T.E., and Spiegelberg, H.L. (1977). Sites of "spontaneous" degradation of IgD. *J. Immunol.* 118: 2138.

Gupta, S., Pahwa, R., O'Reilly, R., Good, K.A., and Siegel, F.P. (1976) Ontogeny of lymphocyte subpopulations in human fetal liver. *Proc. Nat. Acad. Sci.* 73: 919.

Hardy, R.R., Hayakawa, K., Haaijman, and Herzenberg, L.A. (1982). B-cell subpopulations identifiable by two-color fluorescence analysis using a dual-laser FACS.In *Immunoglobulin D: Structure and Function* (G.J. Thorbecke and G.A. Leslie, eds.) *Ann. N.Y. Acad. Sci.* 399: 112.

Havran, W.L., DiGiusto, D.L., and Cambier, J.C. (1984). mIgM:mIgD ratios on B cells: mean mIgD expression exceeds mIgM by 10-fold on most splenic B cells. *J. Immunol.* 132: 1712.

Heiner, D.C., and Rose, B. (1970). A study of antibody responses by radioimmunodiffusion with demonstration of gamma- D antigen-binding activity in four sera. *J. Immunol.* 104: 691.

Heiner, D.C., Saha, A., and Rose, B. (1968). Lability of normal and myeloma IgD. *Fed. Proc.* 27: 489.

Isakson, P.C., Uhr, J.W., Krolick, K.A., Finkelman, F.D., and Vitetta, E.S. (1980). Acquisition of cell surface IgD after in vitro culture of neoplastic B cells from the murine tumore BCL-1. *J. exp. Med.* 151: 749.

Ishikawa, T., Wicher, K., and Arbesman, C.E. (1972). Distribution of immunoglobulins in palatine tonsils. *Int. Arch. Allergy Appl. Immunol.* 43: 801.

Ishizaka, K., (1984). Regulation of IgE synthesis. *Ann. Rev. Immunol.* 2: 159.

Jacobson, E.B., Baine, Y., Chen, Y.W., Flotte, T., O'Neil, M.J., Pernis, B., Siskind, G.W., Thorbecke, G.J., and Tonda, P. (1981). Physiology of IgD: I. Compensatory phenomena in B lymphocyte activation in mice treated with anti-IgD antibodies. *J. exp. Med.* 154: 318.

Jacobson, E.B., Xue, B., O'Neil, M.J., Siskind, G.W., Pernis, B., and Thorbecke, G.J. (1985). Physiology of IgD: V. Enhancement of antibody responses in vivo by allo-anti IgD is due primarily to an indirect effect on B cells. *J. Immunol.* 135: 2293.

Jefferis, R., and Matthews, J.B. (1977). Studies of human IgD myeloma proteins: proteolytic digestion patterns. *Immunochem.* 14: 171.

Jefferis, R., Matthews, J.B., and Bayley, P.M. (1978). Studies of human IgD myeloma proteins. Conformational changes induced in the Fc-delta fragment on heating and exposure to acid pH. *Immunochem.* 15: 19.

Jokipii, A.M.M., and Jokipii, L. (1982). Serum IgG, IgA, IgM and IgD in giardiasis: the most severely ill patients have little IgD. *J. Infect.* 5: 189.

Kantor, G.L., Van Herle, A.J., and Barnett, E.V. (1970). Auto antibodies of the IgD class. *Clin. Exp. Immunol.* 6: 951.

Kearney, J.F., Cooper, M.D., Klein, J., Abney, E.R., Parkhouse, R.M.E., and Lawton, A.R. (1977). Ontogeny of Ia and IgD on IgM-bearing B lymphocytes in mice. *J. exp. Med.* 146: 297.

Keller, M.A., Heiner, D.C., Myers, A.S., and Reisinger, D.M. (1985). IgD in human colostrum. *Pediatr. Res.* 19: 122.

Kermani-Arab, V., Burger, D.R., and Leslie, G.A. (1976). Structure and biological functions of human IgD: X. Enhancement of PHA responsiveness by anti-delta activated lymphocytes. *J. Immunol.* 117: 467.

Kermani-Arab, V., Leslie, G.A., and Burger, D.R. (1977). Structure and biological function of human IgD: IX. Anti-IgD activation of human lymphocytes. *Int. Archs. Allergy appl. Immunol.* 54: 1.

Kessler, S.W., Woods, V.L., Finkelman, F.D., and Scher, I. (1979). Membrane orientation and localization of multiple and distinct allotypic determinants of mouse lymphocyte IgD. *J. Immunol.* 123: 2772.

Kincade, P.W., Lee, G., Scheid, M.P., and Blum, M.D. (1980). Characterization of murine colony forming B cells II. Limits to in vitro maturation, Lyb-2 expression, resolution of IgD+ subsets and further population analysis. *J. Immunol.* 124: 947.

Klapper, D.G., and Mendenhall, H.W. (1971). Immunoglobulin D

concentration in pregnant women. *J. Immunol.* 107: 912.

Knapp, M.R., Severinson-Gronowicz, E., Schroeder, J. and Strober, S. (1979). Characterization of a spontaneous murine B cell leukemia (BCL 1) II. Tumor cell proliferation and IgM secretion after stimulation with LPS. *J. Immunol.* 123: 1000.

Kocher, H.P., and Spiegelberg, H.L. (1979). Tryptic degradation of the C-H1 and V-L regions of IgD and IgE. *J. Immunol.* 122: 1190.

Kohler, P.F., and Farr, R.S. (1967). Quantititative comparison of immunoglobulins in atopic (reaginic) and nonatopic (nonreaginic) individuals: Higher gamma-D levels in atopic sera. *J. Allergy* 39: 311.

Lawrence, D.A., Weigle, W.O., and Spiegelberg, H.L. (1975). Immunoglobulins cytophilic for human lymphocytes, monocytes, and neutrophils. *J. Clin. Invest.* 55: 368.

Layton, J.E., and Howard, M.C. (1978). Distribution of surface IgM and IgD on single cells from lymphocyte subpopulations. *Aust. J. Exp. Biol. Med. Sci.* 56, pt. 4.: 433.

Layton, J.E., Johnson, G.R., Scott, D.W., and Nossal, G.J.V. (1978).

The anti-delta suppressed mouse. *Eur. J. Immunol.* 8: 325.

Layton, J.E., Pike, B.E., Battye, F.L., and Nossal, G.J.V. (1979). Cloning of B cells positive or negative for surface IgD: I. Triggering and tolerance in T-independent systems. *J. Immunol.* 123: 702.

Layton, J.E., Teale, J.M., and Nossal, G.J.V. (1979). Cloning of B cells positive or negative for surface IgD: II. Triggering and tolerance in the T-dependent splenic focus assay. *J. Immunol.* 123: 709.

Lee, S.K., Metrakos, J.D., Tanaka, K.R., and Heiner, D.C. (1980). Genetic influence on serum IgD levels. *Pediatr. Res.* 14: 60.

Lertora, J.J.L., Gomez-Perez, F.J., and Leslie, G.A. (1975). Structure and biological functions of human IgD: V. Insulin antibodies of the IgD class in sera from some diabetic patients. *Int. Arch. Allergy Appl. Immunol.* 49: 597.

Leslie, G.A. (1975). Ontogeny of the chicken humoral immune mechanism. *Am. J. Vet. Res.* 36: 482.

Leslie, G.A. (1980). Idiotypy and IgD isotypy of chicken immunoglobulins. In *Phylogeny of Immunological Memory* (M.J. Manning,

ed.) Elsevier/North Holland Biomedical Press. Amsterdam, the Netherlands. p. 253.

Leslie, G.A. (1973). Structure and biological functions of human IgD: II. Alteration of serum IgD levels during pregnancy. *Proc. Soc. Exp. Biol. Med.* 144: 741.

Leslie, G.A. and Armen, R.C. (1974). Structure and biological functions of human IgD: III. Phylogenetic studies of IgD. *Int. Arch. Allergy* 46: 191.

Leslie, G.A., and Cuchens, M.A. (1982). Developmental aspects of rat IgD expression and the consequences of IgD modulation. In *Immunoglobulin D: Structure and Function* (G.J. Thorbecke and G.A. Leslie, eds.) *Ann. N.Y. Acad. Sci.* 399: 131.

Leslie, G.A., and Martin, L.N. (1977). Structure and function of serum and membrane immunoglobulin D (IgD). *Cont. Topics. Mol. Immunol.* 7: 1.

Leslie, G.A., and Swate, T.E. (1972). Structure and biologic functions of human IgD: I. The presence of immunoglobulin D in human cord sera. *J. Immunol.* 109: 47.

Leslie, G.A., and Teramura, G. (1977). Structure and biological functions of human IgD: XIV. The development of a solid-phase radioimmunoassay for the quantitation of IgD in human sera and secretions. *Int. Arch. Allergy Appl. Immunol.* 54: 451.

Lin, L.C., and Putnam, F.W. (1981). Primary structure of the Fc region of human immunoglobulin D: implications for evolutionary origin and biological function. *Proc. Nat. Acad. Sci.* 78: 504.

Litwin, S.D., Tse-Eng, D., Butler, V.P., and Pernis, B. (1985). Studies on human low serum IgD phenotype and Gm markers. *Clin. Gen.* 27: 134.

Luster, M.I., Armen, R.C., Hallum, J.V., Ahmed, A., Leslie, G.A. (1978). Immunoglobulin class distribution of measles virus antibodies in serum and spinal fluids of patients with subacute sclerosing panencephalitis. *Int. Arch. Allergy Appl. Immunol.* 56: 488.

Luster, M.I., Armen, R.C., Hallum, J.V., and Leslie, G.A. (1976). Measles virus-specific IgD antibodies in patients with subacute sclerosing panencephalitis. *Proc. Nat. Acad. Sci.* 73: 1297.

Luster, M.I., Leslie, G.A., and Bardana, E.J. (1976a). Structure and biological functions of human IgD: Serum IgD in patients with

allergic bronchopulmonary aspergillosis. *Int. Arch. Allergy Appl. Immunol.* 50: 212.

Luster, M.I., Leslie, G.A., and Bardana, E.J. (1976b). Structure and biological functions of human IgD: Antinuclear antibodies in sera of patients with autoimmune disorders. *Int. Arch. Allergy Appl. Immunol.* 52: 212.

Maki, R., Roeder, W., Traunecker, A., Sidman, C., Wabl, M., Raschke, W., and Tonegawa, S.(1981) The role of DNA rearrangement and alternative RNA processing in the expression of immunoglobulin delta genes. *Cell* 24: 353.

Marcolongo, R., and Marsili, C. (1975). Serum IgD and IgE in rheumatoid arthritis. *Z. Immun.-Forsch. Bd.* 148S: 285.

Marshall-Clarke, S., Keeler, K.D., and Parkhouse, R.M.E. (1983). The expression of surface IgD on B cells responsive to thymus-independent and thymus-dependent antigens and its requirement for B cell triggering. *Immunol.* 48: 393.

Martin, L.N., and Leslie, G.A. (1979). In vivo effects of antiserum to IgD on surface immunoglobulins, serum immunoglobulins, and lymphocyte blastogenesis in rhesus monkeys. *Immunol.* 37: 253.

Mather, E.L., Nelson, K.J., Haimovich, J., and Perry, R.P. (1984). Mode of regulation of immunoglobulin mu- and delta-chain expression varies during B lymphocyte maturation. *Cell* 36: 329.

McFadden, S.F., and Vitetta, E.S. (1984). sIgD negative B cells from neonatal mice do not respond to the thymus-independent antigen, TNP-BA in limiting dilution cultures. *J. Immunol.* 132: 1717.

McGregor, I.A., Rowe, D.S., Wilson, M.E., and Billewicz, W.Z. (1970). Plasma immunoglobulin concentrations in an African (Gambian) community in relation to season, malaria, and other infections and pregnancy.*Clin. Exp. Immunol.* 7: 51.

Melcher, U., Vitetta, E.S., McWilliams, M., Lamm, M.E., Phillips-Quagliata, J.M., and Uhr, J.W. (1974). Cell surface immunolglobulin X. Identification of an IgD-like molecule on the surface of murine splenocytes. *J. exp. Med.* 140: 1427.

Mescher, M.F., and Pollock, R.R. (1979). Murine cell surface immunoglobulin: two forms of delta heavy chain. *J. Immunol.* 123: 1153.

Metcalf, E.S., Mond, J.J., and Finkelman, F.D. (1983). Effect of

neonatal anti-delta antibody treatment on the murine immune system: II. Functional capacity of a stable sIgM+ sIa+ sIgD- B cell population. *J. Immunol.* 131: 601.

Moore, K.W., Rogers, J., Hunkapiller, T., Early, P., Nottenberg, C., Weissman, I., Bazin, H., Wall, R., and Hood, L.E. (1981). Expression of IgD may use both DNA rearrangement and RNA splicing mechanisms. *Proc. Nat. Acad. Sci.* 78: 1800.

Mushinski, J.F., Thiele, C.J., Owens, J.D., Blattner, F.R., Shen, A.L., Tucker, P.W., and Fitzmaurice, L. (1982). Genetic aspects of IgD expression: II. Multiple forms of delta chain mRNA in normal mouse spleen, mouse B cell lymphomas, and mous and human myelomas. In *Immunoglobulin D: Structure and Function* (G.J. Thorbecke and G.A. Leslie, eds.) *Ann. N.Y. Acad. Sci.* 399: 15.

Muul, L.M., Mond, J.J., and Finkelman, F.D. (1983). Polyclonal activation of the murine immune system by an antibody to IgD: III. Ontogeny. *Eur. J. Immunol.* 13: 900.

Nerenberg, S.T., and Prasad, R. (1975). Radioimmunoassays for Ig classes G,A,M,D, and E in spinal fluids: normal values of different age groups. Nerenberg *J. Lab. Clin. Med.* 86: 887.

Neuberger, M.S., and Rajewsky, K. (1981). Switch from hapten-specific immunoglobulin M to immunoglobulin D secretion in a hybrid mouse cell line. *Proc. Nat. Acad. Sci.* 78: 1138.

Nordbring, F., Johansson, S.G.D., and Espmark, A. (1972). Raised serum levels of IgE in infectious mononucleosis. *Scand. J. Infect. Dis.* 4: 119.

Olson, J.C., and Leslie, G.A. (1982). IgD: A component of the secretory immune system? In *Immunoglobulin D: Structure and Function* (G.J. Thorbecke and G.A. Leslie, eds.) *Ann. N.Y. Acad. Sci.* 399:97.

Papadopoulos, N.M., and Frieri, M. (1984). The presence of immunoglobulin D in endocrine disorders and diseases of immunoregulation, including the acquired immunodeficiency syndrome. *Clin. Immunol. Immunopathol.* 32: 248.

Parker, D.C. (1980) Induction and suppression of polyclonal antibody responses by anti-Ig reagents and antigen-nonspecific helper factors: A comparison of the effects of anti-Fab, anti-IgM, and anti IgD on murine B cells. *Immunol. Revs.* 52: 115.

Parkhouse, R.M.E., and Cooper, M.D. (1977). A model for the differentiation of B lymphocytes with implications for the biological

role of IgD. *Immunol. Rev.* 37: 105.

Pauwels, R., Bazin, H., Platteau, B., and Van der Straeten, M. (1979). The influence of different adjuvants on the production of IgD and IgE antibodies. *Ann. Immunol.* 131C: 49.

Pearson, T., Galfre, G., Ziegler, A., and Milstein, C. (1977). A myeloma hybrid producing antibody specific for an allotypic determinant on "IgD-like" molecules of the mouse. *Eur. J. Immunol.* 7: 684.

Pernis, B. (1977). Lymphocyte membrane IgD. *Immunol. Revs.* 37: 210.

Pernis, B., Chiappino, G., and Rowe, D.S. (1966). Cells producing IgD immunoglobulins in human spleen. *Nature* 211: 424.

Pernis, B., and Forni, L. (1976). IgD receptors of lymphoid cells. *Immunol. Commun.* 5: 807.

Plebani, A., Mira, E., Mevio, E., Monafo, V., Nortarangelo, L.D., Avanzini, A., and Ugazio, A.G. (1983). IgM and IgD concentrations in the serum and secretions of children with selective IgA deficiency. *Clin. Exp. Immunol.* 53: 689.

Pollock, R.R., Dorf, M.E., and Mescher, M.F. (1980). Genetic control of murine IgD structural heterogeneity. *Proc. Nat. Acad. Sci.* 77: 4256.

Pollock, R.R., and Mescher, M.F. (1980). Murine cell surface immunoglobulin: two native IgD structures. *J. Immunol.* 124: 1668.

Pollock, R.R., and Mescher, M.F. (1982). Structural heterogeneity of murine IgD during ontogeny. *Mol. Immunol.* 19: 1535.

Preud'homme, J., Brouet, J., and Seligmann, M. (1977). Membrane bound IgD on human lymphoid cells, with special reference to immunodeficiency and immunoproliferative diseases. *Immunol. Revs.* 37: 151.

Pure, E., and Vitetta, E.S. (1980). The murine B cell response to TNP-polyacrylamide beads: The relationship between the epitope density of the antigen and the requirements for T cell help and surface IgD. *J. Immunol.* 125: 420.

Putnam, F.W., Takahashi, N., Tetaert, D., Lin, L.C., and Debuire, B. (1982). The last of the immunoglobulins: Complete amino acid sequence of human IgD. In *Immunoglobulin D: Structure and Function* (G.J. Thorbecke and G.A. Leslie, eds.) *Ann. N.Y. Acad. Sci.* 399: 41.

Radl, J., Van den Berg, P., and Jol-Van der Zidje, C.M. (1980). IgD idiopathic paraproteinemia in the aging C57BL/KaLwRij Mouse. *J. Immunol.* 124: 2513.

Reeves, W.G., and Mitchell, J.R.A. (1984). Hyperimmunoglobulinemia D and periodic fever. *Lancet* 1: 1464.

Ritchie, R.F. (1968). Antinuclear antibodies. *Arth. Rheum.* 11: 506.

Rosenberg, Y.J., and Parish, C.R. (1977). Ontogeny of the antibody-forming cell line in mice: IV. Appearance of cells bearing Fc receptors, complement receptors, and surface immunoglobulin. *J. Immunol.* 118: 612.

Rostenberg, I., and Penalosa, R. (1978). Serum IgG and IgD levels in some infectious and non-infectious diseases. *Clin. Chim. Acta* 85: 319.

Roth, P., Tonda, P., and Pernis, B. (1982). Membrane IgD expression and dynamics in clones of human B lymphoblastoid cells. In *Immunoglobulin D: Structure and Function* (G.J. Thorbecke and G.A. Leslie, eds.) *Ann. N.Y. Acad. Sci.* 399: 175.

Rowe, D.S., Crabbe, P.A., and Turner, M.W. (1968). Immunoglobulin D in serum, body fluids, and lymphoid tissues. *Clin. Exp. Immunol.* 3: 477.

Rowe, D.S., Dolder, F., and Welscher, H.D. (1969). Studies of human IgD: I. Molecular weight, and sedimentation coefficient. *Immunochem.* 6: 437.

Rowe, D.S., and Fahey, J.L. (1965a). A new class of human immunoglobulins: I. A unique myeloma protein. *J. Exp. Med.* 121: 171.

Rowe, D.S., and Fahey, J.L. (1965b). A new class of human immunoglobulins: II. Normal serum IgD. *J. Exp. Med.* 121: 185.

Rowe, D.S., Hug, K., Forni, L., and Pernis, B. (1973). Immunoglobulin D as a lymphocyte receptor. *J. Exp. Med.* 138: 965.

Rowe, D.S., McGregor, I.A., Smith, S.J., Hall, P., and Williams, K. (1968). Plasma immunoglobulin concentrations in a West African (Gambian) community and in a group of healthy British adults. *Clin. Exp. Immunol.* 3: 63.

Rudders, R.A., and Anderson, J. (1982). IgD Fc-receptors on normal and neoplastic human B lymphocytes. *Clin. Exp. Immunol.* 50: 579.

Ruddick, J.H., and Leslie, G.A. (1977). Structure and biological functions of human IgD. XI. Identification and ontogeny of a rat lymphocyte immunoglobulin having antigenic cross-reactivity with human IgD. *J. Immunol.* 118: 1025.

Rundle, A.T., Clothier, B., and Sudell, B. (1971). Serum IgD levels and infections in Down's syndrome. *Clin. Chim. Acta* 35: 389.

Salonen, E., Hovi, T., Meurman, O., Veskiari, T., and Vaheri, A. (1985). Kinetics of specific IgA, IgD, IgE, IgG, and IgM antibody responses in rubella. *J. Med. Virol.* 16: 1.

Schmidt, K., and Mueller-Eckhardt, C. (1973). Antinuclear antibodies of IgD class: An analysis of 82 patients. *Z. Immun.-Forsch Bd.* 145: 385.

Scully, C., Yap, P.L., and Boyle, P. (1983). IgE and IgD concentrations in patients with recurrent aphthous stomatitis. *Arch. Dermatol.* 19: 31.

Sell, S., and Gell, P.G.H. (1965a). Studies on rabbit lymphocytes in vitro: I. Stimulation of blast transformation with an anti-allotype serum. *J. Exp. Med.* 122: 423.

Sell, S., and Gell, P.G.H. (1965b). Studies on rabbit lymphocytes in vitro: IV. Blast transformation of the lymphocytes of newborn rabbits induced by anti-allotype serum to a potential IgG allotype not present in the serum of the lymphocyte donors. *J. Exp. Med.* 122: 923.

Sewell, H.F., Chambers, L., Maxwell, V., Matthews, J.B., and Jefferis, R. (1978). The natural antibody response to *E. coli* includes antibodies of the IgD class. *Clin. Exp. Immunol.* 31: 104.

Sewell, H.F., Matthews, J.F., Flack, V., and Jefferis, R. (1979). Human immunoglobulin D in colostrum, saliva, and amniotic fluid. *Clin. Exp. Immunol.* 36: 183.

Sieckmann, D.G., Asofsky, R., Mosier, D.E., Zitron, I.M., and Paul, W.E. (1978). Activation of mouse lymphocytes by anti-immunoglobulins: I. Parameters of the proliferative response. *J. Exp. Med.* 147: 814.

Sire, J., Alcaraz, G., Bourgois, A., and Jordan, B. (1981). Rat IgD myeloma protein: cell-free translation of the delta mRNA and biochemical analysis of intracellular and membrane delta chain. *Eur. J. Immunol.* 11: 632.

Sire, J., Auffray, C., and Jordan, B.R. (1982). Rat immunoglobulin

delta heavy chain gene: nucleotide sequence derived from cloned cDNA. *Gene* 20: 377.

Sire, J., Colle, A., and Bourgois, A. (1979). Identification of an IgD-like surface immunoglobulin on rabbit lymphocytes. *Eur. J. Immunol.* 9: 13.

Sjoberg, O. (1980). Presence of receptors for IgD on human T and non-T lymphocytes. *Scand. J. Immunol.* 11: 377.

Skelly, R.R., Baine, Y., Ahmed, A., Xue, B., and Thorbecke, G.J. (1983). Cell surface phenotype of the lymphoid cells from normal mice and mice treated with monoclonal anti-IgD from birth. *J. Immunol.* 130: 15.

Spiegelberg, H.L., Prahl, J.W., and Grey, H.M. (1970). Structural studies of human gamma-D myeloma protein. *Biochem.* 9: 2115.

Stall, A.M., and Loken, M.R. (1984). Allotypic specificities of murine IgD and IgM recognized by monoclonal antibodies. *J. Immunol.* 132: 787.

Steele, M.G., and Leslie, G.A. (1985). Immunoglobulin D in rat serum, saliva, and milk. *Immunol.* 55: 571.

Steele, M.G., and Leslie, G.A. (1986). IgD and the maternal-fetal interface. In *Immunoregulation and Fetal Survival* (T. Gill and T.G. Wegmann, eds.) Oxford University Press, New York. *in press*.

Steele, M.G. and Leslie, G.A. (1986). IgD antibodies to DNP are induced in ascitic fluid after intraperitoneal immunization. *Fed. Proc.* 45: 257.

Stevens, W.J., de Backer, W., and Vermeire, P.A. (1983). Serum IgA, IgG, IgM, and IgD in allergic (type 1) and non-allergic respiratory diseases. *Clin. Allergy* 13: 11.

Swierczynska, Z., Wozinczko-Orlowska, G., and Maldyk, H. (1976). An IgD myeloma protein with anti-Streptolysin O activity. *Immunochem.* 13: 379.

Tada, T., and Ishizaka, K. (1970). Distribution of gamma E forming cells in lymphoid tissues of the human and monkey. *J. Immunol.* 104: 377.

Tamura, H., Fujinaga, T., and Kuroume, T. (1982). IgD antibody to *Mycoplasma pneumoniae* in human sera as measured by the RAST. *Int. Arch. Allergy Appl. Immunol.* 69: 335.

Tavolato, B., and DeZanche, L. (1968).The IgD in the cerebrospinal fluid. *Acta Neurol. Scand.* 44: 384.

Thiele, C.J., Owens, J.D., Finkelman, F.D., and Mushinski, J.F. (1985). Mouse IgD half molecules with shortened IgD heavy chain result from alterations within C-delta locus. *J. Immunol.* 134: 1251.

Tucker, P.W. (1985). Transcriptional regulation of IgM and IgD. *Immunol. Today* 6: 181.

Tucker, P.W., Cheng, H.L., Richard, J.E., Fitzmaurice, L., Mushinski, J.F., and Blattner, F.R. (1982). Genetic aspects of IgD expression: III. Functional implications of the sequence and organization of the C-delta gene. In *Immunoglobulin D: Structure and Function* (G.J. Thorbecke and G.A. Leslie, eds.) *Ann. N.Y. Acad. Sci.* 399: 26.

Tucker, P.W., Liu, C.P., Mushinski, J.F., and Blattner, F.R. (1980). Mouse immunoglobulin D: Messenger RNA and genomic DNA sequences. *Science* 209: 1353.

Van der Meer, J.W., Vossen, J.M., Radl, J., and Van Nieuwkoop, J.A. (1984). Hyperimmunoglobulinemia D and periodic fever: A new syndrome. *Lancet* 1: 1087.

Van Nieuwkoop, J.A., and Radl, J. (1985). Light chain types of IgD in human bone marrow and serum. *Clin. Exp. Immunol.* 60: 654.

Vasilov, R.G., and Ploegh, H.L. (1982). Biosynthesis of murine immunoglobulin D: heterogeneity of glycosylation. *Eur. J. Immunol.* 12: 804.

Vitetta, E.S. (1982a). Panel discussion in *Immunoglobulin D: Structure and Function* (G.J. Thorbecke and G.A. Leslie, eds.) *Ann. N.Y. Acad. Sci.* 399: 155.

Vitetta, E.S. (1982b). Immunoglobulin receptors revisited. In *Immunoglobulin D: Structure and Function* (G.J. Thorbecke and G.A. Leslie, eds.) *Ann. N.Y. Acad. Sci.* 399: 255.

Vitetta, E.S., Cambier, J.C., Ligler, F.S., Kettman, J.R., and Uhr, J.W. (1977). B cell tolerance: IV. Differential role of surface IgM and IgD in determining tolerance susceptibility of murine B cells. *J. Exp. Med.* 146: 1804.

Vitetta, E.S., and Uhr, J.W. (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 Uhr, J.W. (1975). Immunoglobulin-receptors revisited. *Science* 189: 964.

Wabl, M.R., Johnson, J.P., Haas, I.G., Tenkhoff, M., Meo, T., and Inan, R. (1980). Simultaneous expression of mouse immunoglobulins M and D is determined by the same homolog of chromosome 12. *Proc. Nat. Acad. Sci.* 77: 6793.

Walzer, P.D., and Kunkel, H.G. (1974). The correlation of serum IgD concentration with Gm allotype. *J. Immunol.* 113: 274.

Watson, I., Heiner, D., Rose, B., and Bootello, A. (1969). The demonstration of IgD antinuclear antibody activity in systemic lupus erythematosus. *Clin. Res.* 17: 362.

Weliky, N., Heiner, D.C., Tamura, H., and Anderson, S. (1979). Correlation of tratrazine hypersensitivity with specific hypersensitivity with specific serum IgD levels. *Immunol. Commun.* 8: 65.

White, M.B., Shen, A.L., Word, C.J., Tucker, P.W., and Blattner, F.R. (1985). Human immunoglobulin D: Genomic sequence of the delta heavy chain. *Science* 228: 733.

Wiedermann, D., Widermannova, D., Novotna, D., and Oravcova, V. (1974). On the quantitative rate of immunoglobulin G, immunoglobulin A, immunoglobulin M, and immunoglobulin D of hemeagglutination inhibiting antibodies of transferrin, ceruloplasmin, alpha-2-macroglobulin and C-reactive protein in children with influenza A2 Hong Kong. *Zbl. Bakt. Hyg. I. Abt. Orig.* A229: 141.

Wilder, R.L., Yuen, C.C., Coyle, S.A., and Mage, R.G. (1979). Demonstration of a rabbit cell surface Ig that bears light chain and V-H, but lacks mu-, alpha-, and gamma- allotypes- rabbit IgD? *J. Immunol.* 122: 464.

Winchester, R.J., Fu, S.M., Hoffman, T., and Kunkel, H.G. (1975). IgG on lymphocyte surfaces: technical problems and the significance of a third cell population. *J. Immunol.* 114: 1210.

Wolcott, M., Freeman, D.G., Schrohenloher, R.E., Hammack, W., and Bennett, J.C. (1975). Tryptic cleavage of IgD at elevated temperature and isolation of a Fc-like fragment in high yield. *Immunochem.* 12: 685.

Woods, V.L., Kessler, S.W., Finkelman, F.D., Lieberman, R., Scher, I., and Paul, W.E. (1980). IgD allotypic determinants: I. Determinants

expressed on murine IgD of the a allotype. *J. Immunol.* 125: 2699.

Woods, V.L., Scher, I., Lieberman, R., and Paul, W.E. (1980). IgD allotypic determinants: II. Determinants expressed on IgD of mice of the b, e, and o Igh-C. *J. Immunol.* 125: 2708.

Xue, B., Coico, R., Wallace, D., Siskind, G.W., Pernis, B., and Thorbecke, G.J. (1984). Physiology of IgD: IV: Enhancement of antibody production in mice bearing IgD-secreting plasmacytomas. *J. Exp. Med.* 159: 103.

Yodoi, J., Adachi, M., Teshigawara, K., Miyama-Inaba, M., Masuda, T., and Fridman, W.H. (1983). T cell hybridomas coexpressing Fc receptors (FcR) for different isotypes II. IgA-induced formation of suppressive IgA-binding factor(s) by a murine T hybridoma bearing Fc-gamma-R and Fc-alpha-R. *J. Immunol.* 131: 303.

Yuan, D. (1984). Regulation of IgM and IgD synthesis in B lymphocytes: II. Translational and post-translational events. *J. Immunol.* 132: 1566.

Yuan, D., and Tucker, P.W. (1984). Regulation of IgM and IgD synthesis in B lymphocytes: I. Changes in biosynthesis of mRNA for mu and delta chains. *J. Immunol.* 132: 1561.

Zikan, J., Sima, P., and Tuckova, L. (1983). Cross-reactivity of human and a putative pig IgD: Pig IgD-like molecules as serum and lymphocyte components. *Folia Microbiol.* 28: 474.

Zitron, I.M., and Clevinger, B.L. (1980). Regulation of murine B cells through surface immunoglobulin. I. Monoclonal anti-delta antibody that induces allotype-specific proliferation. *J. Exp. Med.* 152: 1135.

Zitron, I.M., Mosier, D.E., and Paul, W.E. (1977). The role of surface IgD in the response to thymic independent antigens. *J. Exp. Med.* 146: 1707.

PAPER 1

Immunoglobulin D in Rat Serum, Saliva and Milk

Abstract

Previously this laboratory has found very high concentrations of IgD in normal rat milk. Using ELISA methods the relationship between milk, serum, and saliva IgD in lactating and suckling rats was examined. Milk IgD appears to be synthesized in the mammary tissue rather than taken up from the blood because (1) serum IgD remains low and is not significantly different from that of nonlactating females and (2) serum IgD during lactation is poorly correlated with milk IgD. Serum IgD in suckling rats declines in the first seven days following birth and remains relatively low during the remainder of lactation (2-4 μg/ml). The surprisingly high serum IgD is observed at birth (9.3 ± 3.2 μg/ml) is present before suckling begins and is not affected by the onset of suckling. Transient elevations of serum IgD begin to occur following weaning. Rats weaned ten days earlier than normal (day 20 vs. day 30) had significantly higher serum IgD on days 36, 47, and 60. Among 43 adult rats serum IgD was 5.4 ± 3.6 μg/ml and saliva IgD 2.0 ± 1.7 μg/ml. Serum IgD correlates poorly with saliva IgD. The thymus is not required for IgD synthesis since no significant difference in serum IgD was found between nude rats and their euthymic littermates.

Introduction

Although it has been more than 20 years since immunoglobulin D was first discovered in a patient with multiple myeloma and in trace quantities in normal human serum (Rowe and Fahey, 1965a,b), the biological function of secreted IgD remains a mystery. IgD has been found as a major surface immunoglobulin appearing with IgM on B lymphocytes in a wide variety of species including the human, (Rowe et al., 1973), mouse, (Abney and Parkhouse, 1974), monkey, (Martin, Leslie, and Hindes, 1976), rat, (Ruddick and Leslie, 1977), rabbit, (Sire et al, 1979), chicken, (Leslie, 1980), and pig (Zikan, Sima, and Tuckova, 1983). Similarly, serum IgD has been identified in monkeys (Leslie and Armen, 1974), rats (Bazin et al., 1978), mice (Finkelman et al., 1979), and pigs (Zikan et al., 1983) in addition to humans. The evolutionary conservation of serum and membrane IgD in such diverse species itself suggests that the biological function of IgD, however mysterious, is not a trivial one. Recently we provided preliminary evidence that IgD is a secretory immunoglobulin present in rat milk at concentrations between 50 and 300 µg/ml. based on radial immunodiffusion testing (Olson and Leslie, 1982). In this paper we use an ELISA method to confirm these results and extend them to examine (1) the relationship between milk and serum IgD in the lactating mother and her pups, (2) the ontogeny of serum IgD in the pup during suckling and after weaning, and (3) levels of IgD in the

saliva and serum of normal rats, and in the serum and whey of athymic nude rats.

Materials and Methods

Animals. HPR strain rats, bred for a high precipitin response to group A streptococcal carbohydrate and derived from Spraque-Dawley stock, were used unless otherwise noted (Stankus and Leslie, 1974). Athymic nude rats were provided by Carl T. Hansen of NIH and were derived from hooded rats (Festing et al., 1978).

Isolation of saliva, serum, and milk. Anesthetized rats were given 0.25 units of oxytocin intravenously, and milk or colostrum was collected using a vacuum pump. Milk was clarified by centrifugation at 1.1×10^5 *g* for 1 hr at 4°. Blood was collected by tail or cardiac puncture, allowed to clot at 4° overnight, and serum separated by centrifugation at 330 *g* for 15 minutes at 4°. Saliva was collected by suction immediately following ether anesthesia. All samples were stored at -20° until assayed.

Preparation of rabbit anti-IgD enzyme conjugate. Heterologous rabbit anti-rat IgD was generated by immunization with membrane IgD purified from rat lymphocytes (Cuchens, Martin, and Leslie, 1978). A 45%

saturated ammonium sulphate precipate of the serum was prepared and dissolved in 0.01 M Tris buffered saline, pH 7.4 (TBS). The antiserum was absorbed with whole rat serum, rat IgM and '7S' rat immunoglobulin until no detectable reaction occured with IgM, IgG or whole rat serum by double gel diffusion. The absorbed reagent gave a strong precipitin reaction of identity between rat milk and a high-IgD rat serum assayed independently by Dr. Herve Bazin, and with a second antiserum to IgD raised in sheep. IgD-specific antibodies were isolated from an immunoadsorbant made with partially purified milk IgD (see below). After further gel diffusion tests to prove specificity, the antibodies were coupled to alkaline phosphatase (Sigma Type VII-S) (Kearney et al., 1979). The enzyme conjugate bound to vinyl plates coated with partially purified IgD, but not to rat IgM-, '7S' salt-precipiated immunoglobulin- or IgA-coated plates. Demonstration of specificity was shown further by lack of binding inhibition by rat IgM, IgG2c,'7S' salt precipitated immunoglobulin, IgA, or antiserum to IgA, and by the presence of binding inhibition by partially purified IgD and a high-IgD rat serum. Human myeloma sera and murine myeloma ascites fluid with high levels of IgD (the mouse ascites kindly provided by Dr. Fred Finkelman) showed some binding inhibition, while IgD-deficient human and mouse serum did not. This supports studies indicating the presence of cross-reactivity between rat, human and mouse IgD (Golding et al., 1979).

Total IgD assay. Ninety-six well vinyl assay plates (Costar no. 2095) were coated for 2 hr with 1 µg/ml partially purified IgD in pH 9.6 carbonate-bicarbonate buffer (15 mM sodium carbonate, 35 mM sodium bicarbonate, 3mM sodium azide). The plates were rinsed once with pH 7.0 10 mM phosphate buffered saline (PBS), and unreacted sites blocked with 1% BSA in PBS for 2 hrs at 20°. The plates were rinsed four times with PBS containing 0.95% Tween 20 (Sigma, St. Louis, MO) and four times with PBS. Serum to be assayed was diluted in 2% normal rabbit serum/PBS, mixed with the rabbit anti-rat IgD enzyme conjugate, and incubated in wells for 2 hr at 20°. Plates were rinsed as before. Finally, 0.5 mg/ml *p*-nitrophenyl phosphate (Sigma 102) in 1 M diethanolamine, 1mM $MgCl_2$ (pH 9.8) was added to the plates, incubated for 1 hr at 37° and read on an ELISA plate reader. A pooled sample of rat milk containing 120 µg/ml IgD (based on comparison with a high-IgD rat serum assayed by Dr. Bazin) was used as the IgD standard.

Total protein assay. Total protein was measured by optical density at 280 nm of the clarified milk samples (r=0.99 vs protein concentration as assayed by the Bradford method (Bradford, 1976)).

Partial purification of milk IgD. Whole rat milk was passed over a 2.5 x 90 cm Sephacryl 300 gel filtration column (Pharmacia, Piscataway, NJ) equilibrated with pH 7.4 TBS. The IgD-rich fractions were determined by Ouchterlony analysis then pooled and concentrated.

Results

Quantitation of IgD in milk, serum and saliva from lactating rats. Seven lacating rats were milked every other day from day 5 to the end of lactation (Day 30). The IgD concentration in milk ranged from 36 to ~800 µg/ml. Milk IgD ranged from 100 to 300 µg/ml between Days 5 and 21. After Day 21, it tended to increase (Fig. 1). The variation in the levels of IgD in milk mainly appears to be the result of individual differences in milk output versus IgD output. The end-of-lactation increase was accompanied by a parallel increase in milk protein (Fig. 1). Milk IgD (µg/ml of clarified milk protein) varied between 5 and 11 µg/mg, with some rats experiencing an increase around Day 9 as reflected in the mean (Table 1). Serum IgD in lactating females showed considerable variability, but did not differ overall from values seen in non-pregnant and non-lactating rats. Milk IgD concentration (µg/mg clarified milk protein) and serum IgD concentration correlated very poorly ($r=0.13$, $n=40$). Fifteen samples of saliva from five other lacating rats had a mean IgD of 0.9 ± 0.7 µg/ml.

IgD in suckling and weaned rats. Serum was collected from suckling

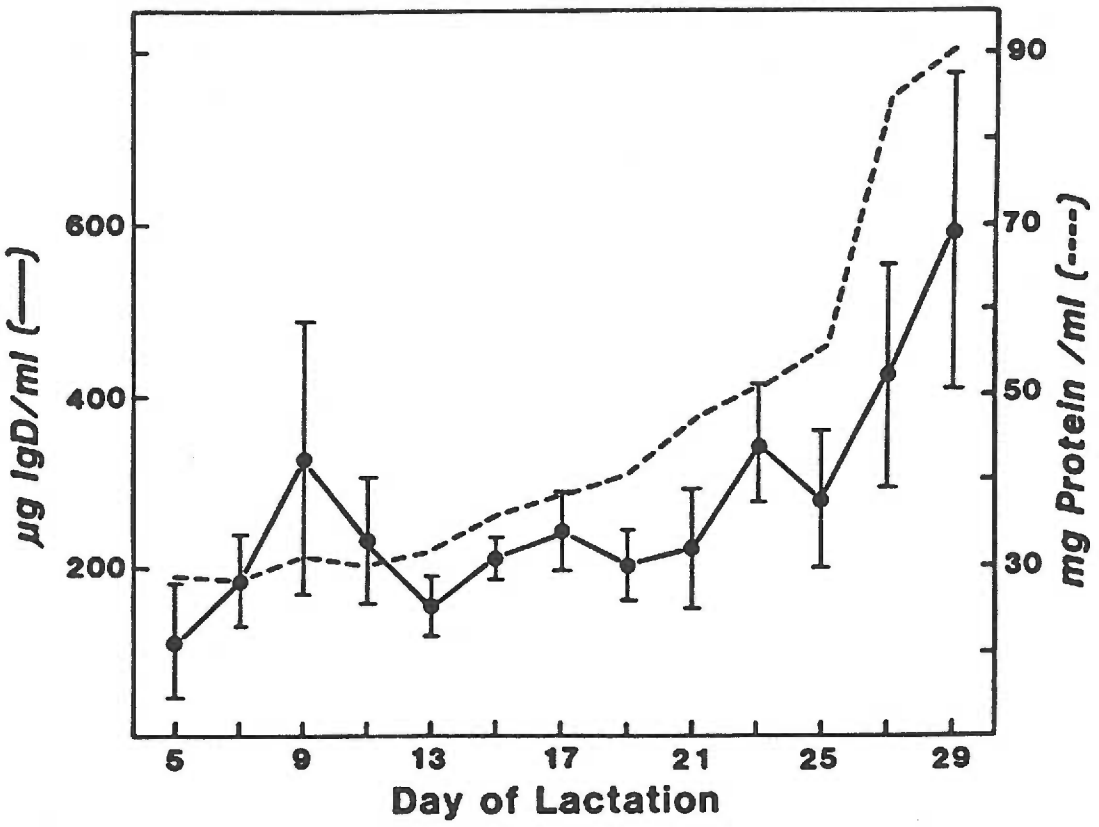


Figure 1. Mean IgD concentration µg/ml (●——●) and mean protein concentration mg/ml (– – – –) in the milk of seven lactating rats during lactation. Values are mean ± SD.

TABLE 1

Serum and Milk IgD Concentrations During Lactation [a]

	Milk IgD	Serum IgD
Day of Lactation	µg/mg Milk protein ± SD (N)	µg/ml ± SD (N)
5	6.6 ± 2.8 (4)	4.5 ± 2.0 (4)
7	7.8 ± 5.1 (6)	5.6 ± 2.7 (3)
9	10.3 ± 9.2 (7)	4.3 ± 1.4 (3)
11	9.7 ± 6.3 (7)	10.5 ± 8.6 (4)
13	6.0 ± 2.4 (5)	5.1 ± 3.9 (4)
15	7.5 ± 1.7 (6)	4.5 ± 2.9 (4)
17	7.0 ± 2.3 (7)	5.2 ± 2.5 (7)
19	5.8 ± 3.1 (7)	5.4 ± 2.0 (5)
21	6.0 ± 3.7 (7)	10.8 ± 10.6 (5)
23	6.9 ± 3.5 (7)	3.6 ± 0.9 (5)
25	6.7 ± 3.7 (5)	6.2 ± 5.1 (4)
27	6.4 ± 2.7 (3)	2.1 ± 0.5 (2)
29	5.9 (1)	6.0 ± 1.0 (2)

[a] µg IgD/mg milk protein was determined for each of N lactating rats and then a mean and SD was calculated.

and weaned rats between birth and 66 days of age. One litter followed between Days 1 and 42 showed a decline of >67% in their serum IgD between Day 1 and Day 7 of lactation (from 9.3 μg/ml to 2.8 μg/ml), remaining at between 2 and 4 μg/ml until the end of lactation at Day 30, when the serum IgD rose sharply in some of the rats, and then declined towards Day 42. A group of August rat sera assayed between Day 21 and Day 66 had serum IgD levels of ~1 ug/ml during the last ten days of lactation, rose to 2.7 μg/ml at Day 30, then declined to 1-2 μg/ml by day 66 (Table 2). A third group (n=20) from three litters was followed from day 1 to Day 60. In the first 8 days of suckling, serum IgD declined from 6.3 μg/ml to 1.8 μg/ml, then remained at 1.8-2 μg/ml to the end of lactation, when the mean level rose owing to transient elevations among individual rats (Table 3).

The relatively high level of serum IgD consistently observed in newborn rats did not appear to be the result of hemoconcentration, since radial immunodiffusion assays of serum transferrin and albumin did not show a similar decline during the first 7 days of life, but instead showed a gradual increase (data not shown). Since IgD in colostrum is high on Day 1 of lactation (Olson and Leslie, 1982), it seemed reasonable to suspect that high serum IgD at birth represented a brief period of transport of milk IgD into suckling rat serum. In order to test for this, serum IgD was assayed in serum taken from half a litter of eight newborn rats on Day 1 before suckling had begun, and

TABLE 2

Ontogeny of Serum IgD in HPR and August Rats [a]

Age (days)	Concentration of IgD (μg/ml ± SD) (N) [b]	
	(HPR rats)	(AUG rats)
1	9.3 ± 3.2 (4)	-
4	5.5 ± 1.4 (4)	-
7	2.8 ± 1.1 (6)	-
10	3.4 ± 1.7 (6)	-
14	2.4 ± 1.3 (11)	-
17	3.6 ± 0.8 (10)	-
21	2.8 ± 0.9 (5)	1.0 ± 0.6 (4)
24	1.9 ± 1.7 (5)	-
28	2.6 ± 2.2 (5)	0.9 ± 0.3 (6)
30	-	2.7 ± 1.4 (6)
32	4.5 ± 3.5 (4)	-
36	10.6 ± 8.9 (4)	-
38	-	1.7 ± 0.5 (5)
42	5.2 ± 2.7 (4)	-
48	-	1.6 ± 1.1 (5)
60	-	1.4 ± 0.6 (5)
66	-	1.1 ± 0.5 (4)

[a] Rats were allowed to wean naturally at 30 days of age.

[b] N = number of individual sera tested.

in serum taken from the remaining half after these rats had suckled for a day.

The Day 1 unsuckled rats had a mean serum IgD of 8.5 µg/ml (SD = 3.9) and the Day 2 suckled rats had a mean serum IgD of 7.9 µg/ml (SD = 0.7), which indicates that the first day of suckling had no apparent positive influence on suckling rat serum IgD. Thus high serum IgD observed in newborn rats is acquired either transplacentally or is synthesized *de novo* by the newborn rat. This does not exclude the possibility that milk IgD makes a contribution to serum IgD in the newborn rat.

If the serum IgD observed in newborn rats were synthesized by the newborn rat and not acquired from the mother via the placenta, then one hypothesis which could explain the low serum IgD in suckling rats is that ingestion of milk suppressed serum IgD. Therefore weaning rats 10 days earlier than normal might result in the earlier appearance of elevated serum IgD in some of the weanlings. In order to test this, rats from three litters were bled periodically between Day 1 and Day 60. At Day 20, half of each litter was weaned and the other half left to suckle naturally for an additional 10 days.

Each group had an equal number of males and females. No significant difference between the groups was observed on Day 24 and Day 28

following early weaning, hence there was no evidence to suggest that early weaning resulted in the earlier appearance of transiently elevated IgD (Table 3). However the early-weaned rats showed a distinctly greater tendency to develop transiently elevated serum IgD of a greater magnitude than that of the late weaned rats. A highly significant difference in serum IgD was observed on Days 36, 47, and 60, with a marginal difference observed on Day 32. On days 47 and 60, one rat in the early-weaned groups showed a serum IgD level of 252 µg/ml and 88.6 µg/ml respectively. Of the 13 serum samples which had > 6 µg/ml IgD (200 tested), one was from the late-weaned group and 12 were from the early-weaned group.

Serum and saliva IgD in adult rats. Normal adult rats (n = 43) with a mean age of 319 days had 5.4 ± 3.6 µg/ml serum IgD and 2.0 ± 1.7 µg/ml of saliva IgD. The 20 female rats with a mean age of 304 days had somewhat more serum IgD than 23 male rats with a mean age 340 days (7.1 ± 3.5 µg/ml vs. 4.1 ± 3.1 µg/ml). Saliva samples taken from 19 of the female rats had 1.9 ± 1.9 µg/ml IgD, while 15 of the male rats had 2.2 ± 1.4 µg/ml. No significant correlation was found between serum IgD and age ($r = 0.17$), saliva IgD and age ($r = 0.09$), or saliva and serum IgD ($r = 0.09$). In adult rats as in young weaned rats, the level of serum IgD is highly variable from individual to individual. Although no longitudinal study was done in this group of rats, tests on other individual sera at different times indicate that, as with

TABLE 3

Influence of Early Weaning on Serum IgD [a]

day (Age)	pup serum IgD (μg/ml ± SD (N))	maternal serum IgD (μg/ml ± SD (N))	Milk IgD (μg/ml ± SD (N))
1	4.2 ± 1.4 (9)	-	-
8	1.8 ± 0.8 (18)	6.3 ± 4.8 (2)	189 ± 170 (3)
15	1.9 ± 0.7 (19)	2.0 ± 0.2 (2)	237 ± 75 (2)
20	1.0 ± 0.4 (19)	2.6 ± 1.0 (3)	213 ± 137 (3)
24	E: 3.1 ± 3.4 (9)		
	L: 1.4 ± 0.3 (9)	3.1 ± 2.6 (3)	200 ± 36 (3)
	$\bar{x}$: 2.3 ± 2.5 (18)		
28	E: 1.8 ± 1.2 (9)		
	L: 1.3 ± 0.7 (10)	2.7 ± 0.8 (3)	
	$\bar{x}$: 1.5 ± 1.0 (19)		
32	E: 7.2 ± 9.9 (8)		
	L: 2.1 ± 1.2 (10)	2.6 ± 0.5 (3)	
	$\bar{x}$: 4.4 ± 6.9 (18)		
	(p = 0.12)[b]		
36	E: 2.4 ± 4.0 (6)		
	L: 1.9 ± 0.5 (5)	9.3 ± 7.7 (2)	
	$\bar{x}$: 7.5 ± 8.0 (11)		
	(p = 0.04)[b]		
40	E: 2.4 ± 4.0 (8)		
	L: 2.8 ± 4.1 (8)	5.7 ± 2.5 (2)	
	$\bar{x}$: 2.6 ± 3.9 (16)		
47	E: 30.9 ± 82.9 (9)		
	L: 1.3 ± 0.3 (7)	2.1 (1)	
	$\bar{x}$: 17.9 ± 62.5 (16)		
	(p = 0.01)[b]		
60	E: 12.1 ± 28.8 (9)		
	L: 1.6 ± 0.2 (8)		
	$\bar{x}$: 7.2 ± 21.1 (17)		
	(p = 0.004)[b]		

[a] Half of three litters of rats were weaned at 20 days of age, the rest allowed to wean naturally at about day 30. The means for the resulting groups are designated "E" and "L" respectively. "$\bar{x}$" is the mean for all the rats in both groups.

[b] Values of p represent the probability that the difference between the E and L groups are not statistically significant, based on the Mann-Whitney U test.

younger rats, serum IgD can fluctatiate considerably over time in any one animal (data not shown).

A group of six 68-day-old nude rats had 5.0 ± 1.1 µg/ml serum IgD, whereas their six euthymic heterozygous littermates had 3.9 ± 1.0 µg/ml. A second group of six 72-day-old nude rats had 2.7 ± 2.6 µg/ml serum IgD, whereas 19 euthymic (rnu/+ and +/+) littermates had a mean serum IgD of 1.4 ± 0.9 µg/ml. These results confirm the findings of Bazin et al. (1980), in that serum IgD in nude rats is not significantly different from euthymic littermates, and indicates that serum IgD does not require the thymus for the maintenance of normal levels. The same appears to be true for IgD production in milk. Milk obtained on Day 2 from a lactating athymic nude rat contained 232 µg/ml IgD, which was not markedly different from that observed in normal lactating rats.

Discussion

High levels of IgD exist normally in rat milk (Olson and Leslie, 1982). Two questions concerning milk IgD are (i) whether IgD occurs in milk as a transudate from serum or is genuinely a secretory immunoglobulin, and (ii) where milk IgD finds its site of action within the suckling pups. In cattle, Dixon, Wiegle and Vanquez (1961)

showed that a prepartum drop in serum IgG1 was accompanied by the selective transport of that immunoglobulin into milk (for review see Butler, 1983). More recently, Halsey et al. (1982) showed that significant changes take place in serum polymeric IgA and in serum albumin in lactating mice. Thus, if milk IgD is the result of transudation from serum, then the start and end of lactation might well be expected to be accompanied by definite changes in serum IgD, especially in light of the roughly one-hundred fold difference in milk and serum IgD concentrations. For milk IgD to be transported from serum without such changes, it would be necessary (i) that the rates of synthesis and mammary uptake complement one another exactly, and (ii) that any non-secretory function of serum IgD be either disrupted or be protected by a mechanism which distinguishes it from secreted IgD. Since there is a poor correlation between serum IgD and milk IgD, and because there is no significant difference between levels of serum IgD between lactating and non-lactating rats, it appears more likely that milk IgD is secreted by cells in the mammary tissue as a genuine secretory immunoglobulin, and that it is regulated independently of serum IgD. Proof of this hypothesis awaits histological or radioactive tracer studies. However it is interesting to note that IgD levels in human milk also correlate poorly with those of serum IgD (Bahna, Keller and Heiner, 1982), and histological studies of IgD-containing cells in human mammary tissue are consistent with a local synthesis model rather than a transudation model for milk

IgD (Brandtzaeg, 1983).

Newborn rats possess high levels of serum IgD before suckling, thus their serum IgD is either synthesized by themselves or acquired from the mother via the placenta. Since serum IgD does not change after a day of sucking and declines in the first week, any contribution of milk IgD to serum IgD is not enough to reverse its decline there. If IgD in milk does pass into the circulation of the sucking rat, it may be an inefficient process, quickly sequestered from the blood, rapidly redistributed to other secretory sites, or catabolized.

There are a number of possible explanations for the decline in serum IgD following birth. One explanation is that the serum IgD observed on Day 1 is acquired via the placenta and is gradually catabolized following birth. Another possibility is that serum IgD in the newborn rat is synthesized by the newborn rat itself and that something in milk (IgD?) acts to suppress its synthesis. However while weaning had the effect of increasing the magnitude of transient elevations of serum IgD, it did not cause the elevations to begin appearing earlier, which argues against such a suppressive mechanism transmitted by milk.

The significantly higher serum IgD observed in rats weaned 10 days early could be the result of either premature cessation of sucking or

the premature exposure of the gut to solid food antigens. The stress of early weaning could have induced the effect, however both groups of rats continued to gain weight at a comparable rate and were in apparently good health until the end of the study.

The cause of elevated serum IgD is not known. In clinical studies, elevated IgD has been associated with a variety of diseases and immunological disorders (for review, see Leslie and Martin, 1977). but it can also appear in apparently healthy individuals. Elevated serum IgD in our rat studies was not associated with any overt disease. As elevations in serum IgD occur in apparently healthy rats and occur as a transient rather than chronic state, such phenomena appear to be a consequence of normal immune function. While it is not known whether the elevations represent specific immune responses or non-specific events, Pauwels et al. (1979) have shown that immunization of rats with ovalbumin with *B. pertussis* and aluminum hydroxide results in a 'non-specific' increase in serum IgD. The somewhat higher level of serum IgD in female rats may reflect an association of serum IgD with the generally higher immune responsiveness among female rats. Since nude rats can develop normal levels of both milk and serum IgD, and can develop elevated serum IgD with at least the same frequency as their heterozygous or non-nude littermates, it is clear that T-cell help is not required for the production of IgD in serum or milk.

An important question which remains is that of the antigen specificity of IgD. A very limited number of clinical studies have gone beyond measuring total IgD levels to detect or measure antigen-specific IgD. The unavailability of an animal model until the last few years has been an obstacle to systematic study. Pauwels et al. (1979) reported that in rats a small amount of IgD anti-ovalbumin could be elicited by intraplantar immunization with ovalbumin and adjuvants.

Owing to the high concentration of IgD in milk, it appeared feasible to induce measurable IgD immune responses in milk by intramammmary immunization. Our preliminary results indicate that IgD antibodies to haptenated protein can be induced in both milk and serum by such an immunization protocol. Currently we are engaged in efforts to characterize further this immune response and to use it as a probe for defining more precisely the sites of IgD synthesis and sites of potential transport in both the mother and the suckling rat.

References

Abney, E.R., and Parkhouse, R.M.E. (1974). Candidate for immunoglobulin D present on murine B lymphocytes. *Nature (Lond.)* 252: 600.

Bahna, S.I., Keller, M.A., and Heiner, D.C. (1982). IgE and IgD in human colostrum and plasma. *Pediatr. Res.* 16: 604.

Bazin, H., Becker, A., Urbain-Vansanten, G., Pauwels, R., Bruyns, C., Tilkin, A.F., Platteau, B., and Urbain, J. (1979). Transplantable IgD immunoglobulin secreting tumors in rats. *J. Immunol.* 121: 2077.

Bazin, H., Platteau, B., Pauwels, R., and Capron, A. (1980). Immunoglobulin production in nude rats with special attention to the IgE isotype. *Ann. Immunol.* 131C: 31.

Bradford, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72: 248.

Brandtzaeg, P. (1983). The secretory immune system of lactating human mammary glands compared with other exocrine organs, In *The Secretory Immune System* (J.R. McGhee and J. Mestecky, eds.) *Ann. N.Y. Acad.*

Sci. 409: 353.

Butler, J.E. (1983). Bovine immunoglobulins: an augmented review. *Vet. Immunol. and Immunopath.* 4: 43.

Cuchens, M.A,., Martin, L.N., and Leslie, G.A. (1978). The effect of anti-IgD on serum immunoglobulins, antibody production, and immunoglobulin bearing cells in adult rats. *J. Immunol.* 121: 2257.

Dixon, F.J., Wiegle, W.O., and Vazquez, J. (1961). Metabolism and Mammary Secretion of Serum Proteins in the Cow. *Lab. Invest.* 10: 216.

Festing, M.F.W., May, D., Connors, T.A., Lovell, D., and Sparrow, S. (1978). An athymic nude mutation in the rat. *Nature (Lond.)* 274: 365.

Finkelman, F.D., Woods, V.L., Berning, A., and Scher, I. (1979). Demonstration of mouse serum IgD. *J. Immunol.* 123: 1253.

Golding, H., Cuchens, M.A., Leslie, G.A., and Rittenberg, M.B. (1979). Cross-reactivity of rat, mouse, and human IgD. *J. Immunol.* 123: 2751.

Halsey, J.F., Mitchell, C., Meyer, R., and Cebra, J.J. (1982).

Metabolism of immunoglobulin A in lactating mice: origins of immunoglobulin A in milk. *Eur. J. Immunol.* 12: 107.

Kearney, J.F., Radbruch, A., Leisegang, B., and Rajewsky, K. (1979). A new mouse myeloma cell line that has lost immunoglobulin expression but permits the construction of antibody secreting hybrid cell lines. *J. Immunol.* 123: 1548.

Leslie, G.A. (1980). Idiotypy and IgD isotypy of chicken immunoglobulins. In *Phylogeny of Immunological Memory* (M.J. Manning, ed.) Elsevier/North Holland Biomedical Press. Amsterdam, the Netherlands. p. 253.

Leslie, G.A. and Armen, R.C., (1974). Structure and biological functions of human IgD III. Phylogenetic studies of IgD. *Int. Arch. Allergy* 46: 191.

Leslie, G.A., and Martin, L.N. (1977). Structure and function of serum and membrane immunoglobulin D (IgD). *Cont. Topics Mol. Immunol.* 7: 1.

Martin, L.N., Leslie, G.A., and Hindes, R. (1976). Lymphocyte surface IgD and IgM in non-human primates. *Int. Arch. Allergy* 51: 320.

Olson, J.C., and Leslie, G.A. (1982). IgD: A component of the secretory immune system?, In *Immunoglobulin D: Structure and Function* (G.J. Thorbecke and G.A. Leslie, eds.) *Ann. N.Y. Acad. Sci.* 399: 97.

Pauwels, R., Bazin, H., Platteau, B., and Van Der Straeten, M. (1979). The influence of different antigens on the production of IgD and IgE antibodies. *Ann. Immunol.* 130C: 49.

Rowe, D.S., and Fahey, J.L. (1965a). A new class of human immunoglobulins I. A unique myeloma protein. *J. exp. Med.* 121: 171.

Rowe, D.S., and Fahey, J.L. (1965b). A new class of human immunoglobulins II. Normal serum IgD. *J. Exp. Med.* 121: 185.

Rowe, D.S., Hug., K., Forni, L., and Pernis, B. (1973). Immunoglobulin D as a lymphocyte receptor. *J. Exp. Med.* 138: 965.

Ruddick, J.H., and Leslie, G.A. (1977). Structure and biological function of human IgD. XI. Identification and ontogeny of rat lymphocyte immunoglobulin having antigenic cross-reactivity with human IgD. *J. Immunol.* 118: 1025.

Sire, J.A., Colle, A., and Bourgois, A. (1979). Identification of an

IgD-like surface immunoglobulin on rabbit lymphocytes. *Eur. J. Immunol.* 9: 13.

Stankus, R.P., and Leslie, G.A. (1974). Nonprecipitating and electrophoretically restricted antibodies to carbohydrate of group A Streptoccocus in the rat. *J. Inf. Disease* 130: 169.

Zikan, J., Sima, P., and Tuckova, L., (1983). Cross-reactivity of human and putative pig IgD. Fig IgD-like molecules as serum and lymphocyte components. *Folia Microbiol.* 28: 474.

PAPER 2

IgD and the Maternal-Progeny Interface

Abstract

IgD has been found in rat milk in ten to hundredfold greater amounts than is observed in normal rat serum (Olson and Leslie, 1982). Using ELISA methods, total and DNP-specific rat IgD, IgA, IgM, and kappa immunoglobulin were assayed in gel filtration fractions, in the milk and serum of lactating rats, and in the serum of the pups. The apparent molecular weight of the major species is 670,000 daltons. A minor species, of 140,000 daltons, can be separated by DEAE fractionation. Maternal serum IgD is low during lactation, and is not different from that of nonlactating females, which favors a glandular synthesis model for milk IgD. Milk IgD does not significantly augment the serum IgD of neonatal rats, whose unexpectedly high serum IgD is either of fetal or transplacental origin. Serum IgD in newborn rats declines following birth and does not begin to increase in rats until after weaning. IgD anti-DNP antibodies can be induced in milk by intramammary immunization with DNP-BGG in FCA. Immunization on days 9-10 and 17-18 of pregnancy produced peak milk concentrations of IgD anti-DNP on day 2 of lactation, while immunization 7 days before breeding and on day 7 of pregnancy produced IgD anti-DNP concentrations which peaked on day 7 of lactation.

Introduction

Since its discovery as a serum protein in a human myeloma patient in 1965 (Rowe and Fahey, 1965a), immunoglobulin D has remained a molecule in search of a function. In the ensuing twenty years it has been identified in evolutionarily diverse mammalian species as both a major B lymphocyte surface immunoglobulin as well as an immunoglobulin found free in serum. Such species include humans (Rowe and Fahey, 1965b; Rowe et al., 1973), monkeys (Martin, Leslie, and Hindes, 1976; Leslie and Armen, 1974), rats (Ruddick and Leslie, 1977; Bazin et al., 1978), mice (Abney and Parkhouse, 1974; Finkelman et al., 1979) and pigs (Zikan, Sima, and Tuckova, 1983). Recently this laboratory identified immunoglobulin D as a major immunoglobulin in rat milk, existing normally in concentrations approximately 100 times that found in the serum of lactating rats (Olson and Leslie, 1982). This observation suggests that one potential function for IgD is as secretory immunoglobulin originating in the mammary gland and having its site of action either within the gland itself, in the suckling rat gut, systemically within the suckling rat, or in a combination of sites. In these sites IgD may serve a protective and/or immunoregulatory role.

The large amounts of IgD in rat milk have led us in this report to (1) examine the structure of milk IgD and compare it to what is known

about rat serum IgD, (2) examine the relationship between maternal serum IgD, milk IgD, suckling rat IgD and IgA, IgM, and kappa immunoglobulin levels, and (3) to determine whether, like IgA and IgG, IgD anti-DNP antibodies could be induced by intramammary immunization.

Materials and Methods

Animals. HPR rats, bred for a high precipitin response to group A streptococcal carbohydrate were used in all experiments (Stankus and Leslie, 1974).

Immunizations. Both primary and secondary immunizations consisted of 250 µg of 2,4 dinitrophenyl bovine gamma globulin (DNP_{51}-BGG), prepared according to the method of Eisen (1964). One ml of a 1:1 emulsion of Freund's complete adjuvant was distributed symmetrically in eight sites in the mammary tissue.

Preparation of Milk and Serum Samples. Under ether anesthesia rats were given 0.25 units oxytocin intravenously and milk was collected by mechanical suction. The whole milk was centrifuged at 1.1×10^5 *g* for 1 hr. at 4°C. Blood was collected by tail or cardiac puncture, allowed to clot at 4°C. overnight, then centrifuged at 330 *g* to

collect serum. Samples were stored at -20°C. until assayed.

Preparation of anti-IgD enzyme conjugate. Heterologous anti-rat IgD was raised in rabbits by immunization with rat membrane IgD (Cuchens, Martin, and Leslie, 1978). After 45% saturated ammonium sulfate fractionation of the whole serum, the immunoglobulin-rich fraction was absorbed by immunoadsorbants containing whole rat serum, rat IgM, and '7s' rat Ig. Finally the antibodies were passed over an immunoadsorbant containing IgD isolated from rat whey and the IgD-specific antibodies eluted with 3M sodium thiocyanate. Ouchterlony tests of specifically purified rabbit anti-rat IgD showed a strong reaction of identity between milk IgD and a high-IgD containing serum (assayed independently by Dr. Herve Bazin) but no reaction to normal rat serum, rat IgA, IgM, 7s rat Ig, or monoclonal rat IgG2c. The enzyme conjugate was then prepared with alkaline phosphatase, (Kearney et al., 1979) and shown to be specific for IgD in that it bound specifically to vinyl assay plates coated with partially purified IgD from either milk or serum, but not IgA, IgM, 7s rat Ig, or IgG2c and in that its binding to partially purified IgD was inhibitable by IgD-containing preparations but not by IgA, IgM, 7s rat Ig, or IgG2c.

Monoclonal mouse anti-rat isotype antibodies. Monoclonal mouse hybridomas producing antibodies specific for rat kappa chain (20.7),

rat IgA (1.14.2), and rat IgM (36.12E) were isolated after immunization of BALB/C mice with rat immunoglobulins and fusion of their spleen cells with SP2 plasmacytoma cells (Oi and Herzenberg, 1980). Hybrids were cloned by limiting dilution two or more times. Specificity was confirmed by direct binding to isotype-coated plates and specificity of binding inhibition by purified isotype preparations. Ascites containing high titers of these antibodies were raised in BALB/C mice by i.p. injection of cells a week or more following i.p. injection of 0.5 ml. 2,4,6 tetramethylpentadecane.

General Protocol for ELISA. All assay steps were performed at room temperature, except for the substrate incubation step which was at 37°C. Wells of 96-well vinyl assay plates (Costar #2095) were coated in each assay with 50 µl antigen or antiserum diluted in carbonate-bicarbonate buffer pH. 9.6 (15 mM. sodium carbonate, 35 mM. sodium bicarbonate, 3 mM. sodium azide) for 2 hours. Plates were rinsed 1x with 10 mM. pH 7.0 phosphate buffered saline (PBS). Unreacted sites were then blocked by incubation with 1% BSA in PBS 1 hour and plates were then rinsed 4x with PBS. Antibody binding incubations were for 2 hours followed by 4 rinses with PBS containing 0.05% Tween 20 (Sigma) and 4 rinses with PBS. The enzyme used in all enzyme conjugates was alkaline phosphatase prepared as described by (Kearney et al., 1979). Phosphatase substrate (Sigma 102) (0.4 mg./ml) in 1M diethanolamine buffer and 1 mM magnesium chloride (pH 9.8) was added to the plates and incubated 15 minutes-1 hour at room

temperature or at 37°C. and the plates read on a Dynatech MR 6000 microelisa reader.

Assay for Total IgD. Plates were coated with a 1 µg/ml solution of partially purified IgD in carbonate-bicarbonate buffer. After rinsing and blocking steps, a mixture of enzyme-labeled rabbit anti-rat IgD and dilutions of serum or IgD standard were added to appropriate wells and incubated for two hours. The amount of enzyme conjugate binding to the plate depends on competitive inhibition with free IgD in the sample dilution. After another rinse, plates were incubated with enzyme substrate for 1 hr at 37°C. and read on a microelisa reader.

Assay for Total IgA, IgM, and kappa antibodies. For the IgM and kappa assays, plates were coated with a 10 µg/ml solution of purified rat IgM (mu-kappa) (gift of Dr. W. McKearn) in carbonate-bicarbonate buffer. For the IgA assay, plates were coated with 10 µg/ml of rat IgA (alpha-kappa) (gift of Dr. B. Peri). After rinsing and blocking steps, dilutions of serum or clarified milk in 2% normal rabbit serum-PBS were mixed with an appropriate dilution of monoclonal mouse antibodies in the same diluent and added to the plates. Competitive inhibition with free antigen in the sample dilution determines the amount of mouse monoclonal antibody which binds. After two hours the plates were rinsed and enzyme-conjugated sheep anti-mouse immunoglobulin in 2% normal rabbit serum-PBS was incubated on the

plates for 2 hours. Plates were rinsed, incubated with substrate for 15-60 minutes at 37°C. and read on a microelisa reader.

Assay for IgD, IgA, IgM, and kappa anti-DNP antibodies. Plates were coated with a 50 µg/ml solution of DNP_7-BSA in carbonate-bicarbonate buffer. After rinsing and blocking steps, duplicate serum dilutions were made, one in 2% BSA and one in 2% BSA plus 300 µg/ml DNP-lysine (a concentration of inhibitor adequate to inhibit >90% of anti-DNP binding of strong anti-DNP antisera) and incubated on the plate for 2 hours. After rinsing, a second antibody diluted in 2% BSA-PBS was added to the plates. To detect IgD anti-DNP antibodies, a 1/50 dilution of the anti-delta enzyme conjugate was incubated on the plates for 2 hours, followed by the substrate incubation step. To detect IgA, IgM, or kappa anti-DNP antibodies, dilutions of monoclonal anti rat IgA, anti-rat IgM, or anti-rat kappa in 2% normal rabbit serum were added to the plates and incubated for 2 hours. Plates were rinsed a second time and a dilution of enzyme-conjugated sheep anti-mouse immunoglobulin in 2% normal rabbit serum was added to the plates and incubated for 2 hours. After another rinse, enzyme substrate was added, and incubated 15-60 minutes at 37°C and plates were read on a microelisa reader.

Results

Apparent Molecular Weight of IgD in Milk. Fractionation of milk over Sephacryl-400 (S-400) or Sephacryl-300 (S-300) gel filtration columns calibrated with human IgM, thyroglobulin, ferritin, catalase, human IgG, and bovine serum albumin, resolved a major peak of IgD which co-eluted with thyroglobulin, giving it an apparent molecular weight of 670 kDa (peak I, Fig.1). A minor peak with an apparent molecular weight somewhat less than IgG (140 kDa) was also occasionally observed (peak II, Fig. 1). The position of the IgG elution peak was confirmed in that it coincides with the position of the major kappa-immunoglobulin peak in the gel filtration profile. IgD of similar size has been identified in preparations of serum IgD and may correspond to the 140 kDa IgD species observed on nonreducing SDS-polyacrylamide gel electrophoresis of the rat IgD plasmacytoma proteins described by Bazin (1978). Rat milk IgA, which has an estimated molecular weight of 400 kDa (Cambier and Butler, 1976) eluted in a symmetrical peak, after the major IgD peak and before the IgG peak.

DEAE Fractionation of Clarified Milk. Clarified milk was passed over a DEAE-cellulose anion exchange column equilibrated with 0.1 M tris buffer pH. 8.1 at 4°C. IgD was detected both in the effluent fraction (0.1 M tris) and in the material eluted by a NaCl gradient at a NaCl

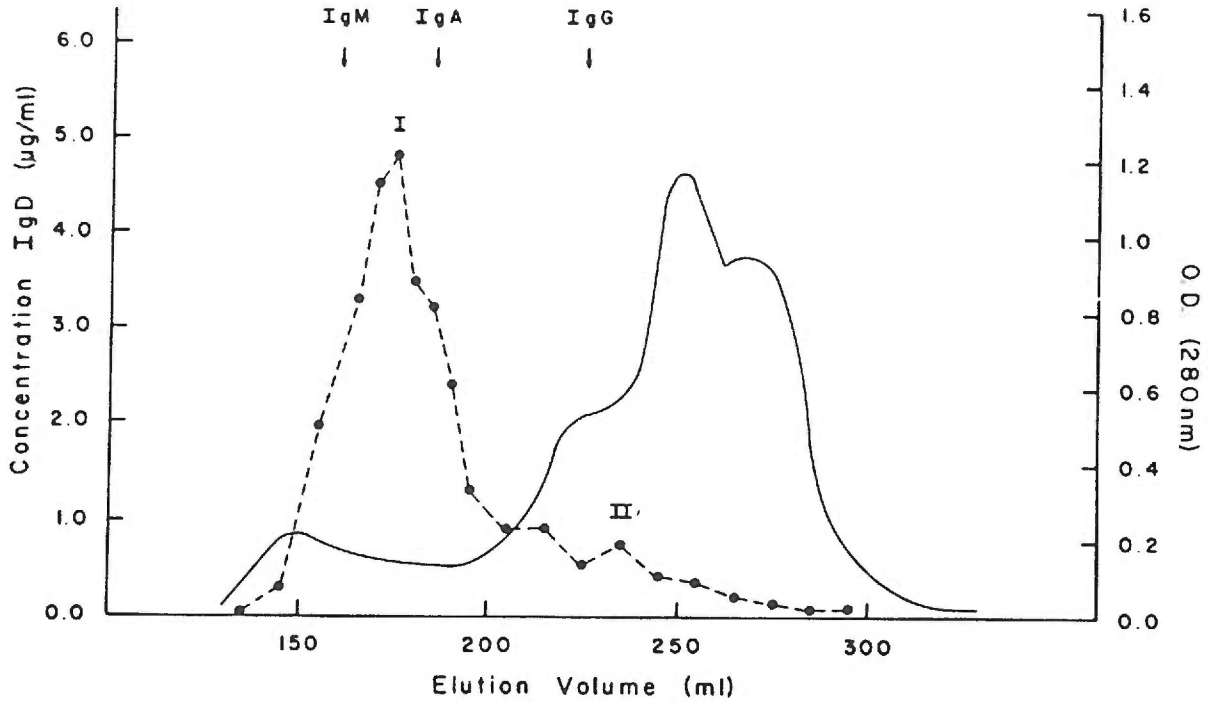


Fig. 1. Elution profile of clarified rat milk on S-300 gel filtration.

concentration of 0.15 M (0.1 M tris plus 0.15 M NaCl). Gel filtration of the dialyzed and concentrated IgD-containing fractions through a calibrated S-300 column yielded an apparent molecular weight for the effluent IgD (0.1 M tris-eluted) of about 140 kDa, which was the same as that of the minor peak observed in gel filtration of clarified milk (peak II, fig. 1), while the apparent molecular weight of the eluted IgD (0.1 M tris + 0.15 M NaCl) was identical to that of the 670 kDa major peak (peak I, fig 1).

Serum and Milk IgD, IgA, IgM, and Milk Protein in the Lactating Rat. The concentration of serum and milk IgD, IgA, IgM, and milk protein for two lactating rats was followed during lactation (Table 1). Serum IgD remained low throughout lactation for both rats in spite of the ten to hundredfold greater amount of IgD appearing in milk. No changes in concentration which would suggest that IgD is being transported in the milk from the serum are apparent either. IgA exists in two-to-tenfold greater concentrations in milk than in serum in the lactating rat. Serum IgA in lactating rats appears to be elevated above that observed in normal rat serum pools, which had IgA concentrations between 0.1-0.2 mg/ml. Milk IgM was present at concentrations which were comparable to that of IgD, (between 20 and 400 µg/ml) although this was 1/3 to 1/60 the concentration of IgM observed in serum. Serum IgM levels in lactating rats were comparable to those observed in normal adult rat serum pools. Kappa

Table 1

Protein, IgM, IgA, and IgD in Two Lactating Rats

Day of Lactation	Protein (mg/ml)	IgM (mg/ml)		IgA (mg/ml)		IgD (µg/ml)	
	Milk	Milk	Serum	Milk	Serum	Milk	Serum
(Rat #1)							
5	18.1	0.23	1.9	4.1	0.33	70	1.8
7	17.8	0.07	1.5	3.0	0.48	31	2.5
9	19.9	0.12	1.1	3.2	0.50	180	2.1
11	21.7	0.16	1.1	3.3	0.62	150	3.0
16	22.4	0.07	1.2	3.6	0.56	52	2.7
18	22.4	0.02	1.2	1.2	0.56	36	2.8
20	26.9	0.09	1.2	1.2	0.86	62	1.5
22	25.6	0.44	-	2.4	-	95	-
24	32.6	0.36	1.1	3.5	1.25	157	2.5
26	39.7	0.43	1.2	2.5	0.69	270	1.7
(Rat #2)							
6	24.4	0.27	0.6	5.9	0.62	126	3.8
10	16.4	0.09	1.0	4.1	0.69	152	4.9
12	18.4	0.08	1.0	4.5	0.86	248	1.8
14	24.8	0.08	0.9	5.0	0.90	73	7.6
16	18.8	0.07	-	3.8	-	128	-
18	20.5	0.07	0.9	3.1	0.90	116	3.6
20	23.2	0.06	1.1	2.5	1.06	119	3.5
22	22.9	0.08	-	3.8	-	142	-
24	22.5	0.07	1.0	2.6	1.18	145	2.5
26	23.1	0.26	1.0	3.4	1.18	164	2.2
30	44.2	-	1.0	-	0.86	757	3.7

immunoglobulins, which represent about 95-99% of the immunoglobulins present in normal rats (Hood *et al.* 1968), are present in milk at between 1/2 and 1/10 that of serum (data not shown). The tendency of milk IgD, IgM, and IgA to reach a minimum at mid lactation and to be higher early and late can be explained by changes both in the quality and quantity of the milk produced in the individual animal. In mid-lactation both the output of milk and its water content is increased.

Serum IgD, IgA, IgM, and Kappa Immunoglobulin in the Suckling Rat. Blood samples from a litter of suckling rats were taken at intervals from birth to 60 days of age and serum IgD, IgA, IgM, and kappa immunoglobulins were quantified (Table 2). As previously reported (Leslie and Cuchens 1982), IgA and IgM are low at birth and gradually increase toward adult levels. Adult levels of IgA are achieved by 28 days, while adult levels of IgM are achieved somewhat later at 47 days. Levels of IgD comparable to that of the lactating mother rat (Table 1) are present at birth and subsequently decline to between 1 and 2 µg/ml until after the end of lactation when transiently elevated serum IgD begins to appear in some rats. This pattern has been confirmed in other experiments. When serum IgD from day 1 unsuckled pups and day 2 suckled pups from the same litter were assayed, no significant difference was observed, serum IgD being relatively high in both groups. Thus the serum IgD seen at birth is primarily acquired

Table 2

Ontogeny of Serum Kappa immunoglobulin (kIg) IgM, IgA, and IgD[a]

Age (days)	no. of sera	kIg (mg/ml)	IgM (µg/ml)	IgA (µg/ml)	IgD (µg/ml)
1	4	0.8	69	28	3.6 (0.8)
8	12	4.0	41	35	1.9 (0.9)
15	13	5.7	177	52	1.8 (0.8)
20	13	6.1	312	82	1.2 (0.3)
24	6	4.4	442	95	1.5 (0.4)
28	7	1.5	466	154	1.6 (0.7)
32	7	1.5	481	190	1.6 (0.9)
36	5	2.7	372	148	1.9 (0.5)
40	5	2.9	604	207	3.7 (5.1)
47	5	2.8	1077	196	1.3 (0.5)
60	5	6.5	1517	176	1.6 (0.2)

[a] Values for IgD represent mean and standard deviation of individual sera. Values for other isotypes represent the mean of duplicate determinations of pooled sera.

either from the placenta or is synthesized *de novo* by the fetus (Steele and Leslie, *in press*) The level of serum kappa immunoglobulins reached a maximum at between day 15 and 20 (mid-lactation) then declined to a low level on days 28 and 32, (about the time of weaning) before rising again. This corresponds to a similar pattern for rat serum IgG1, IgG2a, and IgG2b during lactation observed by Leslie and Cuchens, (1982) and represents mainly the uptake of these isotypes from ingested milk. Since the relatively large amounts of IgD in milk did not likewise enhance serum IgD, it suggests that milk IgD either does not enter the newborn rat circulation, or, if it does, is either rapidly catabolized, or rapidly redistributed to other secretory sites.

Induction of an IgD anti-DNP antibody response in rat milk. To test whether an IgD antibody response could be induced, rats were immunized in the mammary tissue with DNP-BGG in FCA. This procedure has been shown to be effective for inducing IgA and IgG antibodies in rat milk (Cox et al., 1980). Immunization was done at various times prior to parturition. Rats were bled prior to immunizations then milked and bled every five days after the second day of lactation. Milk and serum were assayed for DNP-specific antibodies of the IgD, IgA, IgM, and kappa isotypes (Table 3). IgM and kappa immune responses were higher in serum than in milk. Kappa anti-DNP had a serum/milk ratio of between 6 and 15 during most of lactation, reaching a peak early then

declining gradually thereafter. IgM anti-DNP had a serum/milk ratio of between 14 and 50 and maintained fairly constant levels throughout lactation. IgA anti-DNP, had a much lower serum/milk ratio overall, which varied between 0.33 on day 2 to 5 on day 12. Only the concentration of IgD anti-DNP was generally higher in milk than in serum, with a serum/milk ratio of between 2 and 0.009, reaching a minimum early in lactation and then rapidly rising thereafter. Rats 1 and 2 (Table 3) were immunized and boosted on days 9-10 and 17-18 of pregnancy, (relatively late). The serum IgD anti-DNP immune response was very small, while the milk immune response in both animals reached a peak very early in lactation (day 2) and tapered off rapidly thereafter. A third rat which was primed a week prior to the beginning of pregnancy and boosted two weeks later showed a different timing of the IgD-antibody response, showing a maximum on day 7 rather than on day 2, which suggests that the timing of the intramammary immunization has an important effect on the temporal development of IgD antibodies in milk.

Discussion

IgD exists in milk predominantly as a high molecular weight species of about 670 kDa. Some milk IgD (approximately 140 kDa), however, also exists and may be identical to the 140 kDa moiety

Table 3

IgM, IgA, IgD and Kappa anti-DNP Antibodies in the Serum and Milk of Lactating Rats

			Secondary	Day of Lactation			
Rat #1		Preimmune	Immunization	2	7	17	27
	kIg	0.08 (10)	13.6 (18)	80.0	60.0	9.4	5.6
Serum	IgM	0	7.6	8.4	6.0	10.0	0.0
	IgA	0	1.3	0.7	2.3	2.5	0.1
	IgD	0	0.2	0.3	0.0	0.1	0.0
	kIg	-	-	6.0	6.5	5.6	6.4
Milk	IgM	-	-	0.5	0.5	0.0	0.0
	IgA	-	-	0.6	0.8	0.8	0.4
	IgD	-	-	4.6	0.1	0.6	0.0
Rat #2							
	kIg	0.05 (9)	5.3 (17)	16.0	29.0	41.0	13.5
Serum	IgM	0	2.1	2.2	4.8	0.7	-
	IgA	0	0.5	0.1	0.1	0.4	-
	IgD	0	0.3	0.1	0.0	0.1	0.0
	kIg	-	-	3.4	0.9	3.4	3.5
Milk	IgM	-	-	0.7	0.3	0.2	0.3
	IgA	-	-	0.1	0.0	0.2	3.5
	IgD	-	-	12.4	0.9	0.2	0.2
Rat #3							
	kIg	0.07 (-7)	65.0 (7)	45.0	24.0	17.0	1.1
Serum	IgM	0.3	8.8	26.6	18.7	27.8	14.2
	IgA	0.0	1.1	0.2	7.0	5.3	0.0
	IgD	0.1	0.8	0.4	0.2	0.4	0.2
	kIg	-	-	3.5	0.9	1.4	0.0
Milk	IgM	-	-	1.3	0.0	0.6	0.0
	IgA	-	-	2.2	2.2	1.1	-
	IgD	-	-	1.4	18.0	0.1	0.2

Antibody concentrations for each isotype expressed in terms of a standard isotype anti-DNP antiserum or milk arbitrarily assigned a value of 10 units. An anti-DNP serum was used as the standard for kappa and IgM anti-DNP, an anti-DNP milk was used as the standard for IgA and IgD anti-DNP. Numbers in parenthesis represent the day of pregnancy on which the immunizations were given.

identified by Bazin in serum (1978). The structural and synthetic relationship between the high and low molecular weight IgD's remains to be determined. That is, is the high molecular weight structure a polymer of the low molecular weight unit or is the low molecular weight unit a breakdown product of the high molecular weight unit? If it is an aggregate then it shows remarkable consistency in its physical properties and fractionation characteristics, as no IgD is observed at the void volume on S-300 gel filtration, and DEAE fractionation results in the sharp separation of the high molecular weight major IgD species and the low-molecular weight minor IgD species. The fact that the high molecular weight species binds to DEAE indicates that perhaps a molecular moiety which has adherent characteristics at pH. 8.1 and 0.1 M tris is lost in the breakdown of the molecule into sub-monomeric fragments. Evidence suggests that most of milk IgD is synthesized in the mammary tissue in a manner similar to IgA. If IgD were produced systemically and taken up by the mammary tissue then it would present a formidable task in circulatory logistics to produce several milliliters of milk containing up to several hundred micrograms per ml. each day while maintaining low serum IgD levels no different from that of nonlactating female rats (Steele and Leslie, 1985). Secondly, if there is a biological function of circulating serum IgD which is not connected with secretory immunity, then a mechanism for distinguishing IgD earmarked for secretion from other IgD would be required. Thirdly, the fact that

intramammary immunization led to an IgD immune response predominantly in milk, while more IgA, kappa, and IgM antibodies appeared in serum lends support to a model of intramammary synthesis for milk IgD antibodies. In humans, natural antibodies to IgD have been observed to occur in colostrum at serum/milk ratios which support such a model (Keller *et al.*, 1985).

The ingestion of IgD by the suckling rat does not appear to have much impact on its serum IgD. IgD levels, which are high at birth, decline sharply after the onset of suckling and do not begin to rise (albeit in a highly variable manner) until after lactation is completed. Such high levels of IgD are present before suckling begins, hence are acquired either across the placenta or by de novo synthesis (Steele and Leslie, 1985). In contrast to some rat isotypes, which appear to enter the serum from the milk during lactation, IgD appears to be similar to IgM, IgA, and IgG2c in that it either does not enter the serum at all or enters it very sparingly, or once it enters is quickly sequestered or catabolized.

The high concentration of IgD in newborn serum is itself an interesting question. If it is acquired across the placenta then it is not surprising that after parturition it would decline in serum. Perhaps the large amounts of IgD in milk serve to replace the serum IgD which up until then was provided transplacentally. The apparent

lack of any obvious biological function for IgD and its presence in less than stoichiometric quantities in normal serum suggests that it may be an immunoregulatory rather than an effector molecule. Hence the presence of relatively high concentrations of serum IgD in the fetus would indicate some immunoregulatory role which is played in fetal and neonatal life. Perhaps the IgD which is present in rat milk performs the same function for the lymphoid tissue of the gut that fetal IgD may perform systemically. If IgD is synthesized *de novo* by the fetal rat, then milk may, instead of supplementing serum IgD, actively suppress it, or in some other way modify it. When newborn rats were weaned ten days earlier than normal, subsequent serum IgD levels after lactation were significantly higher than in rats allowed to wean naturally (Steele and Leslie,1985). Hence the effect of milk deprivation or of premature exposure to solid food antigens may have had the effect of stimulating greater serum IgD than normal. While the cause of such an effect is as yet undetermined Chen, Liu, and Katz (1984) reported that i.p. injection of monoclonal IgE into newborn mice caused a class-specific suppression of IgE responses in the adult. A similar suppressive mechanism may apply to passively acquired IgD.

The mammary gland may produce IgD for several reasons: (1) to protect the gland from invasive organisms which enter the mammary tissue, (2) to confer temporary or long-term immunity to the suckling rat, or (3)

to act as a regulatory signal for normal maturation of the neonatal immune apparatus. IgD may function in part as an immunoglobulin for protection since intramammary immunization can produce IgD antibodies specific for the immunizing antigen. It is well known that milk contains antibodies to gut pathogens and can be induced by gut immunization. IgD antibodies to *E. Coli.*(Sewell *et al.*, 1979) and to dietary antigens and grass pollen (Keller *et al.*, 1985) have been shown to occur in human colostrum. In such a case the mother is presumably providing passive gut immunity, and perhaps, by some other means, respiratory immunity. Milk antibodies have been shown to be decisive in the resistance of rat pups to *Trichnella spiralis* larvae (Appleton and McGregor, 1984), and in humans the incidence of diarrheas among infants is greater among formula-fed versus breast-fed infants (Mata and Wyatt, 1971). Therefore it appears likely that IgD participates in the transfer of passive immunity between mother and child.The potential regulatory mechanisms in which milk or serum IgD may participate are unknown. It is of interest to know (1) the specificity of the naturally occuring IgD in rat milk, (2) whether gut or respiratory immunization can lead to a detectable IgD antibody response in milk, (3) the long-term as well as short-term influence of IgD antibodies on the potential of suckling rats to mount antibody responses against the same antigen, (4) the influence of timing of the immunization on the appearance and magnitude of IgD antibody responses in milk.

References

Abney, E.R, and Parkhouse, R.M.E. (1974). Candidate for immunoglobulin D present on murine B lymphocytes. *Nature (Lond.)* 252: 600.

Appleton, J.A., and McGregor, D.D. (1984). Rapid expulsion of *Trichnella spiralis* in suckling rats. *Science* 226: 70.

Bazin, H., Beckers, A., Urbain-Vansanten G. , Pauwels, R., Bruyns, C., Tilkin, A.F., Platteau, B., and Urbain, J. (1978). Transplantable IgD immunoglobulin secreting tumors in rats. *J. Immunol.* 121: 2077.

Cambier, J.C., and Butler, J.C. (1976). Two immunochemically and physicochemically distinct secretory components from rat exocrine secretions *J. Immunol.* 116: 994.

Chen, S.S., Liu, F.T., and Katz, D.H. (1984). IgE class-restricted tolerance by neonatal administration of soluble or cell-bound IgE: cellular mechanisms. *J. Exp. Med.* 160: 953.

Cox, D.S., Taubman, M.A., Ebersole, J.L., and Smith, D.J. (1980). Secretory antibody response of Soluble or Particulate Antigens in Rats. *Mol. Immunol.* 17: 1105.

Cuchens, M.A., Martin, L.N., and Leslie, G.A. (1978). The effects of anti-IgD on serum immunoglobulins, antibody production, and immunoglobulin bearing cells in adult rats. *J. Immunol.* 121: 2257.

Eisen, H.N. (1964). IV. Preparation of purified anti-2,4-dinitrophenyl antibodies. In: *Methods of Biomedical Research, Volume 10* (H. Eisen, ed.) Year Book Medical Publishers, Chicago. p. 94.

Finkelman, F.D., Wood, V.L., Berning, A., and Scher, I. (1979). Demonstration of mouse serum IgD. *J. Immunol.* 123: 1253.

Hood, L., Gray, W.R., Sanders, B.G., and Dreyer, W.J. (1968). Light chain evolution. *Cold Spring Harbor Symp. Quant. Biol.* 32: 133.

Kearney, J. F., Radbruch, A., Leisegang, B., and Rajewsky, K. (1979). A new mouse myeloma cell line that has lost immunoglobulin expression but permits the construction of antibody-secreting hybrid cell lines. *J. Immunol.* 123: 1548.

Keller, M.A., Heiner, D.C., Myers, A.S., and Reisinger, D.M. (1985). IgD in human colostrum. *Pediatr. Res.* 19: 122.

Leslie, G.A., and Armen, R.C. (1974). Structure and biological functions of human IgD. III. Phylogenetic studies of IgD. *Int.*

Arch. Allergy 46: 191.

Leslie, G.A. and Cuchens, M.A. (1982). Developmental aspects of rat IgD expression and the consequences of IgD modulation. In *Immunoglobulin D: Structure and Function* (G.J. Thorbecke and G.A. Leslie, eds.) *Ann. N.Y. Acad. Sci.* 399: 131.

Martin, L.N., Leslie, G.A., and Hindes, R. (1976). Lymphocytes surface IgD and IgM in non-human primates. *Int. Arch. Allergy* 51: 320.

Mata, L.J. and Wyatt, R.G. (1971). Host resistance to infection. *Am. J. Clin. Nut.* 24: 976.

Oi, V.T., and Herzenberg, L.A. (1980). Immunoglobulin-producing hybrid cell lines. In: *Selected Methods in Cellular Immunology* (B.B. Mishell and S.M. Shiigi, eds.) W.H. Freeman Co., San Francisco, p. 351.

Olson, J.C., and Leslie, G.A. (1982). IgD: A component of the secretory immune System?, In *Immunoglobulin D: Structure and Function* (G.J. Thorbecke and G.A. Leslie, eds.) *Ann. N.Y. Acad. Sci.* 399: 97.

Rowe, D.S., and Fahey, J.L. (1965a). A new class of human

immunoglobulins. I. A unique myeloma protein. *J. Exp. Med.* 121: 171.

Rowe, D.S., and Fahey, J.L. (1965b). A new class of human immunoglobulins. II. Normal serum IgD. *J. Exp. Med.* 121: 185.

Rowe, D.S., Hug, K., Forni, L., and Pernis, B. (1973). Immunoglobulin D as a lymphocyte receptor. *J. Exp. Med.* 138: 965.

Ruddick, J.H., and Leslie, G.A. (1977). Structure and biological function of human IgD. XI. Identification and ontogeny of rat lymphocyte immunoglobulin having antigenic cross-reactivity with human IgD. *J. Immunol.* 118: 1025.

Sewell, H.F., Chambers, L., Maxwell, V., Matthews, J.B., and Jefferis, R. (1978). The natural antibody response to *E. coli* includes antibodies of the IgD class. *Clin. Exp. Immunol.* 31: 104.

Stankus, R.P., and Leslie, G.A. (1974). Nonprecipitating and electrophoretically restricted antibodies to carbohydrate of group A Streptococcus in the rat. *J. Inf. Disease* 130: 169.

Steele, M.G., and Leslie, G.A. (1985). Immunoglobulin D in rat serum, saliva, and milk. *Immunology* 55: 571.

Zikan, J., Sima, P., and Tuckova, L. (1983). Cross-reactivity of human and a putative pig IgD. Pig IgD-like molecules as serum and lymphocyte components. *Folia Microbiol.* 28: 474.

Note added in proof. After paper 2 was accepted for publication additional gel filtration studies on milk IgD were done which necessitate a revision of the conclusions about its molecular weight. Briefly (1) while earlier experiments indicated that peak I milk IgD co-eluted with thyroglobulin (667 kDa), more recent experiments with better column resolution suggest that it elutes somewhat slower than thyroglobulin and hence possesses a somewhat lower apparent molecular weight and (2) re-calibration of the S-300 gel filtration column using immunoglobulin standards produces a Log MW vs. elution volume curve which is distinct from that derived from using the gel filtration standards recommended by Pharmacia (see appendix 3). Based on these observations the apparent molecular weight of peak I milk IgD is now estimated to be 300-400 kDa. Other molecular weight estimates presented in this thesis also use the immunoglobulin standard curve. (See appendices 1 and 3).

PAPER 3

IgD Antibodies to DNP Are Specifically Induced in Ascitic Fluid After Intraperitoneal Immunization

Abstract

Three groups of 5-6 female rats were primed with s.c. 1:1 Freund's complete adjuvant (FCA), followed a week later by weekly i.p. immunizations with 9:1 FCA, in which the adjuvant was given along with either DNP-GASV, DNP-OVA, or saline. 11/16 rats produced some ascitic fluid within 2 weeks after the beginning of i.p. immunizations, with 15/16 producing by 3 weeks. Using an ELISA, total and DNP-specific IgD, IgM, and kappa light chain-bearing immunoglobulin (kIg, used as a measure of total rat Ig) were quantified. No significant differences in levels of total IgD, IgM, or kIg were observed in serum or ascitic fluid between the immunization groups. Treatment with 1:1 FCA s.c. resulted in a significant two to sixfold increase in serum IgD by day 7 ($p < 0.01$), while no significant change was observed in the levels of serum IgM or kIg. On day 21, IgD in ascitic fluid was significantly higher than serum IgD ($p < 0.01$) reaching levels up to 33 times that of serum IgD in individual rats, with a mean level for 11 rats of 68.4 µg/ml. In contrast total IgM and kIg were significantly lower in ascitic fluid than in serum, as was total protein (all $p < 0.05$). On day 28 ascitic fluid IgD dropped to roughly 1/8 its previous level, whereas IgM and kIg were relatively unchanged. IgD antibodies to DNP were elicited in small amounts in serum and in larger amounts in ascitic fluid by both DNP-GASV and DNP-OVA, with an ascitic fluid/serum (A/S) ratio of 9.8 and 2.6 on day 21 respectively, while

A/S ratios for IgM and kIg antibodies to DNP were generally much lower (0.1-1.4). This study thus demonstrates a potentially useful method for investigating the conditions under which IgD antibody responses are induced and offers the potential for the purification of antigen-specific IgD.

Introduction

In the last few years serum IgD has been identified as a normal serum immunoglobulin in a number of phylogenetically diverse species. In addition to its early identification in human (Rowe and Fahey, 1965) and non-human primate serum (Leslie and Armen, 1974), it has now been found in the serum of rats (Bazin *et al.*, 1978), mice (Finkelman *et al.*, 1979) and swine (Zikan *et al.*, 1983). In all of these species serum IgD is an immunoglobulin normally found at very low concentrations, and sensitive to proteolysis (Spiegelberg *et al.* 1970; Alcaraz *et al.*, 1980; Neuberger and Rajewsky, 1981; Zikan *et al.*, 1979). Serum IgD thus appears be a regular a component of the humoral immune system.

Recently it has been shown that mice given IgD-secreting plasmacytomas or injected with purified plasmacytoma IgD are markedly enhanced for 7S and 19S PFC responses (Xue *et al.*, 1984), a phenomenon which has subsequently been shown to be mediated through delta receptor bearing T lymphocytes (Coico *et al.*, 1985a,b). Therefore at least one biological function of serum IgD may be as a modulator of antibody responses by way of its constant heavy chain determinants.

Although clinical studies of human serum IgD have demonstrated an association between elevated serum IgD levels and/or IgD antibodies

with a number of syndromes and diseases, most notably atopic, viral, bacterial and autoimmune disease, such studies, owing to the limitations of human research, have only been suggestive of the factors determining the induction of IgD antibodies. At present there is only one report in the literature showing induction of IgD antibodies following experimental immunization (Pauwels *et al.*, 1979). In this study we show that total IgD and IgD antibodies to DNP can be regularly and specifically induced in rat ascitic fluid following immunization with Freund's complete adjuvant (FCA). This discovery should provide a useful experimental system for examining the regulation of IgD antibody responses and render more feasible the isolation and and purification of antigen-specific IgD.

Material and Methods

Animals. Female HPR rats, a strain derived from Sprague-Dawley stock and originally bred for a high precipitin response to group A streptococcal carbohydrate were used in these experiments (Stankus and Leslie, 1974). All animals were over six months of age. Female rats were used as they have been shown to be more amenable than males to ascitic fluid induction (Douglas *et al.*, 1979; Olson, 1981).

Immunizations. On day 0, three groups of 5-6 rats were immunized

subcutaneously (s.c.) in 4 sites with a 1:1 suspension of antigen and Freund's complete adjuvant (FCA) (Difco) distributed symmetrically in 4 sites in the shoulders and hindquarters. On days 7, 14, 28, and 35, animals were immunized intraperitoneally (i.p.) with 1 ml of a 1:9 suspension of antigen to FCA, following a protocol for ascitic fluid induction in mice (Tung *et al.*, 1976) modified for use in rats (Olson et al., 1977).Group I received 230 mg and 460 mg (s.c. and i.p. immunizations respectively) 2,4 dinitrophenylated group A Streptococcal vaccine (DNP-GASV) prepared as described (Osterland *et al.*, 1966; Montgomery and Pincus, 1973). Group II received 250 µg and 500 µg, respectively, of 2,4 dinitrophenylated ovalbumin $DNP_{12.5}$OVA, prepared as described (Eisen, 1964). Group III received saline in lieu of antigen in all immunizations.

Isolation of Serum and Ascitic Fluid. Blood was collected either from the tail artery or from the heart. Paracentesis was performed using an 18 gauge needle with rats under ether anesthesia. Serum and ascitic fluid were allowed to clot overnight at 4°C, and spun at 330 g for 15 minutes at 4°C to remove blood and clotted material. Residual fibrin was removed by passing the ascitic fluid through a wire mesh. All samples were stored at -20°C until assayed.

General Protocol for ELISA. All the ELISA's described below used the following general protocol. Incubation steps were performed at room

temperature unless otherwise indicated. Plates were incubated in a humid environment to minimize evaporation and temperature differences. 96-well polyvinyl assay plates (Costar #2095) were coated with 50 µl of an antigen or antibody dissolved in coating buffer (15 mM sodium carbonate, 35 mM sodium bicarbonate, 3 mM sodium azide, pH 9.6) for 2 hours. Plates were rinsed 1x with phosphate buffered saline (PBS) (10 mM pH 7.0) and blocked with 200 µl 1% BSA in PBS for 1 hour, and then rinsed 4x with PBS. All antibody-binding incubations used 2% normal rabbit serum in PBS (2% NRaS) as diluent, unless otherwise indicated. Incubations in antibody-binding steps were 2 hours with a well volume of 100 µl followed by rinsing 4x with PBS-Tween (PBS with 0.05% Tween 20, (Sigma, St.Louis, MO)) followed by 4 rinses with PBS. After the last antibody incubation step, in which alkaline phosphatase-labeled reagents were used, prepared as described (Kearney et al., 1979), plates are incubated with 100 µl/well of a 0.4 mg/ml solution of p-nitrophenyl phosphate (Sigma #102) in substrate buffer (1 M diethanolamine, 1 mM magnesium chloride, pH. 9.8) at 37 °C. for 30-90 minutes and read on a Dynatech MR 6000 Microelisa reader.

Assays for Total IgD, Total Kappa Immunoglobulin (kIg), and Total IgM. All the assays in this group were based on competitive inhibition of the binding of isotype-specific antibodies to isotype-coated plates. The assay for total IgD was performed as described (Steele and Leslie, 1985). For the IgM and total kappa immunoglobulin (kIg) assays,

plates were coated with 5 µg/ml of an affinity purified rat IgM kappa hybridoma protein (11c). In the IgM assay, sample and standard IgM dilutions were prepared and then mixed with a 1/15,000 dilution of monclonal mouse anti-rat IgM ascitic fluid (GALM 36.12E). In the next step the plates were incubated with alkaline phosphatase-labeled sheep anti-mouse Ig diluted in the same buffer and followed by incubation with enzyme substrate. In the kIg assay, samples and standard kIg dilutions are mixed with a 1/125 dilution of alkaline phosphatase-labeled monoclonal mouse anti-rat kappa (GALK 20.7),followed by incubation with enzyme substrate. The determination of kappa light chain-bearing immunoglobulins was used here as an approximation of total rat immunoglobulins, since kappa-bearing immunoglobulins make up > 95% of rat immunoglobulins.

Assay for Total Protein. Total protein in serum and ascitic fluid samples was assayed by using the dye-binding method of Bradford (1976) (Biorad, Torrance,CA).

Assay for IgD, IgM, and Kappa immunoglobulin (kIg) anti-DNP antibodies. Plates were coated with a 50 µg/ml solution of DNP$_7$ BSA in coating buffer and blocked with 1% BSA (See general protocol). In the first antibody incubation step, duplicate serum dilutions were made, one in 1% BSA plus inhibitor (1 mg/ml DNP-BSA, a concentration found adequate to inhibit > 95% of DNP-specific binding by a high-titer

anti-DNP antiserum at 1/10 dilution) the other in 1% BSA without inhibitor. After rinsing, a second antibody to detect binding of a given isotype was diluted in 2% NRaS and added to the plates. To detect IgD or kIg anti-DNP antibodies, alkaline phosphatase conjugates of either polyclonal rabbit anti rat delta prepared as described, (Steele and Leslie, 1985) or monoclonal mouse anti-rat kappa (GALK 20.7) were used. To detect IgM anti-DNP antibodies a dilution of monoclonal mouse anti-rat IgM ascitic fluid (GALM 36.12E) was added, followed by incubation with alkaline phosphatase-conjugated sheep anti-mouse immunoglobulin. Anti-DNP antibodies were determined from the difference in binding between inhibited and uninhibited wells as compared to a standard antiserum. kIg anti-DNP antibodies were used as an approximation of total rat antibodies to DNP (see above).

Monoclonal anti-isotype reagents. Mouse hybridomas producing antibodies to rat kappa chain (GALK 20.7) and rat IgM (GALM 36.12E) were isolated after immunization of BALB/c mice with rat immunoglobulins followed by fusion of their spleen cells with SP2 plasmacytoma cells (Oi and Herzenberg, 1980). GALK 20.7 bound specifically to kappa bearing monoclonal IgM, IgA, and IgG2c, was inhibited in its binding by purified rat Fab' fragments and by kappa-bearing immunoglobulins. GALM 36.12E bound specifically to IgM and was specifically inhibitable by preparations of monoclonal IgM, and by IgM-containing S-300 gel filtration fractions of 40% SAS

precipitated rat serum immunoglobulins. Ascites containing high titers of these antibodies were raised in BALB/c mice by i.p. injection of cells a week or more following i.p. injection of 0.5 ml 2,4,6 tetramethylpentadecane (Aldrich, Milwaukee, WI).

Results

Induction of Ascites. Each group of female rats (5-6 rats) was primed s.c. with 1:1 FCA and boosted weekly thereafter with 9:1 FCA i.p. (*see Materials and Methods*). Visually apparent ascites developed between 7 and 21 days after the initial i.p. immunization, and most of the animals had collectable fluid by day 14 (Table 1). 15/16 animals eventually produced some ascitic fluid and individual animals showed wide variability in the amount of ascitic fluid produced (2 ml. to 56 ml. per tapping). In the subsequent weeks animals developed considerable fibrosis in the peritoneum with diminution and eventual cessation of ascitic fluid production. There were no marked differences between the experimental groups in the volume of ascitic fluid produced.

Induction of IgD by FCA in Serum and Ascitic Fluid. No significant differences were observed between the immunization groups with respect

TABLE 1

Yield of Ascitic Fluid in Rats Given
Freund's Complete Adjuvant Intraperitoneally[a]

Immunization group		days following first i.p. immunization day 14	day 21	day 28	Cumulative
Saline	Rats with ascites	2/5	3/5	3/5	5/5
	Total Volume	34 ml.	59 ml.	45 ml.	138 ml.
DNP-GASV	Rats with ascites[b]	5/5	2/4	2/4	5/5
	Total Volume	48 ml.	53 ml.	45 ml.	131 ml.
DNP-OVA	Rats with Ascites	4/6	2/6	1/6	5/6
	Total Volume	27 ml.	77 ml.	55 ml.	159 ml.

[a]Rats were given 1 ml 9:1 FCA i.p. on day 0, 7, 14, 21, 28.

[b]One animal in this group died.

to total IgD, IgM, or kappa light chain bearing immunoglobulins (kIg, used throughout this study as an estimate of total antibody. See *Materials and Methods*). A week following s.c. immunization with 1:1 FCA, serum IgD showed a significant two to sixfold increase over its pre-immunization levels ($p < 0.01$) and remained significantly elevated over day 0 levels ($p<.01$) for the remainder of the experiment (Table 2). No further increases in serum IgD over day 7 levels were found, although some animals showed markedly elevated serum IgD on day 14 before ascites developed (e.g. 89.0 µg/ml and 55.3 µg/ml). Total serum IgM or kappa immunoglobulin did not change significantly between day 0 and day 7 ($p>0.05$) (data not shown). A significant decrease in serum kIg was observed between day 14 and day 21 ($p < 0.05$), when a majority of the rats had developed ascites, perhaps reflecting the transudation of serum immunoglobulins into ascitic fluid. A similar decrease in serum IgM and serum total protein was not present.

On day 21, the first day of ascitic fluid collection, the concentration of IgD in ascitic fluid was on the average 9 times greater than it was in rat serum ($p < 0.01$) and ranged from 0.9 µg/ml to 461 µg/ml in a group of 11 rats (Table 2). Serum IgD in rats with ascitic fluid was not significantly different from non-producing rats. IgM and kIg concentrations were significantly greater in serum than in ascitic fluid ($p < 0.05$) (data not shown). A week later, when 6 of 7 rats yielding ascitic fluid received their second tapping, IgD levels

TABLE 2

Effect of Treatment With Freund's Complete Adjuvant on Levels of IgD in Rat Serum and Ascitic Fluid

		μg. IgD/ml. ± SD (N)[a]	
day	Treatment	Serum IgD	Ascitic Fluid IgD
0	1 ml. 1:1 FCA s.c.	1.9 ± 2.0 (15)[b]	-
7	1 ml. 9:1 FCA i.p.	7.2 ± 8.2 (13)[b]	-
14	1 ml. 9:1 FCA i.p.	14.9 ± 25.4 (14)	-
21	1 ml. 9:1 FCA i.p.	7.3 ± 3.9 (15)[c]	68.4 ± 133 (11)[c]
28	1 ml. 9:1 FCA i.p.	6.9 ± 6.5 (15)	8.2 ± 8.5 (8)

Mean of μg/ml IgD determined for individual rats ± SD (number of rats in sample).

day 7 IgD > day 0 IgD, $p < 0.01$ by Mann-Whitney U test.

Ascites IgD > Serum IgD, $p < 0.01$ by Mann-Whitney U test.

in the peritoneal fluid were no longer significantly different from that of serum. This may have either been an artifact produced by removal of a large fraction of the ascitic fluid present on previous tappings or may have been due to a regulatory event. The concentrations of ascitic fluid IgM and kIg did not similarly decrease in secondary tappings. This suggests that the appearance of IgD in ascitic fluid was either less dependent on transudation from serum than IgM and kIg, or that a regulatory event in the peritoneum resulted in the selective suppression of IgD-secreting plasma cells. The ascitic fluid/serum (A/S) ratio of IgD for day 21 ranged from 0.2 to 75.3 with a geometric mean of 3.6 (Table 3). In contrast to this the geometric mean A/S ratio for IgM, kIg, and total protein were all < 1. The closeness to the (A/S) ratio for total protein suggests that IgM and kIg in the ascitic fluid were primarily the result of transudation. However while individual levels for kIg and total ascitic fluid protein for days 21 and 28 had a relatively strong positive correlation ($r = 0.82$ and 0.92 respectively), the correlation between IgM and total ascitic fluid protein for days 21 and 28 were much weaker ($r = -0.35$ and 0.08). This indicates that while kIg, which is composed predominantly of 7S immunoglobulins, may have accumulated in ascitic fluid mainly through transudation, much of the IgM in ascitic fluid may have been synthesized locally. In spite of the depletion of ascitic fluid after the first tapping, the A/S ratio for IgM was 1.1 on day 28.

TABLE 3

Ascitic Fluid/Serum ratio of IgD, IgM, kIg and Total Protein in Rats given Freund's Complete Adjuvant Intraperitoneally[a]

Isotype	day 21	day 28
IgD	3.6 (11)	0.5 (7)
IgM	0.5	1.1
kIg	0.6	0.6
protein	0.6	0.6

[a] Numbers represent geometric mean of individual ratios for rats with ascites on days 21 and 28. Numbers in parentheses represent number of ratios used to calculate geometric mean.

IgD, IgM, and kIg antibodies to DNP in Serum and Ascitic Fluid. IgD antibodies to DNP were elicited both by DNP-GASV and DNP-OVA by the immunization protocol (Table 4). Primary immunization (s.c.) with DNP-GASV did not elicit significant levels of anti-DNP antibodies of any isotype, while the second immunization (i.p.) was followed on day 14 by modest levels of antibodies of all three isotypes. A third immunization (i.p.) on day 14 and subsequent i.p. immunizations thereafter did not significantly increase serum IgD antibody levels above that of day 14, while the day 14 immunization was followed by a an 8-fold increase in serum IgM antibodies and a roughly two-fold increase in serum kIg antibodies. On day 21, the first day of ascitic fluid collection, IgD antibodies to DNP in ascitic fluid averaged about 14 times their level in serum, while by day 28 they had decreased to about 1/7 their level the week before. In the same interval, ascitic fluid IgM and kIg anti-DNP antibodies had remained the same or increased.

DNP-OVA elicited a modest increase in serum IgD anti-DNP after primary s.c. immunization, and elicited relatively high levels of serum IgM anti-DNP and kIg anti-DNP. The second immunization (i.p.) did not increase serum IgD anti-DNP levels further, while IgM anti-DNP and kIg anti-DNP were both increased in serum about threefold. Subsequent i.p. immunizations on day 14 and thereafter did not increase levels of

TABLE 4

IgD, IgM, and kIg anti-DNP Antibodies in Rat Ascitic Fluid and Serum after immunization with DNP-GASV and DNP-OVA

day	Immunization		IgD anti-DNP[a] units/ml±SD(n)	IgM anti-DNP µg/ml±SD(n)	kIg anti-DNP µg/ml±SD(n)
	DNP-GASV				
0	1:1 FCA s.c.	S:	5 ± 2 (5)	0 ± 0 (5)	23 ± 11 (5)
7	9:1 FCA i.p.	S:	9 ± 5 (5)	0 ± 0 (5)	17 ± 11 (5)
14	9:1 FCA i.p.	S:	25 ± 11 (5)	7 ± 0 (5)	118 ± 88 (5)
21	9:1 FCA i.p.	S:	21 ± 8 (5)	56 ± 70 (5)	224 ± 112 (5)
		A:	298 ± 231 (5)	49 ± 63 (5)	826 ± 725 (5)
28	9:1 FCA i.p.	S:	32 ± 46 (4)	21 ± 21 (4)	241 ± 135 (4)
		A:	42 ± 34 (2)	70 ± 42 (2)	908 ± 501 (2)
	DNP-OVA				
0	1:1 FCA s.c.	S:	7 ± 2 (5)	7 ± 14 (5)	53 ± 59 (5)
7	9:1 FCA i.p.	S:	23 ± 15 (5)	392 ± 497 (5)	401 ± 318 (5)
14	9:1 FCA i.p.	S:	19 ± 10 (5)	1365 ± 1316 (5)	1244 ± 667 (5)
21	9:1 FCA i.p.	S:	23 ± 16 (5)	1204 ± 1715 (5)	1150 ± 501(5)
		A:	59 ± 11 (4)	126 ± 98 (4)	365 ± 165(4)
28	9:1 FCA i.p.	S:	17 ± 9 (6)	770 ± 427(6)	1168 ± 300 (6)
		A:	358 ± 350 (2)	210 ± 203(2)	625 ± 377(2)

[a] Units of IgD anti-DNP antibodies expressed relative to a standard IgD anti-DNP ascitic fluid assigned a value of 100 units. S = serum, A = ascitic fluid.

any isotype over the day 14 levels. The concentration of IgD anti-DNP in ascitic fluid was 2-3 times its concentration in serum on day 21 (4 rats), while on day 28, 2/6 rats with ascitic fluid had about 20 times more IgD anti-DNP in ascitic fluid than in their serum. Serum levels of IgD anti-DNP in rats with ascites were not significantly different from those in rats not producing ascitic fluid.

As with the A/S ratio for total IgD, the A/S ratio for IgD anti-DNP antibodies was generally higher in ascitic fluid than in serum, while the A/S ratio for IgM and kIg anti-DNP antibodies was much lower (Table 5). In the DNP-GASV immunization group, IgD antibodies were more sharply elevated on day 21 than in the DNP-OVA immunization group. The effect of prior ascitic fluid collection did not appear to affect the IgD anti-DNP response as it did the level of total IgD in ascitic fluid on day 28. The fact that the A/S ratio for IgM and kIg were in some cases greater than 1 suggests that some local production of IgM and IgG had occurred in the peritoneal cavity.

Discussion

The very low concentration of IgD in normal serum and the early observations that it lacked cytophilic activity (Lawrence *et al.*,

TABLE 5

Ascitic Fluid/Serum Ratio of IgD, IgM, and kIg anti-DNP antibodies[a]

Antigen	Isotype	day 21	day 28
DNP-GASV	IgD	9.8 (5)	2.0 (3)
	IgM	0.8	0.4
	kIg	1.4	3.3
DNP-OVA	IgD	2.6 (4)	23.9 (2)
	IgM	0.1	2.3
	kIg	0.3	2.3

[a] Geometric mean of ratios calculated for individual rats.
Numbers in parentheses represent number of rats in each sample.

1975), that it was found only in human and primate serum (Rowe and Fahey 1965, Leslie and Armen 1974) and only rarely and in unusual situations had detectable antibody activity made it appear to be a molecule without a biological function and perhaps merely an anomaly of primate evolution. However, since IgD is now known to be both a serum and membrane immunoglobulin in rats (Bazin et al, 1978), mice (Finkelman *et al.*, 1979), and swine (Zikan *et al.*, 1983), it is apparent that secreted IgD has been sufficiently important to be preserved during the evolution of each of these species. The more recent observations that it possesses cytophilic activity for some T cells in humans (Sjoberg, 1980) and in mice (Coico *et al.*, 1985), and, especially the observation that it enhances antibody responses *in vivo* and *in vitro* through its interaction with delta receptor bearing T cells (Xue *et al.* 1985; Coico *et al.*, 1985a,b) argue in favor of the existence of at least one potential biological function for secreted IgD. Nevertheless, at present very little is known regarding the factors influencing an IgD antibody response or the effector function of such antibodies.

In this study we have shown that treatment of rats with FCA by the protocol described caused an accumulation of ascitic fluid containing up to 33 times the level of IgD found in the serum of the same rats, and up to 160 times the level of IgD found in preimmune rats. Examination of pools of ascitic fluid from other strains of rats

immunized with other antigens showed that 19/19 had sufficient IgD present to be detectable by Ouchterlony gel diffusion. We have also shown that an easily measurable IgD antibody response, which is clearly dissociated from IgM and kappa anti-DNP responses, occured in ascitic fluid following i.p. immunization. The discovery of a method for producing relatively large quantities of antigen-specific IgD may provide an opportunity both to investigate the function of antigen-specific IgD and to examine the structure of IgD, which in preliminary studies appears to be similar to rat plasmacytoma IR-731 IgD with respect to trypsin sensitivity and to serum IgD with respect to molecular weight by gel filtration (Bazin *et al.*, 1979; Alcaraz *et al.*, 1980, 1981). It also provides an opportunity to investigate the special conditions existing in the peritoneum or in any site of inflammation which may lead to a preferential accumulation of IgD. Provided IgD plasma cell precursors can be induced in sufficient numbers in the peritoneum cell hybridization procedures may enable investigators to isolate antigen specific monoclonal rat IgD.

Human IgD is known to have a relatively short half-life in serum as compared to IgM, IgA, and IgG (Rogentine *et al.* 1966). In view of this, the accumulation of IgD in ascitic fluid may be the result of its protection in the environment of the peritoneum from processes of uptake or degradation. Alternatively, precursors of IgD-secreting cells are either induced to migrate into the peritoneum or are already

present in the peritoneum in especially high concentrations. The decrease in ascitic fluid IgD on day 28, when 6 of 7 rats were tapped for the second time, may be explained in a number of ways: (1) the removal of a large portion of the ascitic fluid and cells the previous week may have removed most of the IgD-secreting cells or their precursors, (2) the additional i.p. immunizations given after ascites had developed may have suppressed further IgD antibody responses, or (3) ongoing changes in the regulatory environment of the peritoneum which were independent of previous tapping or additional immunizations may have shut down the production of IgD antibodies. In support of (1), is the observation that the one rat which had not previously been tapped for ascitic fluid on day 21, had an ascitic fluid IgD concentration of 34.1 µg/ml whereas the remaining rats had 13 µg/ml or less. In support of (3), is the observation that total IgM and kIg remained relatively high in day 28 ascitic fluid, suggesting that there was a selective suppression of IgD production.

Most of the literature dealing with ascitic fluid induction has examined the phenomenon primarily as a laboratory method, and has not examined the underlying cellular mechanisms. Using a similar protocol to ours in F344 rats, Olson (1981) reported that, between 2 and 4 weeks following the induction of ascites, the fluid contained predominantly polymorphonuclear leukocytes, with lymphocytes and atypical lymphocytes making up most of the remainder. In experimental

inflammation in the peritoneal cavity of the guinea pig, Van Dinther-Janssen *et al.* (1985) observed plasma cell infiltration with production of specific antibodies to DNP-BSA in chronic but not acute inflammation. The cellular mechanisms resulting in the production of IgD-containing ascitic fluid clearly requires further study.

Freund's complete adjuvant (FCA) appears to enhance the production of IgD. In this respect it is of interest to note that tuberculosis and leprosy, both caused by members of the genus *Mycobacteriae*, have been associated with elevated serum IgD (Buckley and Trayer, 1972; Rowe *et al.*, 1968). Pauwels *et al.*, (1979) observed that IgD antibodies to ovalbumin were induced in rat serum following immunization with either FCA or *B. pertussis* with aluminum hydroxide, but not with aluminum hydroxide alone, while IgE responses were observable only in rats immunized with aluminum hydroxide or *B. pertussis* with aluminum hydroxide. FCA has subsequently been well established as a down-regulator of IgE synthesis through its induction of IgE suppressive factors (for review see Ishizaka, 1984).

A major difficulty in the investigation of IgD antibody responses has been the very low concentration of serum IgD, and consequently the even lower concentrations of IgD antibodies to a given antigen. Therefore it is desirable to devise methods of amplifying the IgD response. It is also desirable to find a way to obtain relatively

large quantities of the antibodies in order to perform molecular and *in vitro* studies of IgD antibody function. In part both these objectives have been realized in the present study The much higher concentrations of IgD found in other samples of ascitic fluid not connected with this study suggest that the IgD response could be further optimized. Recently we have reported that IgD is normally present in high concentrations in rat milk (Steele and Leslie, 1985), and, by intramammary immunization with FCA, have shown IgD antibodies to DNP are inducible in milk (Steele and Leslie, *in press*). Current research efforts are directed toward a more complete physical characterization of milk and ascitic fluid IgD and toward the purification of antigen-specific IgD.

References.

Alcaraz, G., Bourgois, A., Moulin, A., Bazin, H., and Fougereau, M. (1980) Partial structure of a rat IgD molecule with a deletion in the heavy chain. *Ann. Immunol.* 131 C: 363.

Alcaraz, G., Colle, A., Boned A., Kahn-Perles, B., Sire, J., Bazin, H., and Bourgois, A. (1981) Tryptic and plasmic cleavage of a rat myeloma IgD. *Mol. Immunol.* 18: 249.

Bargellesi, A., Corte, G., Corsulich, E., and Ferrarini, M. (1979) Presence of serum IgD and IgD-containing plasma cells in the mouse. *Eur. J. Immunol.* 9: 490.

Bazin, H., Beckers, A., Urbain-Vansanten, G., Pauwels, R., Bruyns, C., Tilkin, A.F., Platteau, B., and Urbain, J. (1978) Transplantable IgD immunoglobulin-secreting tumors in rat. *J. Immunol.* 121: 2077.

Bradford, M. (1976) A rapid and sensitive method for the quantitation of microgram quantitites of protein utilizing the principle of protein-dye binding. *Analyt. Biochem.* 72: 248.

Buckley, C.E., and Trayer, H.R. (1972) Serum IgD in sarcoidosis and tuberculosis. *Clin. exp. Immunol.* 10: 257.

Coico, R.F., Xue, B., Wallace, D., Pernis, B., Siskind, G.W., and Thorbecke, G.J. (1985a) T cells with receptors for IgD. *Nature, Lond.* 316: 744.

Coico, R.F., Xue, B., Wallace, D., Siskind, G.W., and Thorbecke, G.J. (1985b) Physiology of IgD IV. Transfer of the immunoaugmenting effect of IgD with T-delta containing helper cell populations. *J. exp. Med.* 162: 1852.

Douglas, E.G., Hamada, Y., McKearn, T.J. (1979) Ascites production in 9 rat strains. *J. Immun. Meth.* 26: 69.

Eisen, H.N., (1964) IV. Preparation of purified anti-2,4-dinitrophenyl antibodies. In *Methods of Biomedical Research, Volume 10* (H. Eisen ed.) p. 94. Year Book Medical Publishers, Chicago.

Finkelman, F.D., Woods, V.L., Berning, A., and Scher, I. (1979) Demonstration of mouse serum IgD. *J. Immun.* 123: 1253.

Ishizaka, K. (1984) Regulation of IgE synthesis. *Ann. Rev. Immunol.* 2: 159.

Kearney, J.F., Radbruch, A, Leisegang, B., and Rajewsky, K. (1979) A

new mouse myeloma cell line that has lost immunoglobulin expression but permits the construction of antibody-secreting hybrid cell lines. *J. Immunol.* 123: 1548.

Lawrence, D.A., Weigle, W.O., and Spiegelberg, H.L. (1975) Immunoglobulins cytophilic for human lymphocytes, monocytes, and neutrophils. *J. Clin. Invest.* 55: 368.

Leslie, G.A., and Armen, R.C. (1974) Structure and biological functions of human IgD. III. Phylogenetic studies of IgD. *Int. Arch. Allergy* 46: 191.

Montgomery, P.C., and Pincus, J.H. (1973) Molecular restriction of anti-DNP antibodies induced by dinitrophenylated type III pneumococcus. *J. Immunol.* 111: 42.

Neuberger, M.S., and Rajewsky, K. (1981) Switch from hapten-specific immunoglobulin M to immunoglobulin D secretion in a hybrid mouse cell line. *Proc. Nat. Acad. Sci.* 78: 1138.

Oi, V.T., and Herzenberg (1980) Immunoglobulin-producing hybrid cell lines. In *Selected Methods in Cellular Immunology* (B.B. Mishell and S.M. Shiigi, eds.) W.H. Freeman Co., San Francisco. p. 351.

Olson, J.C., (1981) Utilization of rat ascitic fluid for monitoring immune regulation in vivo. In *The Immune Network: Endogenous and Exogenous Influences on Idiotype Expression* (doctoral dissertation) p. 212.

Olson, J.C., Dawson, D.E., and Leslie, G.A. (1977) Rat ascitic fluid as a source of large quantities of antibody. *Fed. Proc.* 36: 1198.

Osterland, C.K., Miller, E.J., Karakawa, W.W., and Krause, R.M. (1966) Characterisitics of streptococcal group-specific antibody isolated from hyperimmune rabbits. *J. exp. Med.* 123: 599.

Pauwels, R., Bazin, H., Platteau, B., and Van Der Straeten, M. (1979) The influence of different antigens on the production of IgD and IgE antibodies. *Ann. Immunol.* 130 C: 49.

Rogentine, G.N., Rowe, D.S., Bradley, J., Waldmann, T.A., and Fahey, J.L. (1966) Metabolism of human immunoglobulin D (IgD). *J. Clin. Invest* 45: 1467.

Rowe, D.S., and Fahey, J.L., (1965) A new class of human immunoglobulins. II. Normal serum IgD. *J. exp. Med.* 121: 185.

Rowe, D.S., McGregor, I.A., Smith, S.J., Hall, P., and Williams, K.

(1968) Plasma immunoglobulin concentrationin a West African (Gambian) community and in a group of healthy British adults. *Clin. exp. Immunol.* 10: 257.

Sjoberg, O. (1980) Presence of receptors for IgD on human T and non-T lymphocytes. *Scand. J. Immun.* 11: 377.

Spiegelberg, H.L., Prahl, J.W., and Grey, H.M. (1970) Structural studies of human gamma-D myeloma protein. *Biochem.* 9: 2115.

Stankus, R.P., and Leslie, G.A. (1974) Nonprecipitating and electrophoretically restricted antibodies to carbohydrate of group A *Streptococcus* in the rat. *J. Infect. Dis.* 130: 169.

Steele, M.G., and Leslie, G.A. (1985) Immunoglobulin D in rat serum, saliva, and milk. *Immunol.* 55: 571.

Steele, M.G. and Leslie, G.A. (1986) IgD and the maternal-fetal interface. In *Immunoregulation and Fetal Survival* (T.Gill and T.G. Wegmann, eds.) Oxford University Press, New York. *in press.*

Tung, A.S., Ju, S., Sato, S., and Nisonoff A. (1976) Production of large amounts of antibodies in individual mice. *J. Immunol.* 116: 676.

Van Dinther-Janssen, A.C.M.H., Hofland, L., and Scheper, R.J. (1986) Specific plasma cell accumulation in antigen-induced chronic inflammation in the guinea pig peritoneal cavity. *Int. Archs. Allergy appl. Immun.* 51: 320.

Xue, B., Coico, R., Wallace, D., Siskind, G.W., Pernis, B. and Thorbecke, G.J. (1984) Physiology of IgD IV. Enhancement of antibody production in mice bearing IgD-secreting plasmacytomas. *J. exp. Med.* 159: 103.

Zikan, J., Sima, P., and Tuckova, L. (1983) Cross-reactivity of human and a putative pig IgD. Pig IgD-like molecules as serum and lymphocyte components. *Folia. Microbiol.* 28: 474.

SUMMARY AND CONCLUSIONS

Summary. In spite of the very low levels of IgD in the serum of lactating rats, IgD is consistently elevated in milk and the onset and cessation of lactation are not marked by noticeable changes in serum IgD, as might be expected if IgD produced in the mammary gland were the result of transudation. Nor is serum IgD in lactating rats significantly different from that of nonlactating and non-pregnant female rats. This provides circumstantial but nevertheless strong evidence for local synthesis of IgD in the mammary gland tissue itself. The fact that intramammary immunization is an effective means of producing IgD antibodies in milk reinforces this conclusion.

High levels of IgD in the milk of lactating rats (36 - ~800 µg/ml) are consistently observed, and remain fairly constant through lactation when expressed in terms of µg IgD/mg total protein. Milk IgD appears to be a molecule which exists predominantly as a multimeric species, which, based on column calibration with immunoglobulin standards appears to be between 300 and 400 kDa by gel filtration. The molecule therefore appears to be a dimer, assuming the monomeric unit is about 150 kDa, as reported by Alcaraz et al., (1980). Partial reduction and alkylation of milk IgD resulted in ~150 kDa and 50 kDa species (see appendix 1).

In spite of the fact that suckling rats ingest large quantities of milk IgD, it has little effect on their serum IgD level, which remains relatively low through lactation. The relatively high concentration of IgD in newborn rat serum is therefore not the result of milk ingestion, and so its presence must be the result of either transplacental transfer or fetal synthesis.

As has been reported for the human, serum IgD levels in rats are subject to considerable individual variation, with no readily apparent ill effects accompanying either high or low concentrations of IgD. At least in the period following weaning to age 60 days there is considerable temporal variation in serum IgD as well, and is characterized by transient elevations in serum IgD which last from a few days to several weeks in duration. T cells do not appear to be responsible for the maintenance of serum IgD levels, since serum IgD levels in athymic nude rats is not significantly different from that of euthymic heterozygous or euthymic homozygous littermates.

Subcutaneous immunization of HPR rats with Freund's complete adjuvant (FCA) result in marked elevation in serum IgD levels, an effect which is independent of the use of antigen with the adjuvant. Intraperitoneal immunization with FCA results in a marked accumulation of IgD in ascitic fluid. While the effect on serum and ascitic fluid

IgD levels by other adjuvants was not tested, Pauwels, (1979) observed a similar tendency of FCA to increase serum IgD concentrations which was not observed with other adjuvants.

Rat IgD in serum and ascitic fluid shows marked size heterogeneity, appearing in at least three and perhaps more size species by gel filtration. In contrast milk IgD appears to be almost entirely of one size species. The susceptibility of IgD to proteolysis may explain some of the heterogeneity. The heterogeneity may also represent the synthesis of monomeric, dimeric, and other polymeric forms in serum and ascitic fluid similar to that observed with IgA or of subclasses of IgD which preferentially assume different sizes. Some of the size heterogeneity, may represent random aggregates of IgD or degradative intermediates. The low molecular weight IgD species migrating between IgG and BSA by gel filtration may be either F(ab') fragments shed from B lymphocytes or Fc and/or F(ab') fragments resulting from artifactual or *in vivo* proteolysis of IgD.

IgD antibodies to DNP may be induced in milk by intramammary immunization with Freund's complete adjuvant (FCA) and dinitrophenylated ovalbumin (DNP-OVA), bovine gamma globulin (DNP-BGG), or group A *Streptococcal* vaccine (DNP-GASV). While kIg and IgM antibodies were induced mainly in serum by such an immunization protocol, the IgD antibody response appeared mainly in milk during the

early part of lactation. Such antibodies appear also to be inducible by drinking water immunization with 0.1% DNP-OVA during the breeding and pregnancy period (see Appendix 4).

IgD antibodies may also be induced in ascitic fluid following weekly intraperitoneal immunization with DNP-OVA or DNP-GASV with FCA. While kappa light chain bearing antibodies and IgM antibodies to DNP occurred in both the ascitic fluid and serum, the initial ascitic fluid/serum (A/S) concentration ratios of these antibodies were generally ~1 or less, while in initial ascitic fluid the (A/S) concentration ratio was generally >1 and ranged from 2 to as much as 23.9. The cause of the relatively high concentrations of both total IgD and IgD antibodies in ascitic fluid is not known, but a plausible explanation would be the accumulation in ascitic fluid of IgD-secreting cells. If this is true then it may be possible to isolate IgD-secreting hybridomas by fusion of cells from peritoneal washes or ascitic fluid.

Conclusions. The research presented in this dissertation represent an initial characterization of the qualitative and quantitative nature of rat IgD in serum and secretions, and as such sets the stage for the use of the rat as a model system for the investigation of the biological function of IgD. It seems likely that IgD serves both as a secretory and serum immunoglobulin, serving similar or very different

functions in those two compartments. As a secretory immunoglobulin which is especially rich in rat milk, it may be that rat IgD is important to the passive immunity which is conferred by milk. Alternatively it may merely be an immunoglobulin which is protective to the mammary tissue. However such a role seems unlikely to represent its sole function since human milk IgD antibodies have been found with specificity to gut organisms (Sewell et al., 1978) and to antigens of likely upper respiratory tract exposure (Keller et al. 1985) which do not seem relevant to the protection of the mammary gland itself. As has been suggested by the work of Bazin et al. (1978) it may be possible to selectively suppress serum IgD and IgE by chronic anti-delta treatment. It would be of interest therefore to see if IgD is selectively suppressed in the milk, and if so, what effect the suppression of milk IgD would have on the immunological status of the suckling rat. Following purification of IgD and radiolabeling of IgD from rat milk it would be interesting also to examine the tissue localization of IgD within the suckling rat.

The presence of adult levels of serum IgD in the newborn rat indicates that IgD may play a role in the fetal development of the immune system just as IgD in milk may play a parallel role in the suckling rat. There is some controversy as to whether IgD crosses the placenta in humans (Leslie and Swate, 1972; Salonen et al., 1985). It would be of interest therefore to determine through radioactive tracer studies

whether IgD derived from ascitic fluid will cross the placenta.

Recent work by Xue et al. (1984) and Coico et al. (1985 a,b) in the murine system provide the first indication that one biological function of IgD may be its enhancement of immune responses via an interaction with IgD Fc receptors on T cells. Xue et al. found that IgD in the form of TEPC 1017 and 1033 plasmacytoma proteins can enhance splenic immune responses in euthymic but not athymic nude mice. Subsequently Coico et al. observed that a population of Lyt 1+2- and IgD Fc receptor positive T cells increased in number following exposure to IgD. When such cells were adoptively transferred they could similarly enhance splenic PFC responses. The parallel to the regulatory mechanism elucidated for IgE is striking. There it has been shown that the presence of IgE increases the numbers of *IgE* Fc receptor-positive Lyt 1+2- T cells which in turn secrete IgE binding factors. Such factors, depending on the regulation of their glycosylation, serve to suppress or enhance IgE antibody responses (Ishizaka, 1984). In light of this it would be of great interest to determine if IgD-binding factors are produced by the Lyt 1+2- IgD Fc receptor positive T cells identified by Coico. Provided such IgD-binding factors exist it presents some interesting theoretical possibilities as to the function of both secreted and lymphocyte membrane IgD. If such factors bind to lymphocyte membrane IgD, then clearly it must act in concert with other stimuli, otherwise the

result would be polyclonal activation of IgD+ cells. Perhaps sIgD, in binding to antigen, allows an IgD-binding factor to bind with greater affinity, or if not, perhaps only cells which have received a second stimulus will respond to the binding of the factor. In this way the regulatory impact of an IgD binding factor could be restricted to B cells responding to antigen.

In a similar scenario, secreted IgD, bound to antigen or to anti-delta antibody might serve to trigger IgD Fc receptor-bearing T cells to proliferate and secrete IgD-binding factors locally to stimulate responding B cells. In the experiments by Xue et al. (1984) the injection of large amounts of non-antigen-specific plasmacytoma IgD may have had an effect merely because the resultant high concentration of serum IgD overcame a hypothetical low affinity of T cell IgD Fc receptors for such IgD. It would be of interest therefore to determine the relation between a range of serum IgD levels and the IgD enhancing effect on PFC responses reported by Xue et al. (1984).

The enhancement of antibody responses by anti-delta treatment may have as much to do with the binding of such anti-delta to serum IgD as to its binding to lymphocyte membrane IgD. The binding of anti-delta to serum IgD might have the same effect on its affinity for IgD Fc receptors as the binding of antigen, thus stimulating Fc receptor-bearing T cells throughout the body and in this way

transmitting an enhancing effect on antibody responses. This would allow an explanation for the "indirect effect" of anti-delta on B cells reported by Jacobson et al., (1985).

Since antibody responses take place in athymic nude mice and rats and in chronically anti-delta suppressed mice which are greatly depleted of IgD+ cells (Finkelman, Mond, and Metcalf, 1983; Metcalf, Mond, and Finkelman, 1983), it is clear that the proposed regulatory circuit for IgD and IgD-binding factors would not be the only means whereby antibody responses might take place, but may be supplemented by alternative or salvage pathways. Such pathways notwithstanding, the depletion of IgD+ cells appears to seriously debilitate the immune system, as has been suggested by Metcalf, Mond and Finkelman (1983), citing studies in which anti-delta suppressed mice and CBA/N mice are killed by normally nonlethal infections of *Salmonella typhimurium* (Metcalf, Mond, and Finkelman, 1982) and *Plasmodium yoelii* (Hunter, Finkelman, and Smith, 1981).

Very little if anything is yet known concerning the cellular aspects of IgD induction. Histological studies have found IgD-producing cells in a variety of tissues both secretory and other locations (see section IV of literature review) under different conditions. However little has been done to determine the kinetics of the appearance of these cells, the possible migration of blasts, etc. It would be

desirable therefore to conduct detailed studies of IgD-containing cells in mammary tissue, milk, ascitic fluid, the peritoneum, and other sites in an effort to identify the source of the IgD which has been observed in these studies. If IgD-containing cells appear in the peritoneum in sufficient numbers it may be possible using cell hybridization techniques to produce IgD-secreting hybridomas of a given specificity, which would greatly assist efforts to understand the biological function of IgD in the context of its antigen specificity.

Our preliminary studies as well as those of Tamura, Fujinaga, and Kuruome, (1982) have found that IgD responses tend to occur relatively early as compared to other isotype responses. This would be in agreement with the concept of a regulatory molecule with the function of amplifying an initial response to antigen. The low concentration of IgD in serum could be explained by the fact that it represents a molecule with a primarily regulatory function and not an effector one. Consequently high concentrations of IgD are simply not required for this function to be carried out.

Much remains to be done before the biological function of IgD is completely understood. The directions which I believe that the research should take involve studies of the tissue location of IgD-producing cells and the conditions under which they appear,

examination of the parameters of IgD antibody responses in serum and milk in response to different antigens, routes of immunization, and dosage levels and their relationship to responses of other isotypes, the investigation of whether IgD is transported into the fetus during pregnancy or synthesized *de novo*, and finally the isolation of IgD antibodies of a given specificity which then can be used to test the impact of antigen-specific IgD on the immune system in *in vivo* and *in vitro* experiments. The work presented in this thesis has elucidated some of the basic characteristics of secreted IgD and IgD responses in the rat. It is hoped that it will prove useful in providing a basis for further research using the rat as a model.

References

Bazin, H, Platteau, B., Becker, A., and Pauwels, R. (1978). Differential effect of neonatal injections of anti-mu or anti-delta antibodies in the synthesis of IgM, IgD, IgE, IgA, IgG1, IgG2a, and IgG2c immunoglobulin classes. *J. Immunol.* 121: 2083.

Coico, R.F., Xue, B., Wallace, D., Pernis, B., Siskind, G.W., and Thorbecke, G.J. (1985). T cells with receptors for IgD. *Nature* 316: 744.

Coico, R.F., Xue, B., Wallace, D, Siskind, G.W., and Thorbecke, G. J. (1985). Physiology of IgD: VI. Transfer of the immunoaugmenting effect of IgD with T-delta containing helper cell populations. *J. exp. Med.* 162: 1852.

Finkelman, F.D., Mond, J.J., and Metcalf, E.S. (1983). Effect of neonatal anti-delta antibody treatment on the murine immune system. I. Suppression of development of surface IgD+ B cells and expansion of a surface IgM+IgD- B lymphocyte population. *J. Immunol.* 131: 593.

Hunter, K.W., Finkelman, F.D., and Smith , L.P. (1981) Modulation of the immune response by antibodies to IgD. *Fed. Proc.* 40: 1071.

Ishizaka, K., (1984). Regulation of IgE synthesis. *Ann. Rev. Immunol.* 2: 159.

Jacobson, E.B., Xue, B., O'Neil, M.J., Siskind, G.W., Pernis, B., and Thorbecke, G.J. (1985). Physiology of IgD: V. Enhancement of antibody responses in vivo by allo-anti-IgD is due primarily to an indirect effect on B cells. *J. Immunol.* 135: 2293.

Keller, M.A., Heiner, D.C., Myers, A.S., and Reisinger, D.M. (1985). IgD in human colostrum. *Pediatr. Res.* 19: 122.

Leslie, G.A., and Swate, T.E. (1972). Structure and biologic functions of human IgD: I. the presence of immunoglobulin D in human cord sera. *J. Immunol.* 109: 47.

Metcalf, E.S., Mond, J.J., and Finkelman, F.D. (1982). Abnormal B cell function in mice treated with anti-IgD from birth. *Fed. Proc.* 41: 424.

Metcalf, E.S., Mond, J.J., and Finkelman, F.D. (1983). Effect of nonatal anti-delta antibody treatment on the murine immune system: II. Functional capacity of stable sIgM+ sIa+ sIgD- B cell population. *J. Immunol.* 131: 601.

Pauwels, R., Bazin, H., Platteau, B., and Van der Straeten, M. (1979). The influence of different adjuvants on the production of IgD and IgE antibodies. *Ann. Immunol.* 131C: 49.

Salonen, E., Hovi, T., Meurman, O., Vesikari, T., and Vaheri, A. (1985). Kinetics of specific IgA, IgD, IgE, IgG, and IgM antibody responses in rubella. *J. Med. Virol.* 16: 1.

Sewell, H.F., Matthews, J.F., Flack, V., and Jefferis, R. (1979). Human immunoglobulin D in colostrum, saliva, and amniotic fluid. *Clin. Exp. Immunol.* 36: 183.

Tamura, H., Fujinaga, T., and Kuruome, T. (1982). IgD antibody to *Mycoplasma pneumoniae* in human sera as measured by the RAST. *Int. Arch. Allergy Appl. Immunol.* 69: 335.

Xue, B., Coico, R., Wallace, D., Siskind, G.W., Pernis, B., and Thorbecke, G.J. (1984). Physiology of IgD: IV. Enhancement of antibody production in mice bearing IgD-secreting plasmacytomas. *J. exp. Med.* 159: 103.

APPENDIX 1

Structural Studies of Rat IgD in Milk, Saliva, Serum and Ascitic Fluid

Introduction

Studies of the structure of rodent immunoglobulin D have relied mainly on isolation of the molecule from the surface of B lymphocytes or from a limited number of plasmacytoma proteins and their nucleic acids. The characterization of rat IgD is no exception, in which the bulk of structural studies have relied on the IR-731 protein (Bazin et al., 1978). It is questionable however whether such plasmacytomas can provide an entirely reliable picture of the molecule as it exists normally *in vivo* owing to the frequently observed mutation of structure which has been reported both for the IR-731 protein (Sire et al., 1981) and for the mouse TEPC 1017 plasmacytoma (Thiele et al., 1985). Studies of the structure of IgD isolated from normal rats is hampered by its low concentrations in serum and the extreme lability and proteolytic sensitivity of the molecule. Recently however, in this laboratory we have shown that IgD concentrations are greatly enhanced in rat milk (Olson and Leslie, 1982; Steele and Leslie, 1985) and in rat ascitic fluid following intraperitoneal immunization with Freund's complete adjuvant (Steele and Leslie, *submitted*). This has allowed more extensive physical characterization of IgD as it exists in these body fluids. More limited characterization studies have been performed on rat serum and saliva IgD.

Apparent Molecular Weight of IgD as Determined by Gel Filtration

Rat Milk IgD. Clarified rat milk was prepared by ultracentrifugation as described (Steele and Leslie, 1985), and fractionated on Sephacryl 300 and Sephacryl 400 gel filtration columns in 0.01 M TBS buffer. Assay of eluting fractions for total IgD revealed a major size species (milk IgD peak I) with an apparent molecular weight between 300-400 kDa based on an S-300 calibration curve generated using immunoglobulin molecular weight standards (Figure 1a; see Appendix 2). Previously it was reported that milk IgD peak I co-migrated with thyroglobulin and that IgA eluted somewhat after thyroglobulin and IgD (Steele and Leslie, *in press*). Subsequent calibration studies indicate that IgA (400 kDa) co-migrates with thyroglobulin on the S-300 gel filtration column. While IgD appears to co-elute with IgA (Figure 1a), the resolution of the peak is relatively poor. An S-400 gel filtration run of the same material (Figure 1b) showed an IgD peak which appeared to elute after IgA as assayed in the same fractions using GALA 1.14.2, a monoclonal mouse anti-rat IgA (see appendix 2). A minor IgD peak (milk IgD peak II) was also occasionally observed, and appeared to elute after IgG with an apparent molecular weight of between 100 and 150 kDa. When a sample of clarified milk was precipitated with 40% saturated ammonium sulfate and the redissolved precipitate passed over a different S-300 gel filtration column with better resolution, the

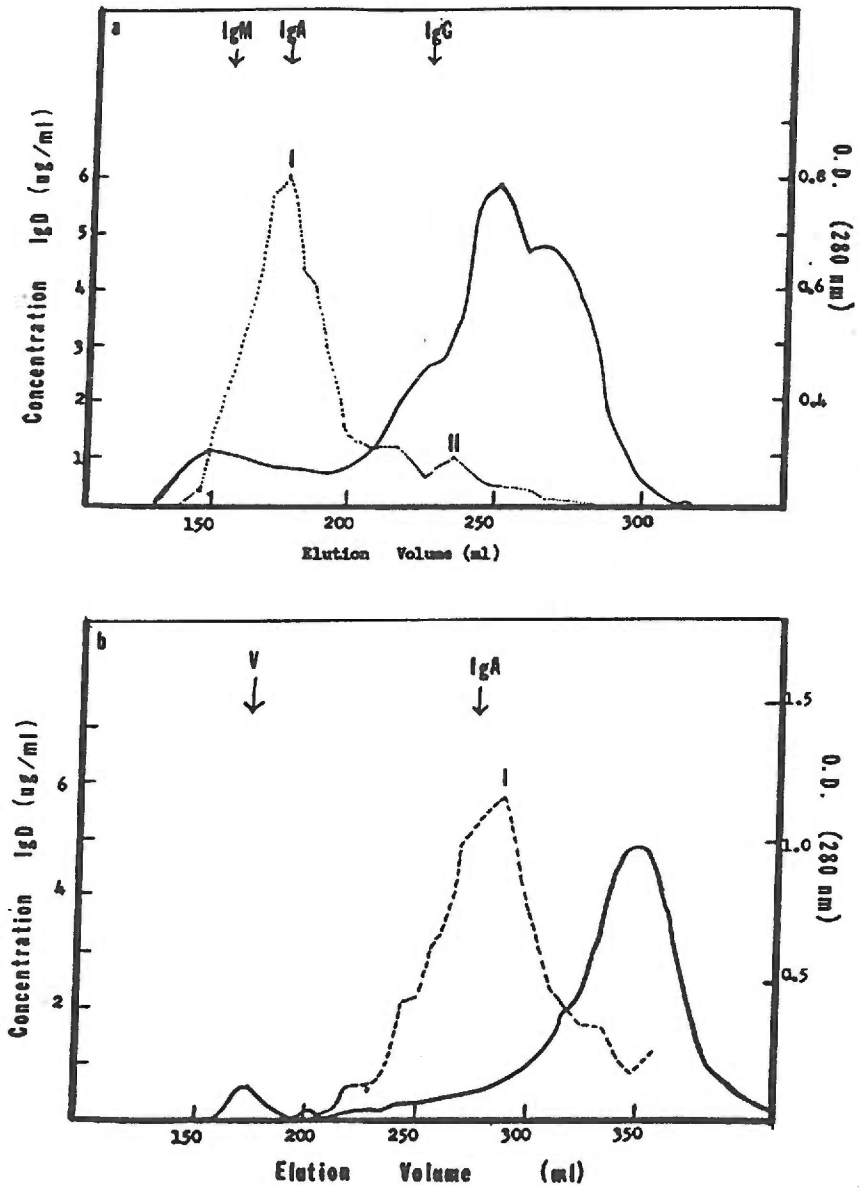


Figure 1 . Elution profile of rat milk by gel filtration on (a) S-400 and (b) S-300. OD 280 (solid line) and IgD (dotted line). (V: void volume).

major peak of IgD was found to elute with an apparent molecular weight of 340 kDa and a minor peak at ~ 97-110 kDa (Figure 2a). In another S-300 run in which clarified milk was fractionated in the presence of 0.1% NP40, the major IgD peak eluted at about 310 kDa (Figure 2b). The results of these experiments and a report that the IgD monomeric unit in rats is 150 kDa (Alcaraz et al., 1980) suggests that milk IgD exists primarily as a dimer. Whether the molecule is associated with secretory component has not yet been conclusively determined. In order to determine the likely monomeric structure of milk IgD, clarified rat milk was partially reduced and alkylated (Leslie and Benedict, 1969) and again fractionated on the S-300 column (Figure 3). The results indicate that partial reduction produced three peaks of IgD, one eluting at about 300 kDa and which may be unreduced milk IgD, a second peak appeared at 160 kDa and would appear to correspond to monomeric units of IgD, while a third, larger peak was observed at about 50-60 kDa and appears to correspond to delta chains. In addition some IgD appeared at the void volume suggesting that some random aggregation of IgD had occurred.

Serum IgD. Two different rat serum pools chosen for their high concentration of IgD based on ELISA were fractionated on an S-300 gel filtration column. A pool of serum collected from (rnu/rnu) and (rnu/+) rats apparently had five IgD size species, peak I eluting between the void volume and the elution position of IgM, peak II eluting between IgM and thyroglobulin (~560 kDa), peak III eluting

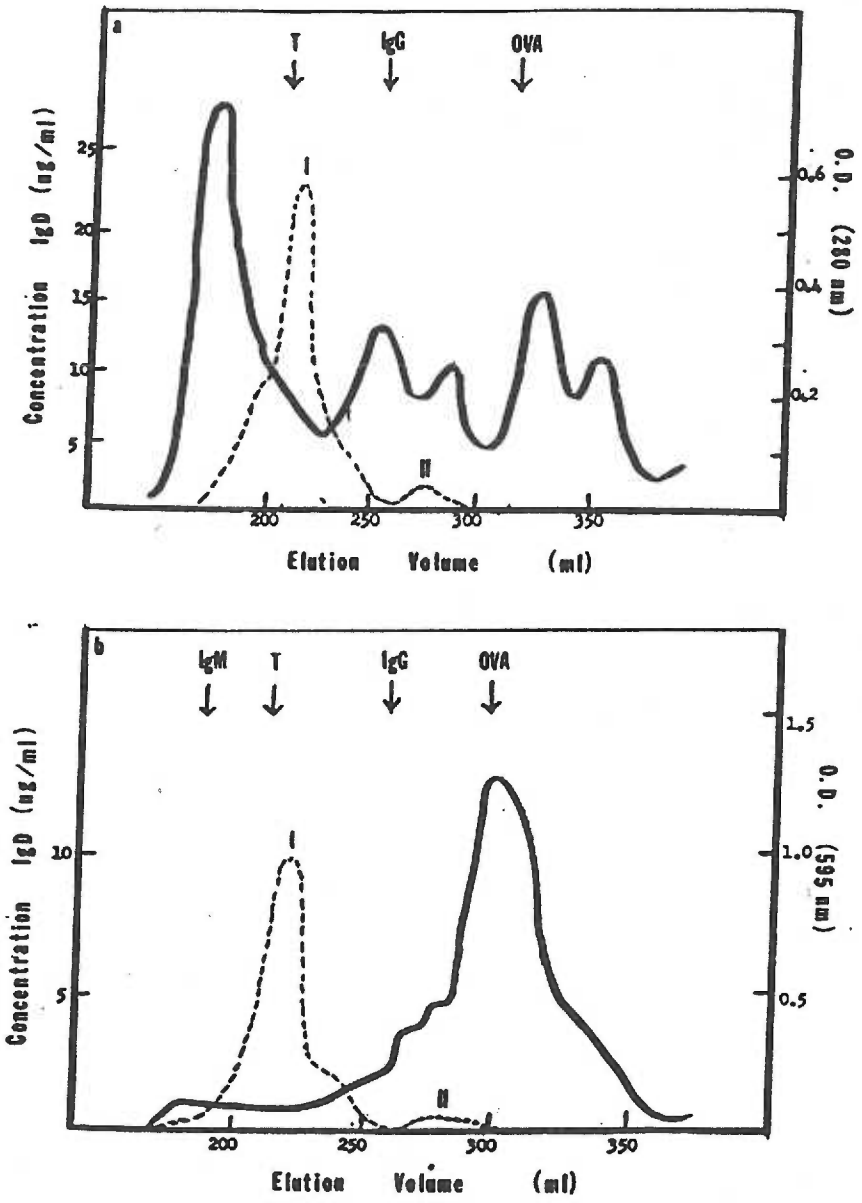


Figure 2 . Elution profile of S-300 gel filtration of (a) milk following salt fractionation with 40% saturated ammonium sulfate, (b) milk with 0.1% NP40 in the eluting buffer. OD 280 nm. (solid (line), IgD (dotted line).

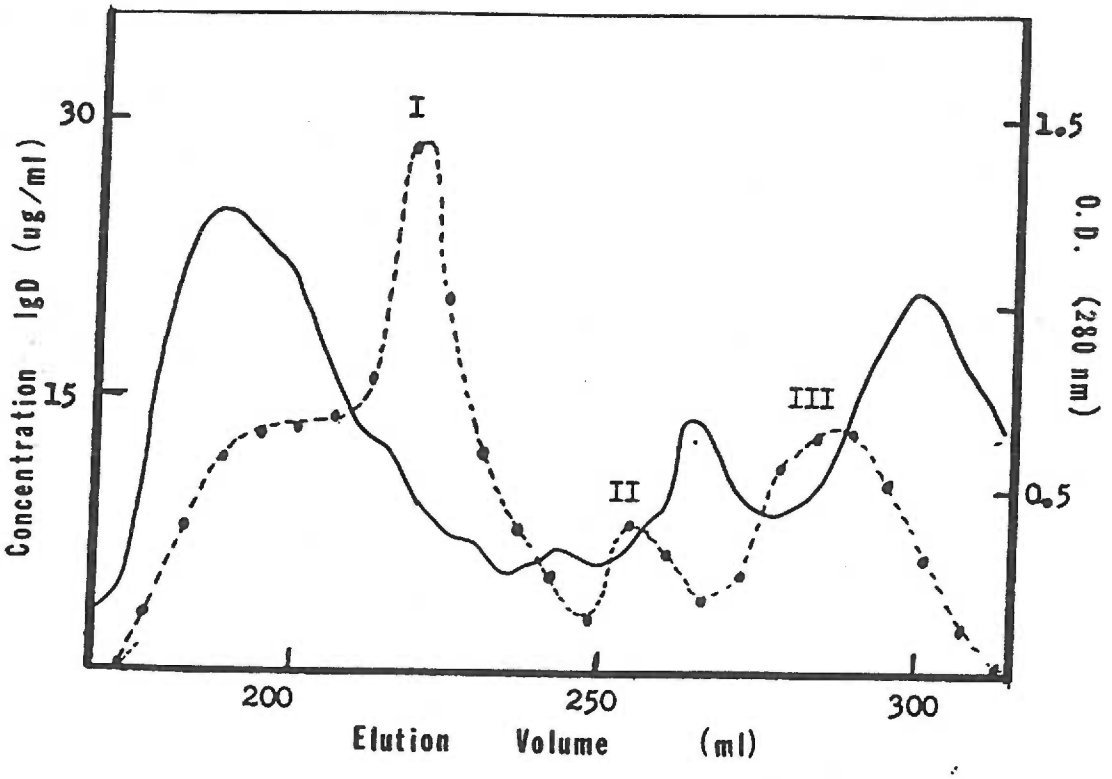


Figure 3 . Partial reduction and alkylation of clarified rat milk.

OD 280 nm (solid line), IgD (dotted line).

between thyroglobulin and IgG (~310 kDa), peak IV eluting somewhat slower than IgG (~140 kDa) and a minor peak V eluting well beyond IgG and probably representing breakdown products (Figure 4a). The largest portion of the IgD present in the pool was in peak IV. A second pool of rat serum collected from two male HPR rats found to have high serum IgD and similarly fractionated produced the first four species with very similar apparent molecular weights (peak I eluting between void volume and IgM, peak II, ~540 kDa; peak III, ~300 kDa; peak IV, ~130 kDa), but in different proportions, with only a small amount of peak IV IgD and larger amounts of IgD in peaks I, II, and III (Figure 4b). The significance of the variation in the proportions of the representation of the four size species of IgD is not known. This second pool was fractionated within 24 hours of collection from the rats while the first pool was fractionated after several months of storage at -20°C. Hence the difference may reflect the differing degree of breakdown of native IgD molecules. The fact that the (rnu/rnu) and (rnu/+) rat pool had a fifth minor peak in a size range which presumably represents IgD fragments may indicate that a greater degree of degradation of the IgD present had taken place (Figure 4a). Similar heterogeneity and differences in the proportions of IgD species in rat ascitic fluid have also been observed (see below).

Another pool of F344 rat serum, lower in IgD content, was precipitated with 40% saturated ammonium sulfate, redissolved in 0.01 M TBS and

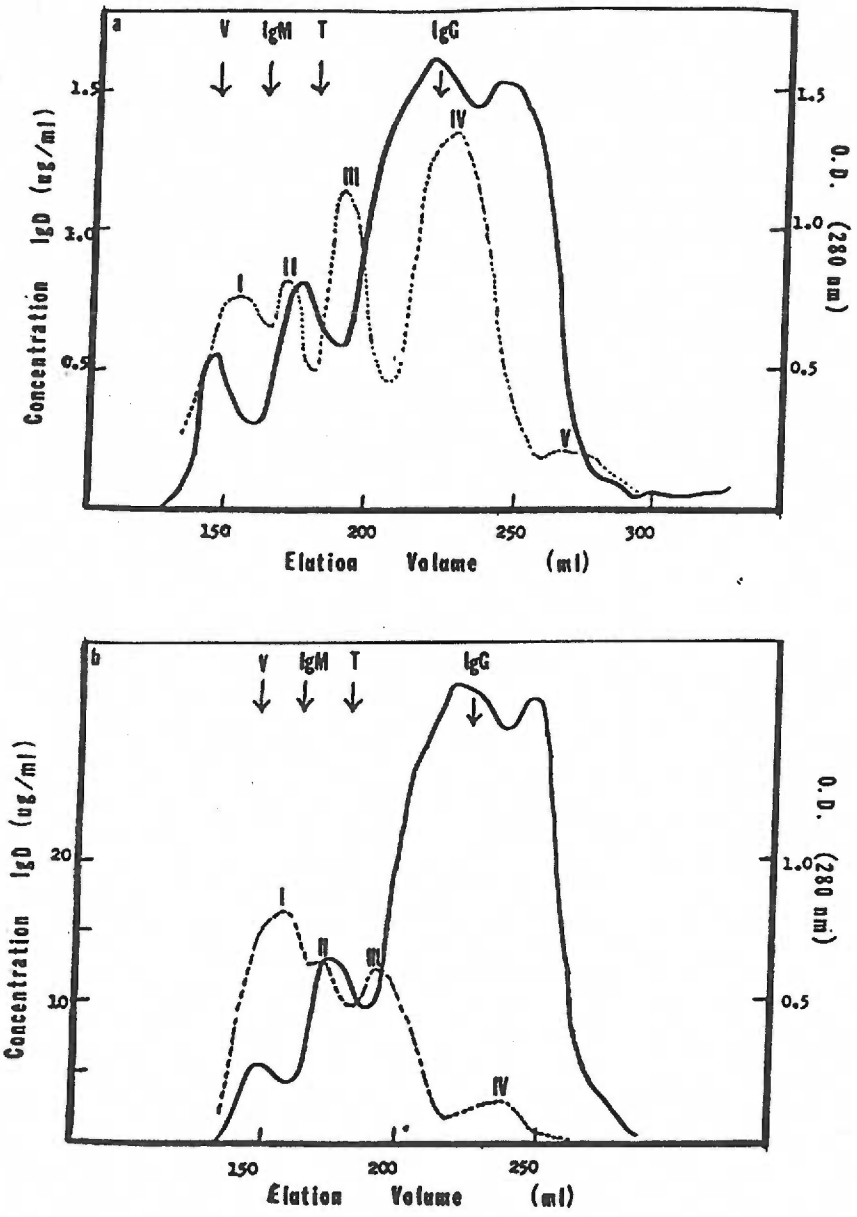


Figure 4 . Elution profile of S-300 gel filtration of (a) pooled rnu/rnu and rnu/+ rat serum (b) pooled HPR rat serum. V: void volume, T: thyroglobulin.

similarly fractionated on the S-300 gel filtration column. Assay of gel filtration fractions revealed only a single IgD peak which appears to correspond to peak II serum IgD (Figure 5a). When fractions from this peak were pooled and concentrated and partially reduced and alkylated (Leslie and Benedict, 1969), the resulting IgD in two separate experiments was found to be ~135-~180 kDa, the second of which is shown in Figure 5b. While there is perhaps more variation in these results than one would desire, they suggest a monomeric unit for serum IgD near the 150 kDa value demonstrated for rat IR-731 IgD. While the molecular weight of peak I IgD is too high to assign a value to and may represent randomly aggregated IgD, the results for peak II, peak III, and peak IV IgD suggest IgD trimers, dimers, and monomers.

Ascitic Fluid IgD. A heterogeneity similar to that of serum IgD has been found in rat ascitic fluid resulting from weekly intraperitoneal injection into rats with 1 ml of a 9:1 Freund's complete adjuvant/saline suspension. In examining gel filtration fractions from several different fractionation experiments the following size species have been observed: (~500-1000 kDa, ~200-300 kDa, and ~60 kDa (Figure 6a); ~440 kDa, ~260 kDa, and ~90 kDa (Figure 6b). Several other gel filtration experiments have shown a similar three-peak pattern. Based on the less than optimal resolution of these three size species it is difficult to say with certainty whether such peaks represent three homogeneous populations of IgD or mixtures of

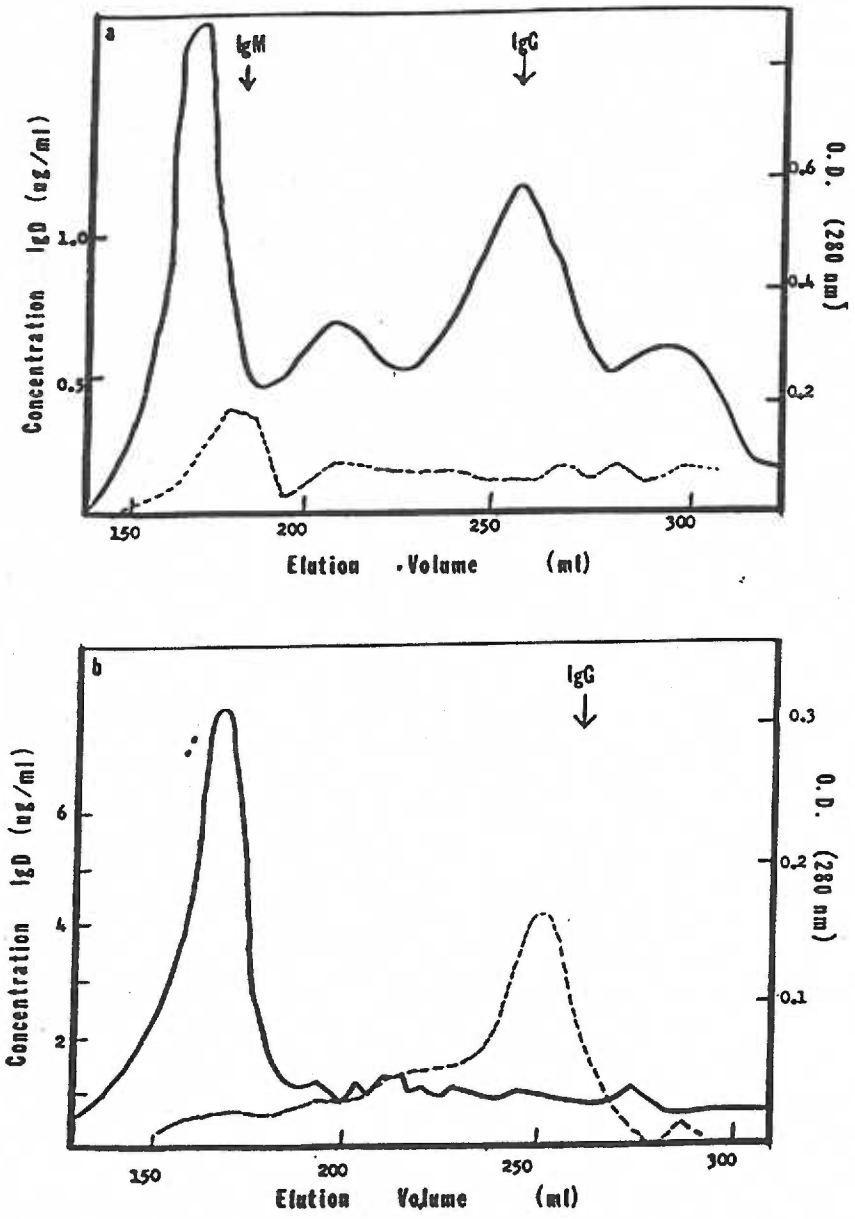


Figure 5 . Elution profile of S-300 gel filtration of (a) pooled F344 rat serum (b) partially reduced and alkylated F344 rat serum. IgD (dotted line), OD 280 nm (solid line).

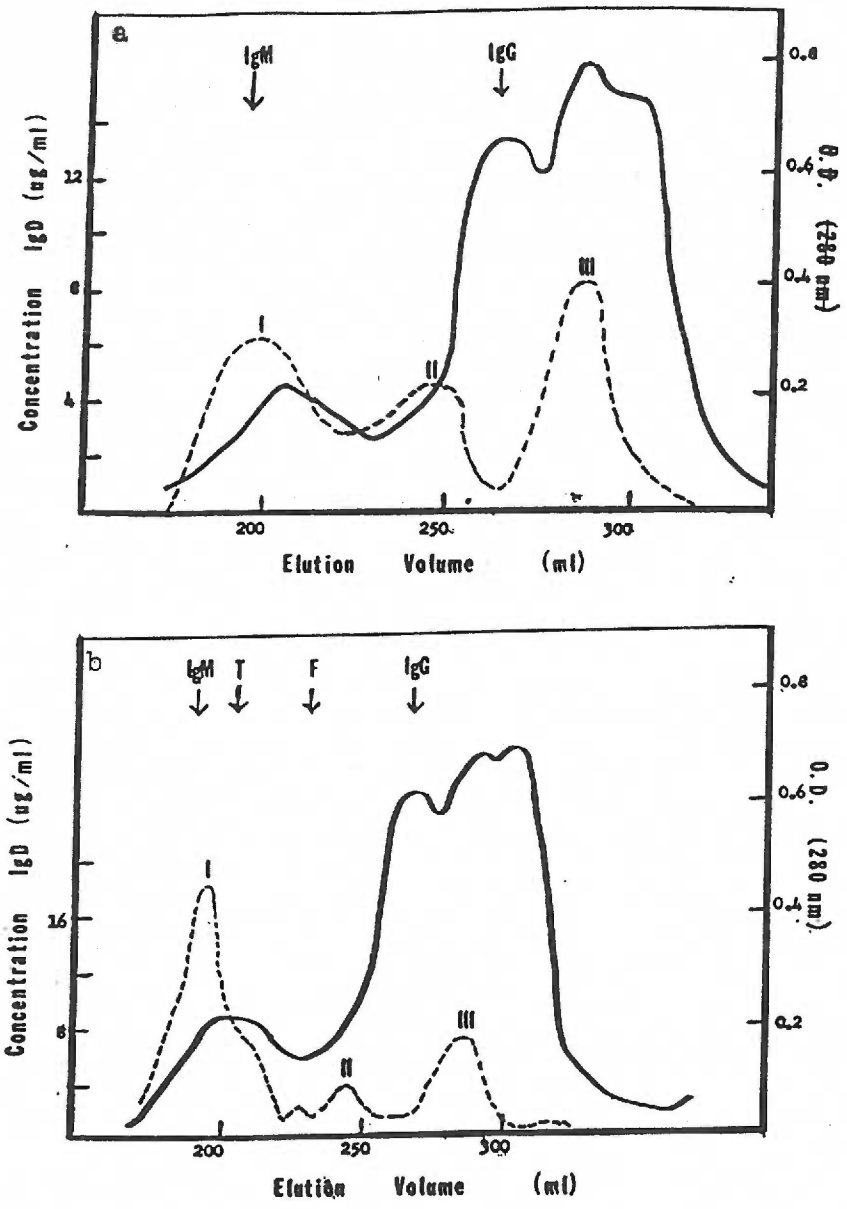


Figure 6 . Elution profile of S-300 gel filtration of two different samples of ascitic fluid from FCA treated rats (a and b).

T: thyroglobulin, F: ferritin.

different size species which the column did not resolve, however the species have provisionally been identified as peak I, II, and III ascitic fluid IgD. The first peak would appear to represent either trimers and/or tetramers, the second dimeric IgD, and the third apparently submonomeric IgD.

Saliva IgD. Saliva was collected from adult male rats under ether anesthesia after administration of 0.8 ml. i.v. of a 1% solution of pilocarpine (Pilocar opthalmic solution). Saliva was pooled and concentrated then passed over an S-300 gel filtration column. When fractions were assayed for IgD and IgA, IgA was found to elute, as expected, after IgM (Figure 7). IgD was detected as eluting after BSA but before ovalbumin, which would suggest a molecular weight of between 66 and 45 kDa. This is about the size expected of IgD tryptic Fab and Fc fragments (Alcaraz et al., 1980) or of IgD heavy chains. It seems likely that proteolysis occurred. It would be of interest to repeat the experiment, adding a serine protease inhibitor such as phenylmethylsulfonylfluoride (PMSF) to the saliva samples shortly after sample collection. It is also possible that gastric juice may have contaminated the saliva.

Behavior of IgD on Ion Exchange chromatography

Milk IgD. Clarified milk was fractionated at 4°C on a DEAE-cellulose

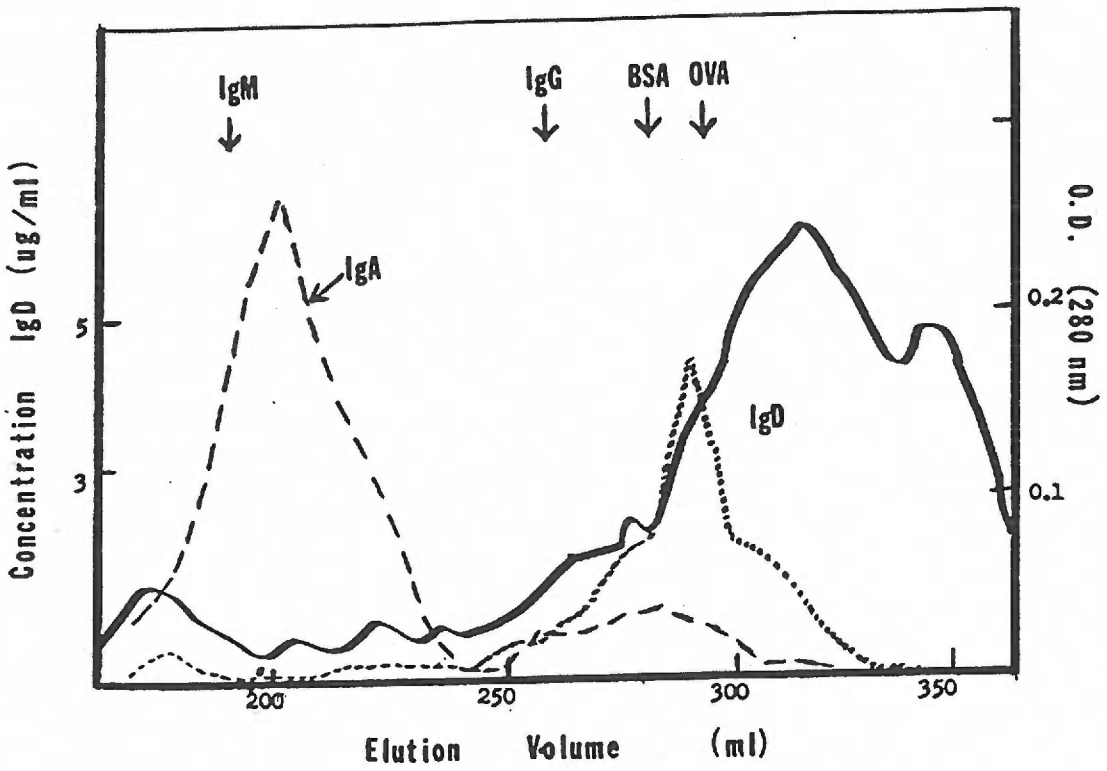


Figure 7 . Elution profile of S-300 gel filtration of pooled HPR rat saliva. OD 280 nm (solid line), IgA (dashed line), IgD (dotted line). BSA: bovine serum albumin, OVA: ovalbumin.

anion exchange column equilibrated in 0.1 M tris buffer as described (Finkelman et al., 1981; Steele and Leslie, *in press*). IgD corresponding to peak I milk IgD was retained on the column, and was eluted by 0.1 M tris buffer + 0.15 M NaCl, while IgD corresponding to peak II IgD was not retained on the column. The respective fractions of IgD were identified by S-300 gel filtration and assay of filtration fractions for IgD.

Ascitic Fluid IgD. An aliquot of 40 ml of whole pooled IgD-containing rat ascitic fluid was fractionated using the same procedure described for milk IgD. As with milk IgD, ascites IgD was found both in effluent and NaCl-elution fractions with most of the IgD being retained on the column. The IgD which bound to the DEAE was eluted by relatively low concentrations of NaCl and resolved into two distinct peaks (DEAE peaks I and II, Figure 8). S-300 gel filtration and IgD assay of the effluent IgD produced a low molecular weight species (~45 kDa, Figure 9), while DEAE peak I and peak II IgD on gel filtration produced three peaks of IgD resembling that of unfractionated ascitic fluid IgD (Figures 10a and 10b) plus other peaks. Apparently but for a portion of low molecular weight IgD approximately the size of delta Fc fragments or delta chains the IgD in ascitic fluid was retained on an ion exchange column. The DEAE fractionation of ascitic fluid IgD did not appear to be an effective method of separating the size species observed there. The low molecular weight IgD peaks observed in the DEAE fractions I and II gel filtration experiments may have

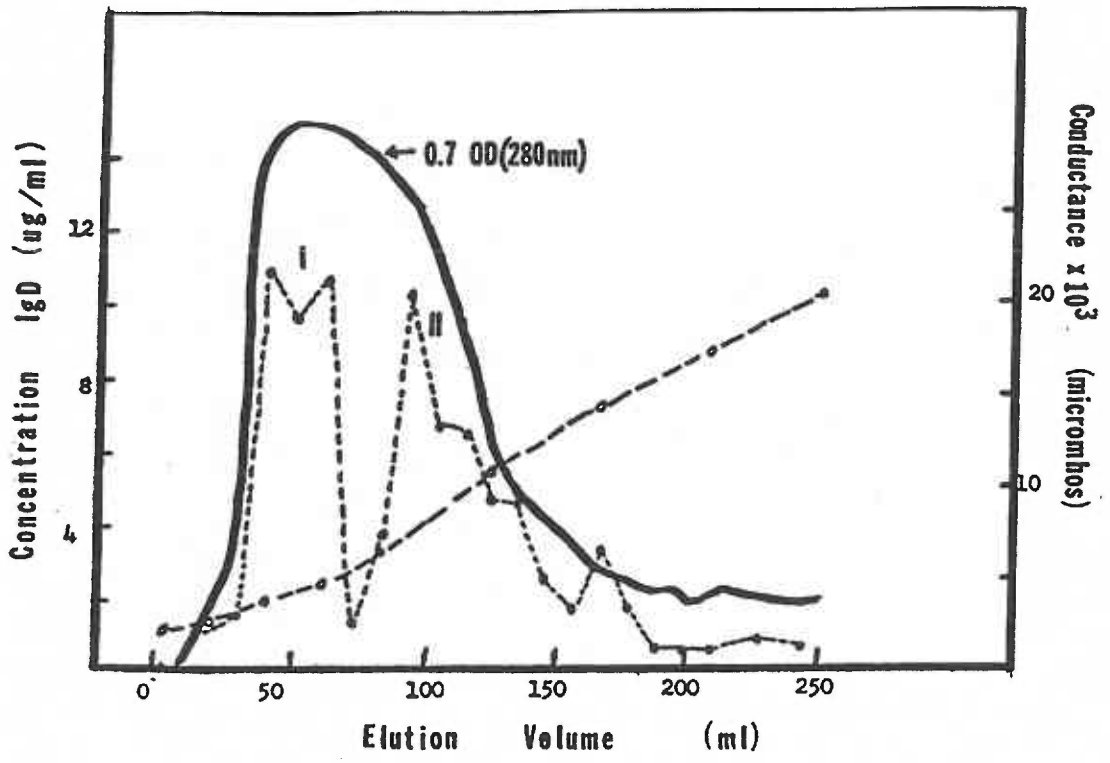


Figure 8 . Elution profile of DEAE ion exchange column with a 250 ml gradient from 0 to 0.5 M NaCl in 0.1 M tris buffer at 4°C. OD 280 nm (solid line), IgD (dotted line). Conductance (dashed line).

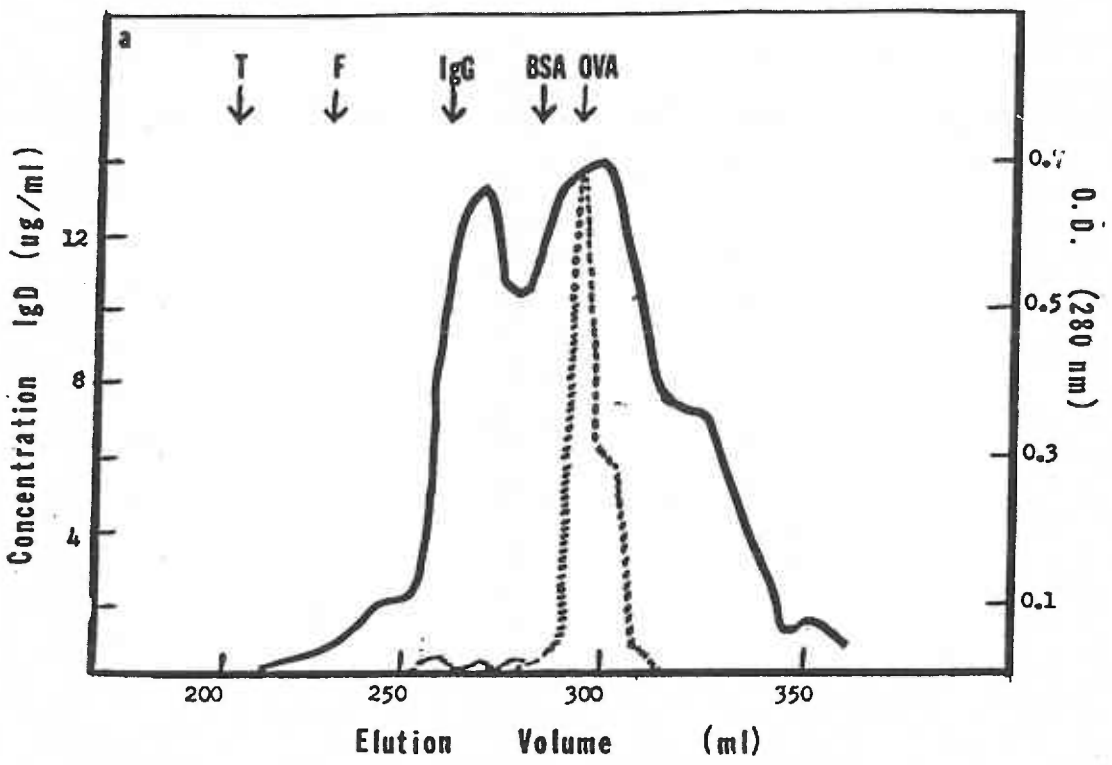


Figure 9. Elution profile of S-300 gel filtration effluent IgD from DEAE ion exchange column (equilibrated with 0.1 M tris buffer pH 8.1 after passage of pooled rat ascitic fluid. OD 280 nm (solid line), IgD (dotted line). T: thyroglobulin F: ferritin, BSA: bovine serum albumin, OVA: ovalbumin.

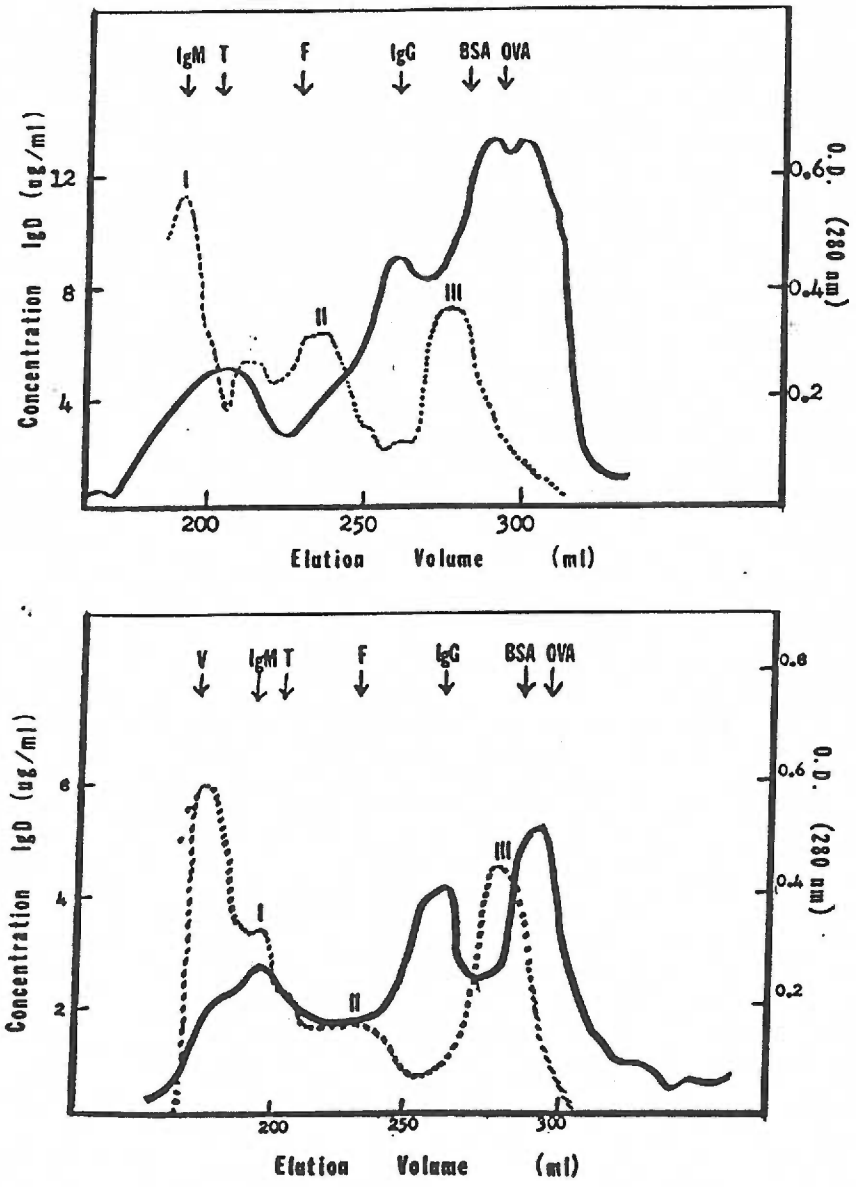


Figure 10 . Elution profile of S-300 gel filtration of DEAE ion exchange elution peaks I and II (a and b respectively). V: void volume, T: thyroglobulin, F: ferritin, BSA: bovine serum albumin, OVA: ovalbumin.

been the result of spontaneous breakdown of higher molecular weight IgD following elution from the column.

Ouchterlony gel diffusion test of IgD species

Sheep antiserum to rat membrane IgD, prepared as described (Cuchens, Martin, and Leslie, 1978) gave an apparent reaction of identity with adjacent wells between ascitic fluid IgD gel filtration peaks I, II, and III, and a high IgD rat serum (89 µg/ml) and a standard clarified milk (120 µg/ml, Figure 11). A similar pattern was observed using rabbit anti-IgD. These results indicate that the sheep antiserum recognized either a single species (or multiple species with very similar diffusion rates) between adjacent wells with the possible exception of unfractionated rat ascitic fluid (well #6). Such a test does not eliminate the possibility that antigenic differences exist in the IgD of these six preparations, but only that the sheep antiserum, for the most part did not distinguish any.

Sensitivity of rat IgD to trypsin

Peak I ascites IgD, partially purified by gel-filtration, was digested with trypsin at a 1/100 enzyme/substrate ratio at room temperature. At various time intervals between 1 and 30 minutes samples were taken from the digestion mixture and added to tubes containing trypsin

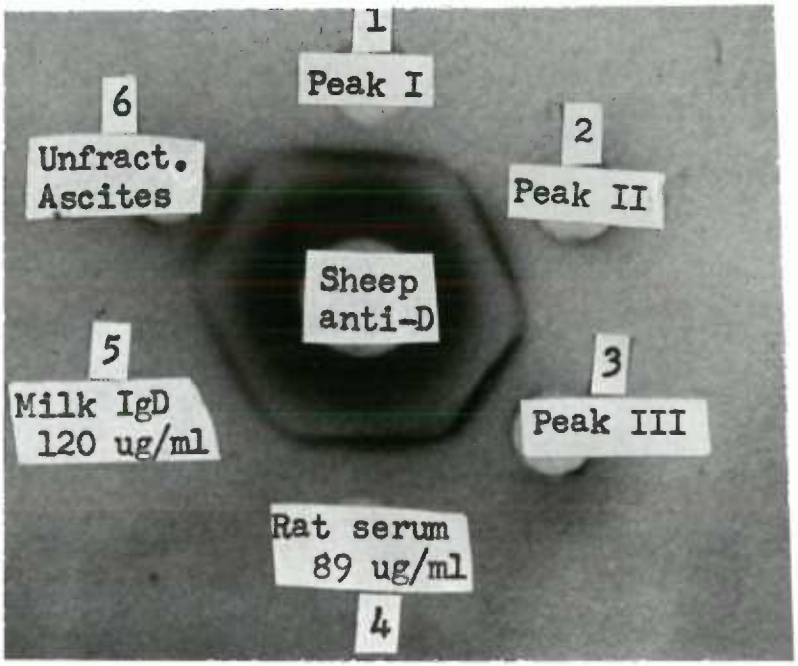


Figure 11 . Ouchterlony gel diffusion test of different preparations of IgD tested against sheep anti-rat IgD in center well. 1: Peak I ascitic fluid IgD, 2: Peak II ascitic fluid IgD, 3: peak III ascitic fluid IgD, 4: a rat serum with 89 µg/ml IgD, 5: rat milk with 120 µg/ml IgD, and 6: whole unfractionated rat ascitic fluid.

inhibitor for a final 10/1 inhibitor/enzyme concentration. Samples from the different time points were immediately transferred to an agarose immunoelectrophoresis gel, performed as described (Olson, 1981). Troughs were filled with sheep anti-rat IgD. Degradation is apparent within 1 minute with conversion of a faster-migrating species to a slower one (Figure 12). Conversion to this slower-migrating species was nearly complete by 9 minutes and complete by 15 minutes. Gel filtration on S-300 of a 9 minute trypsin digest showed that the IgD eluted in a single peak in an elution volume close to that of the OVA calibration standard, suggesting a fragment of a size similar to that reported for IR-731 plasmacytoma protein tryptic Fc and Fab fragments: 36 and 50 kDa respectively (Alcaraz et al., 1981) and similar to that of effluent IgD on DEAE fractionation. Preliminary results with rat milk IgD suggested a similar sensitivity to trypsin.

Sensitivity of Rat IgD to heat and chemical denaturation

Samples of pooled ascitic fluid and clarified milk were heated for 30 minutes at 56°C or allowed to stand for 30 minutes at room temperature, then assayed for IgD by ELISA (Steele and Leslie, 1985). After 30 minutes at 56°C, IgD content had been reduced in both preparations by roughly 2/3 over the control samples (Table 1). In a test of IgD anti-DNP sensitivity to heat denaturation, the same experiment was performed using a sample of high IgD anti-DNP immune

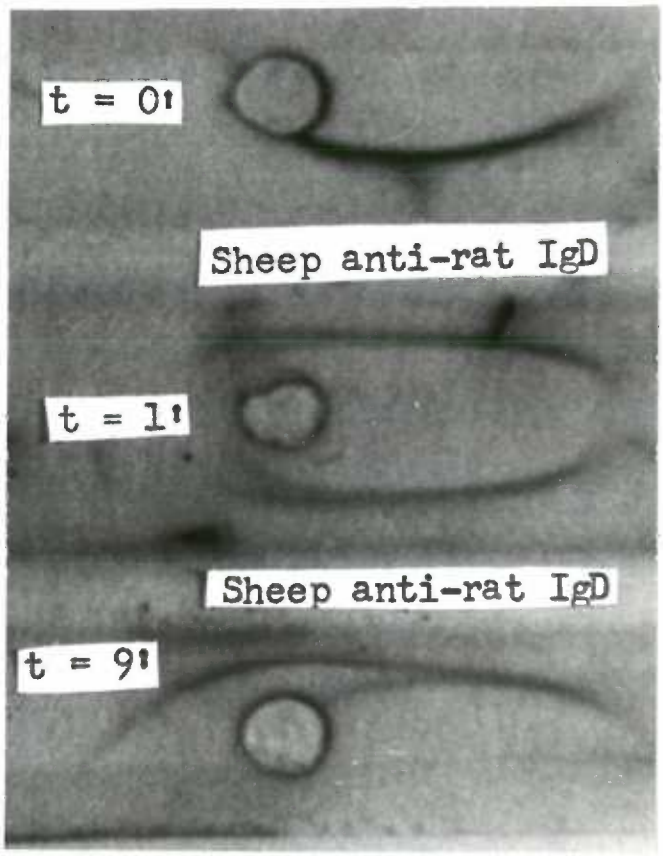


Figure 12 . Immunoelectrophoresis of trypsin-digested rat ascitic fluid peak I IgD after 0, 1, and 9 minutes of digestion.

TABLE 1

Effect of Heating on Total IgD in Milk and Ascitic Fluid

IgD preparation	unheated control (µg/ml)	heated 56°C 30 minutes (µg/ml)	Per Cent change
Ascitic Fluid	178	58.4	- 67.1%
Milk	506	162	- 67.3%

ascitic fluid. IgD anti-DNP concentration was reduced by more than 80%, while kappa light chain bearing anti-DNP (used here as an approximation of total anti-DNP) was decreased by 20%.

In a test of the extent to which IgD is denatured by treatment with sodium thiocyanate, samples of ascitic fluid IgD peak I, peak II, and peak III, whole ascitic fluid, and whole clarified milk were dialyzed for 24 hrs. at 4°C in either 3M sodium thiocyanate in 0.01 M TBS (pH 7.4) or 0.01 M TBS (pH 7.4). All samples were next dialyzed another 24 hours at 4°C in 0.01 M TBS, then assayed for IgD. Thiocyanate-treated samples from all sources showed between 62% and 76% loss of IgD as compared to control-dialyzed samples (Table 2).

Polyacrylamide Gel Electrophoresis of Rat IgD

SDS-polyacrylamide gel electrophoresis of partially purified rat IgD reveals a multipilicity of possible heavy chain bands which could represent intact or degraded delta chains, or heavy chains from other immunoglobulins. Since rat IgD is much more sensitive to trypsin than most other immunoglobulin classes one approach to identifying likely IgD bands was to observe the effect of trypsin digestion on the pattern of heavy chain bands in a 12% gel. The results of this experiment is shown in figure 13. Samples of trypsin-digested IgD from peak I ascitic fluid IgD and peak I milk IgD, partially purified

TABLE 2

Effect of 3 M Sodium Thiocyanate on Total IgD [a]

IgD preparation	Control dialyzed (µg/ml)	Sodium Thiocyanate dialyzed (µg/ml)	Per Cent change
Ascitic Fluid	188.7	49.4	- 75.1%
Peak I Ascitic fluid IgD	67.8	16.5	- 75.6%
Peak II Ascitic fluid IgD	50.0	22.0	- 66.0%
Peak III Ascitic fluid IgD	110.4	41.6	- 62.3%
Milk	68.0	25.4	- 62.6%

[a] Samples of each IgD preparation were dialyzed overnight at 4 degrees in either 3M thiocyanate in 0.01 M TBS or in 0.01 M TBS (pH. 7.4) for 24 hours followed by 24 hours dialysis in 0.01 M TBS.

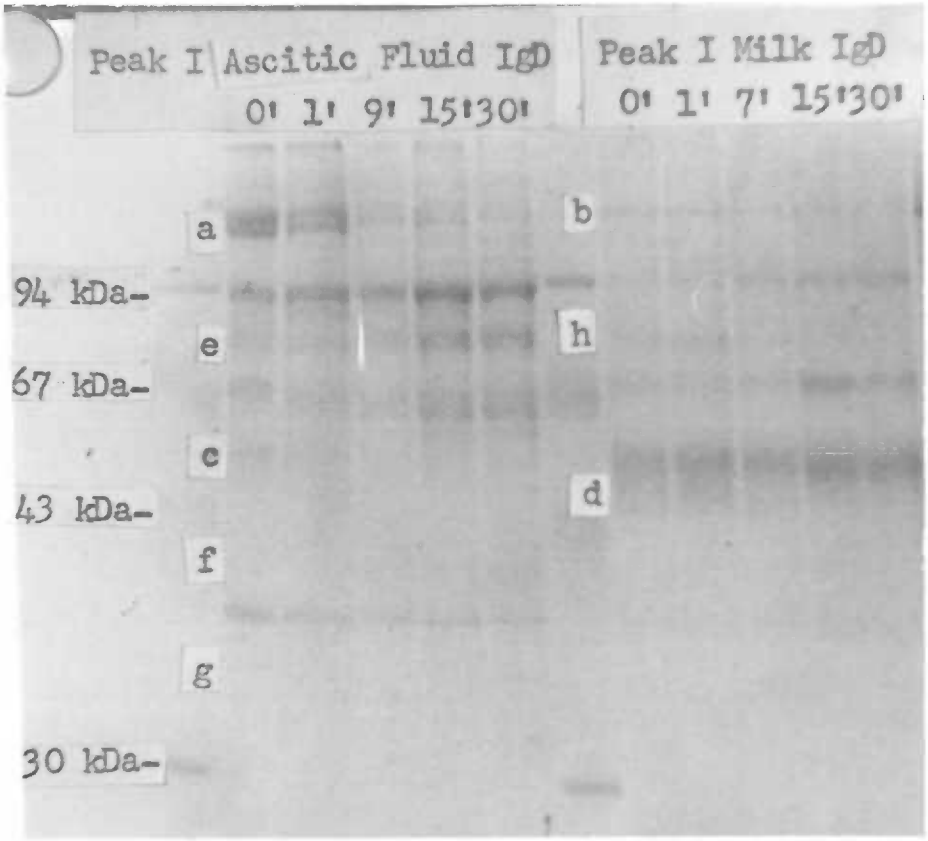


Figure 13 . 12% polyacrylamide gel electrophoresis of trypsin-digested peak I milk IgD and peak I ascitic fluid IgD.

by gel filtration, were digested for 0, 1, 7 or 9, 15, and 30 minutes as described above, then run on a 12% SDS-polyacrylamide gel. A number of bands showed susceptibility to trypsin. In both milk and ascitic fluid IgD a high molecular weight band > 94 kDa disappeared which may represent incompletely reduced IgD monomers or delta dimers (bands a and b). Two other bands (bands c and d) estimated to be ~49 kDa also disappeared in both preparations and which, based on studies of the structure of the IR-731 rat plasmacytoma protein, would be likely candidates for the intact rat delta chain. In rat ascitic fluid the > 94 kDa band (a) appears to be a doublet while the > 94 kDa single band (b) seen in the rat milk digest appears to correspond to the slower migrating of the doublet bands > 94 kDa in rat ascitic fluid. A band estimated to be 76 kDa increases in intensity in the rat ascitic fluid IgD digest (e), and likewise two other bands increase in intensity, with estimated molecular weights of 39 and 34 kDa respectively (f and g). Another band in rat milk IgD (h), co-migrating with the 76 kDa band in rat ascitic fluid IgD (e), increases in intensity. Of course delta chains may not be the only trypsin-sensitive peptides present in the partially purified preparations. Positive identification of delta chains and their fragments could be achieved either by immunoblotting, (provided delta determinants are not destroyed in the procedure), or by iodination of IgD preparations, immunoprecipitation with anti-delta antiserum, and SDS-polyacrylamide electrophoresis (Ruddick and Leslie, 1977).

Approaches to Purification of Rat IgD from milk and ascitic fluid

The marked lability of IgD coupled with its paucity in normal rat serum has made it difficult to purify. It is desirable therefore to use a starting material which is plentiful and rich in IgD. Although occasionally a rat serum with very high IgD concentrations were found, usually only 1-5 ml of it were available. The high concentrations of IgD consistently found in rat milk, and in rat ascitic fluid induced by the methods described (Steele and Leslie, *submitted*) provide two good alternative sources.

The most effective method for purifying IgD from rat ascitic fluid combines salt fractionation and gel filtration. In a typical experiment the following results were obtained in purifying rat ascitic fluid IgD (Table 3). 75 ml of rat ascitic fluid which was determined to contain 120 µg/ml IgD was precipitated with 40% saturated ammonium sulfate at 4 degrees for 3 hours with stirring then centrifuged in a Sorvall RC2-B centrifuge at 10,000 rpm in an SS-34 rotor at 4 degrees for 10 minutes. Precipitate was resuspended in 40% saturated ammonium sulfate/ 0.01 M tris buffer and again pelleted. After being redissolved in 30 ml 0.01 M TBS (pH 7.4) 92.6% of the IgD was recovered in the precipitate with the remainder present in the supernatant, a 7.8-fold purification over the starting

TABLE 3

Purification of IgD from Rat Ascitic Fluid

Purification Intermediate	Vol.	IgD µg/ml	OD (280 nm) per ml	Total IgD	Fold Purification
1. Pooled Ascitic Fluid	75 ml	120	65	~9000	1
2. 40% SAS cut of Ascitic Fluid	30 ml	314	21.6	~9400	7.8
3. 40% SAS Ascitic Fluid Supernatant	8 ml	94.6	125	746	0.4
4. Gel Filtration Peak I	6.6 ml	94.5	2.86	624	17.8
5. 1.5 M NaSCN eluate from Sheep anti IgD IA	1 ml	26.8	0.25	26.8	58.0

material. A portion of this material is then aliquotted to 2.7 ml volumes and successive gel filtration runs were made over an S-300 gel filtration column in 0.01 M TBS pH plus 0.01 M epsilon-aminocaproic acid as a protease inhibitor. Peak gel filtration fractions from peak I ascitic fluid IgD were then pooled and concentrated in the presence of 1 µg/ml PMSF (phenylmethylsulfonyl fluoride), a serine protease inhibitor). At the end of this procedure, a 17.8-fold purification has been achieved (#4). In one experiment 5 ml. of gel filtration peak I (#4) was passed over a sheep anti-rat IgD immunoadsorbant column, then eluted with 1.5 M sodium thiocyanate, dialyzed overnight against 0.01 M TBS at 4°C, then concentrated. The resulting purification of IgD was roughly 3.2-fold, with a 58-fold purification over the pooled ascitic fluid. A similar procedure was followed for purification of IgD from rat milk, with similar results.

References

Alcaraz, G., Bourgois, A., Moulin, A., Bazin, H., and Fougereau, M. (1980). Partial structure of a rat IgD molecule with a deletion in the heavy chain . *Ann. Immunol. (Inst. Pasteur)* 131C: 363.

Alcaraz, G., Colle, A, Boned, A., Kahn-Perles, B., Sire, J., Bazin, H., and Bourogis, A. (1981). Tryptic and plasmic cleavage of a rat myeloma IgD. *Mol. Immunol.* 18: 249.

Bazin, H., Beckers, A., Urbain-Vansanten, G., Pauwels, R., Tilkin, A.F., Platteau, B., and Urbain, J. (1979). Transplantable IgD immunoglobulin-secreting tumors in rat. *J. Immunol.* 121: 2077.

Cuchens, M.A., Martin, L.N., and Leslie, G.A. (1978). The effect of anti-IgD on serum immunoglobulins, antibody production, and immunoglobulin-bearing cells in adult rats. *J. Immunol.* 6: 2257.

Finkelman, F.D., Kessler, S.W., Mushinski, J.F., and Potter, M. (1981). IgD-secreting murine plasmacytomas: Identification and partial characterization of two IgD myeloma proteins. *J. Immunol.* 126: 680.

Leslie, G.A., and Benedict, A.A. (1969). Structural and antigenic

relationships between avian immunoglobulins I. The immune responses of pheasants and quail and reductive dissociation of their immunoglobulins. *J. Immunol.* 103: 1356.

Olson, J.C. (1981). The immune network: endogenous and exogenous influences on idiotype expression. (doctoral dissertation, Oregon Health Sciences University) p. 23.

Olson, J.C., and Leslie, G.A. (1982). IgD: A component of the secretory immune system? In *Immunoglobulin D: Structure and function* (G.J. Thorbecke and G.A. Leslie, eds.) *Ann. N.Y. Acad. Sci.* 399: 97.

Ruddick, R.A., and Leslie, G.A. (1977). Structure and biological functions of human IgD. XI. Identification and ontogeny of a rat lymphocyte immunoglobulin having antigenic cross-reactivity with human IgD. *J. Immunol.* 118: 1025.

Sire, J., Alcaraz, G., Bourgois, A., and Jordan, B. (1981). Rat IgD myeloma protein: cell-free transaltion of the delta mRNA and biochemical analysis of intracellular and membrane delta chain. *Eur. J. Immunol.* 11: 632.

Steele, M.G., and Leslie, G.A. (1986). Immunoglobulin D in rat serum, saliva and milk. *Immunol.* 55: 571.

Steele, M.G., and Leslie, G.A. (1986). IgD antibodies to DNP are induced in ascitic fluid after intraperitoneal immunization. *submitted*

Thiele, C.J., Owens, J.D., Finkelman, F.D., and Mushinski, J.F. (1985). Mouse IgD half molecules with shortened IgD heavy chain result from alterations within C-delta locus. *J. Immunol.* 134: 1251.

APPENDIX 2

Demonstration of the Epitope specificity of the GALK 20.7 (anti-rat kappa), GALM 36.12E (anti-rat IgM), GALA 1.14.2 (anti-rat IgA) and Rabbit anti rat IgD

Introduction

Hybridoma cell lines producing antibodies to rat kappa light chain, rat IgM, and rat IgA were isolated following cell hybridization of SP2 plasmacytoma cells with splenocytes from BALB/c mice immunized and boosted with gel filtration fractionated HPR rat milk (Oi and Herzenberg, 1980). Monoclonal antibodies were tested for their binding and inhibition specificity, and to a limited extent their strain-specificity. Binding specificity was tested using an ELISA plate assay method as described (Steele and Leslie, *submitted*, see pp. 130-131.) with slight modifications. 5-10 µg/ml solutions of rat isotype preparations in carbonate-bicarbonate buffer were used to coat wells of vinyl assay plates, followed by blocking with 1% BSA-PBS. Dilutions of culture supernatant in 2% NRaS-PBS were incubated in the wells for 2 hrs at room temperature, followed by incubation with alkaline phosphatase-labeled Sheep anti-mouse Ig, which had previously been thoroughly absorbed with rat serum Ig to remove cross-reacting anti-mouse Ig antibodies. Following this step, color reagent was added, incubated for 30-90 minutes at either room temperature or at 37°C, then read on a microelisa reader.

Inhibition specificity was tested by first titering the putative anti-isotype reagent to determine the highest dilution of anti-isotype antibody which produced a satisfactory background color reagent

density after a standard incubation of 1 hour at 37°C. This was generally about 0.5 to 0.6 at 420 nm using the Dynatech microelisa reader. The titer of monoclonal antibody-containing ascitic fluid used in such tests typically was between 1/10,000 to 1/30,000, while culture supernatant had to be used at much lower concentrations, typically being used at about 100x that concentration. Such titering is necessary to maximize the sensitivity of the antibody to binding inhibition, by small amounts of the epitope in solution.

Once the ideal titer of the antibody preparation has been determined, an assay procedure analogous to that used for the total IgD assay was performed (Steele and Leslie, 1985). Plates were coated with an isotype preparation for which the monoclonal antibody showed binding specificity as before. Dilutions of different isotype-containing preparations mixed with the proper dilution of the monoclonal antibody and added to assay plate wells to test the ability of the isotype-containing preparations to competitively inhibit the binding of the monoclonal antibody to the coated plates. By showing a correlation between the concentration of the epitope as shown in the ELISA assay and the isotype concentrations determined by other assay methods, such as radial immunoassay or other methods, the isotype specificity of the antibody is established. To test for the possible allotype specificity of the monoclonal antibody, assays are performed with isotype preparations or serums from a variety of rat strains.

The complete absence or markedly lower binding inhibition by an isotype preparation from a given rat strain or strains in the face of a known concentration of the isotype determined by other methods would be an indication of allotypic specificity for the anti-isotype reagent. No such allotypic specificity was found in any of the rat sera for any of the anti-isotype reagents tested against Lou/Min, Brown Norway, F344, or HPR rat serum. Additional methods for testing the isotype specificity of the reagents are (1) to assay gel filtration fractions for the isotype and showing the detection of molecular size species characteristic of the isotype examined and (2) the ability to specifically purify the monoclonal antibody by affinity chromatography using isotype-containing immunoadsorbants.

GALK 20.7. The GALK 20.7 hybridoma was isolated following screening assays in which culture supernatant showing binding to plates coated with an IgM-kappa myeloma (Lou/Wsl), IgA-kappa (IR-22) (Lou/Wsl), 7S HPR rat serum Ig (40% saturated ammonium sulfate fraction of whole HPR rat serum followed by gel filtration and isolation of the Ig peak corresponding to 7S HPR rat Igs, predominantly IgG), 19S HPR rat Ig (prepared as with 7S HPR rat Ig with isolation of Ig peak corresponding to 19S HPR rat Ig, predominantly IgM), and to gel-filtration fractionated IgD-rich milk from HPR rats. Following two successive clonings of the hybridoma it was grown up in culture medium and vials frozen. Subsequently it was transplanted into BALB/c

mice pre-treated with 2,4,6 tetramethylpentadecane (Pristane) (Aldrich, Milwaukee, WI) and ascitic fluid collected and pooled. Specifically purified GALK 20.7 was isolated by passage of whole ascitic fluid over a rat IgA-kappa (IR-22) immunoadsorbant and eluted with 3 M sodium thiocynanate. The purified protein was shown to have retained its ability to bind the panel of rat immunoglobulin preparations as before, and following enzyme labeling with alkaline phosphatase as described (Steele and Leslie, 1985) and titering for the minimum concentration yielding suitable background binding, it was tested for inhibition specificity. The ascitic fluid was used directly in some assays with a dilution of 1/15,000. To test the specificity for light chains, a trypsin digest of 7S HPR rat serum Ig was prepared, and Fab' fragments purified from it by gel filtration and ion exchange chromatography. Such fragments were then tested for their ability to inhibit the binding of GALK 20.7 to plates coated with rat IgM-kappa (Figure 1). Both intact rat IgM-kappa and 7S HPR rat serum Ig Fab' fragments were capable of inhibiting > 80% binding at a protein concentration of between 40 and 50 µg/ml. The ability of the reagent to bind monoclonal kappa-bearing IgA and IgM as well as Fab' fragments of 7S HPR rat serum Ig, and to be inhibited by Fab' fragments of 7S HPR rat serum Ig indicates a specificity for kappa light chains. Subsequently the reagent has been shown to identify multiple peaks in gel fractions of rat serum corresponding to IgM, IgA, and IgG. Using a panel of alkaline phosphatase mouse

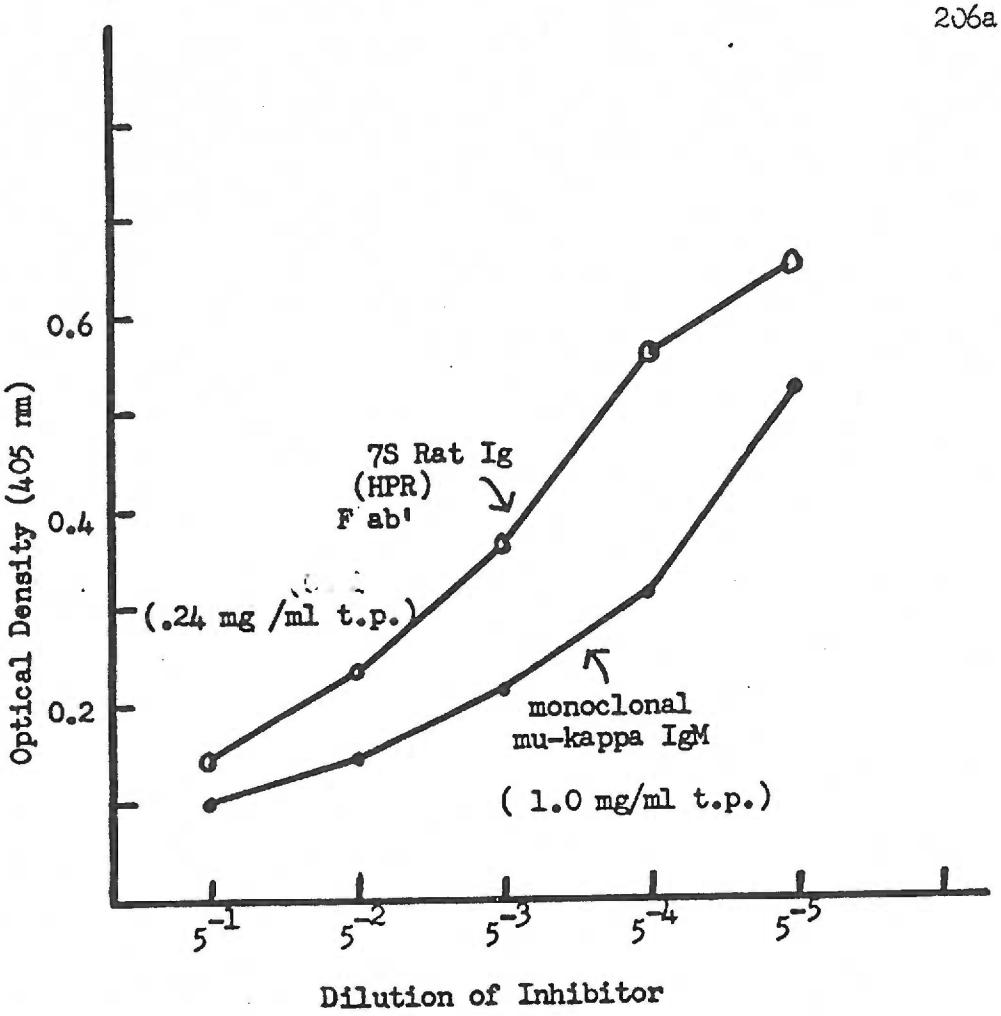


Figure 1. Binding inhibition of GALK 20.7 anti-rat kappa light chain to plates coated with rat Lou/Wsl plasmacytoma IgM-kappa by Fab' fragments of 40% SAS precipitated 7S HPR rat serum Ig and the rat Lou/Wsl plasmacytoma IgM-kappa. Abbreviations: t.p., total protein.

isotype-specific reagents (gift of Dr. M. B. Rittenberg) GALK 20.7 was identified as mouse IgG1-kappa immunoglobulin in an direct binding ELISA.

GALM 36.12E. The GALM 36.12E hybridoma was isolated following observation of specific binding to IgM-kappa bearing plates and to 19S HPR rat serum Ig plates in a like manner (see above). Following its isolation it was grown in BALB/c mice and ascitic fluid was collected and titered. It was specifically purified from ascites following passage over a rat IgM-kappa immunoadsorbant. In a test of its isotype identity as described above, it was found to be an IgG1-kappa immunoglobulin. Following titering of the ascitic fluid, it was used in tests of inhibition specificity in its binding to rat IgM-kappa by specifically purified rat IgG2c (monoclonal rat anti-SACHO 90.1), 7S HPR rat serum Ig, 19S HPR rat serum Ig, monoclonal IgA (IR-22), and monoclonal IgM (Figure 2). The ascitic fluid was used routinely in assays with a dilution of 1/30,000. The binding of GALM 36.12E or the inhibition thereof was detected using an additional incubation step using alkaline phosphatase-labeled sheep anti mouse Ig. Significant inhibition was observed only in 19S HPR rat serum Ig (3.1 mg/ml total protein) and in monoclonal IgM (2.14 mg/ml total protein) (both highly enriched preparations of IgM) while rat IgG2c (1.4 mg/ml IgG2c), monoclonal IgA (IR-22) (1 mg/ml IgA), 7S HPR rat serum Ig (1 mg/ml total protein) did not show inhibition. In trial assays of

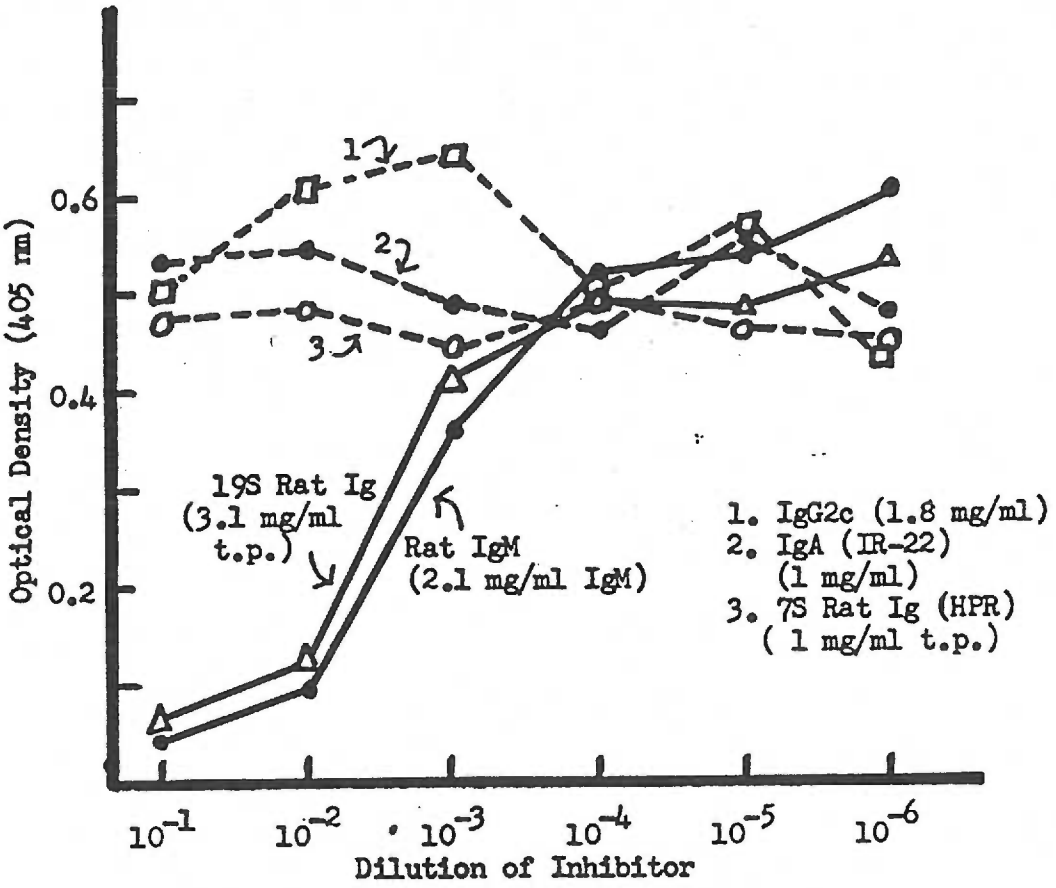


Figure 2. Binding inhibition of GALM 36.12E anti-rat IgM to plates coated with rat Lou/Wsl plasmacytoma IgM-kappa by the rat IgM (2.1 mg/ml) (●——●), 40% SAS precipitated 19S HPR rat serum Ig (3.1 mg/ml total protein (t.p.)) (△——△), monoclonal rat IgG2c anti-SACHO (90.1, 1.8 mg/ml) (□- -□), rat Lou/Wsl plasmacytoma IgA (IR-22, 1 mg/ml) (●---●), and 40% SAS precipitated 7S HPR rat serum Ig (1 mg/ml total protein (t.p.)) (○----○).

preparations with quantities of IgM determined by radial immunodiffusion, the ELISA assay for IgM was shown to correlate well with previously determined values in serum from HPR, Copenhagen, and F344 rats. Assay of gel filtrations fractions of 40% saturated ammonium sulfate precipitated rat serum Ig identified a single peak corresponding to 19S rat Ig.

GALA 1.14.2. The GALA 1.14.2 hybridoma was isolated following the observation of specific binding to monoclonal IgA (IR-22) coated plates. As with the other hybridomas it was grown in BALB/c mice and ascitic fluid collected. The ascitic fluid was titered and tested for inhibition specificity. The ascitic fluid was used routinely in assays with a dilution of 1/30,000. Attempts to specifically purify the antibodies intact from ascitic fluid on an IgA immunoadsorbant were unsuccessful. Although the reduction of anti-IgA activity in the effluent material showed that the antibody apparently did bind to the immunoadsorbant, following elution with 3 M sodium thiocyanate the eluted antibodies had lost their ability to bind IgA. The isotype of the specifically purified antibody was identified as IgG2b-kappa. A test of the inhibition specificity of the antibody showed that IR-22 plasmacytoma IgA (8.4 mg/ml IgA) inhibited the antibody to an equivalent degree as 7S HPR rat serum Ig (1 mg/ml total protein, mostly IgG) and monoclonal rat IgM (2.14 mg/ml total protein, mostly IgM) at between a 600 and 3000 fold higher dilution (Figure 3). A

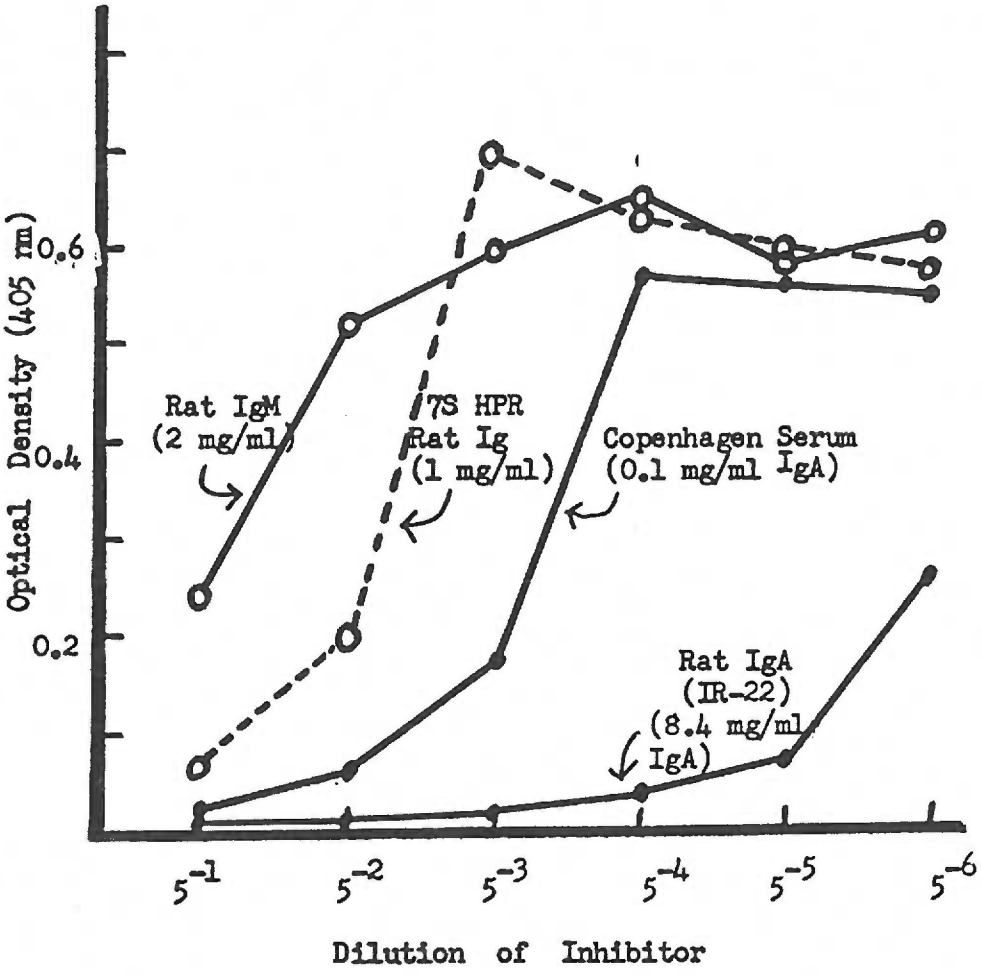


Figure 3. Binding inhibition of GALA 1.14.2 anti-rat IgA to plates coated with rat plasmacytoma IgA (IR-22) the rat Lou/Wsl plasmacytoma IgA (8.4 mg/ml) (●——●), standard Copenhagen rat serum (0.1 mg/ml IgA) (●——●), 40% SAS precipitated 7S HPR rat serum Ig (1 mg/ml total protein (t.p.)) (o----o), and rat IgM (2 mg/ml) (o——o).

standard Copenhagen rat serum pool containing 0.1 mg/ml IgA showed an intermediate range of inhibition. Using the antibody in an IgA assay of gel filtration fractions of the IR-22 plasmacytoma identified two peaks representing monomeric and dimeric IgA (Figure 4). Assays of the same fractions using polyclonal goat anti-rat IgA by radial immunodiffusion confirmed the results.

Rabbit anti rat membrane IgD. The preparation of this reagent is described in detail in the materials and methods section of paper 1 (Steele and Leslie, 1985). Examples of tests of the binding specificity and inhibition specificity of the reagent are shown in figures 5 and 6 respectively. The anti-delta conjugate showed binding specificity for plates coated with (1-5 µg/ml) IgD prepared by gel filtration from rat milk and ascitic fluid, while negligible binding occurred to plate wells coated with 5-10 µg/ml of rat IgA (IR-22), rat IgM, or 7S HPR rat serum Ig (Figure 5). For tests of inhibition specificity, plates were coated with milk IgD peak I prepared by gel filtration at between 1-2 µg/ml in bicarbonate buffer as described (see paper 1) and the enzyme conjugate was diluted 1/50. HPR rat milk (120 µg/ml IgD) and a 1:1 dilution of a high IgD rat serum (138 µg/ml IgD, previously assayed independently by H. Bazin), inhibited the enzyme conjugate at >16x the dilution of preparations of 3.75 mg/ml rat IgA (IR-22), 7.25 mg/ml 7S HPR rat serum Ig, and 2 mg/ml 19S HPR rat serum Ig (Figure 6). As these preparations are all 40% SAS

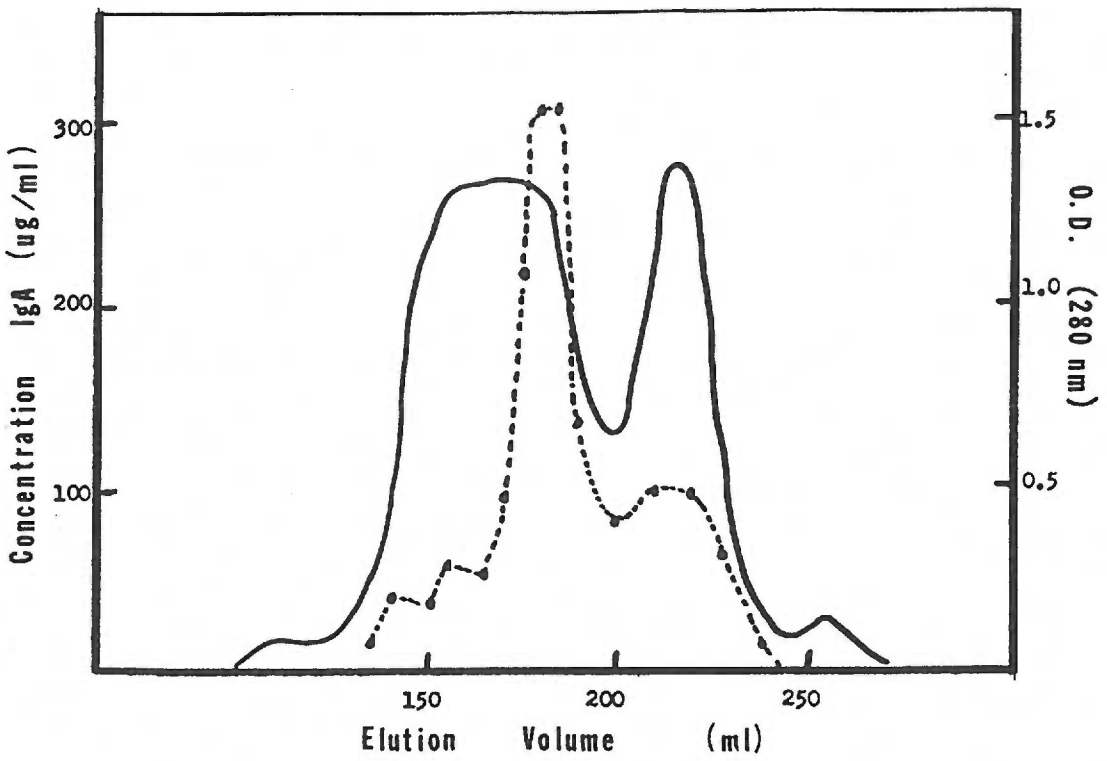


Figure 4. Elution profile of rat plasmacytoma IgA purified from 40% SAS precipitated ascitic fluid on an S-300 gel filtration column as detected by GALA 1.14.2 anti-rat IgA.
OD 280 nm (———), IgA (●-----●).

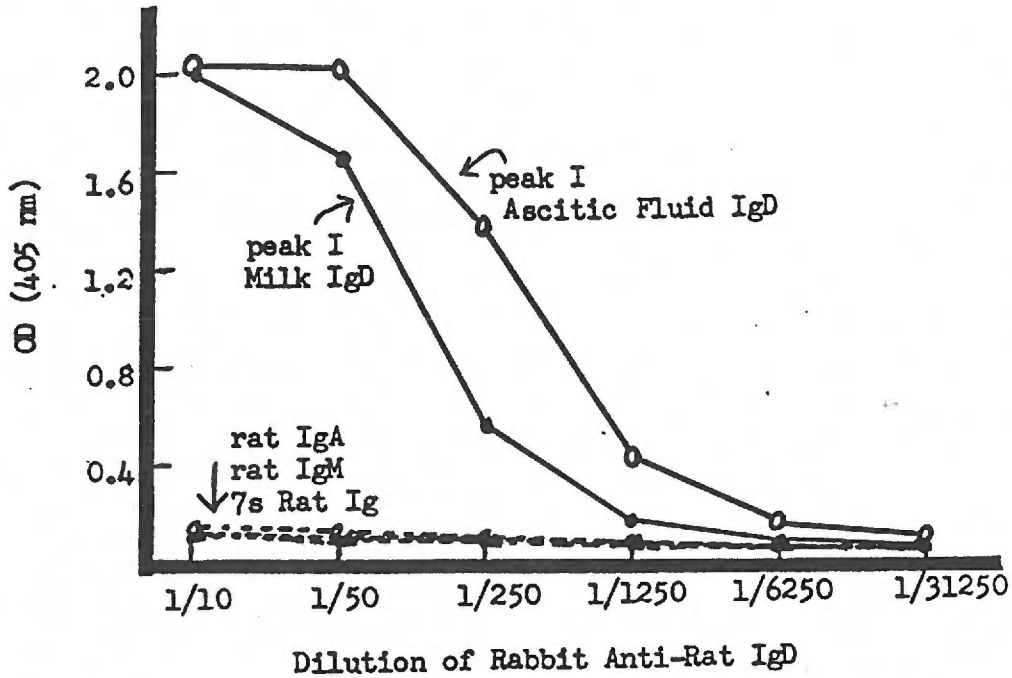


Figure 5. Binding of rabbit anti-rat IgD to assay plates coated with peak I ascitic fluid IgD (O——O), peak I milk IgD (●——●), rat IgA (IR22) (O····O), rat Lou/Wsl plasmacytoma IgM (●·····●), and 40% SAS precipitated HPR 7S rat serum Ig (Δ----Δ).

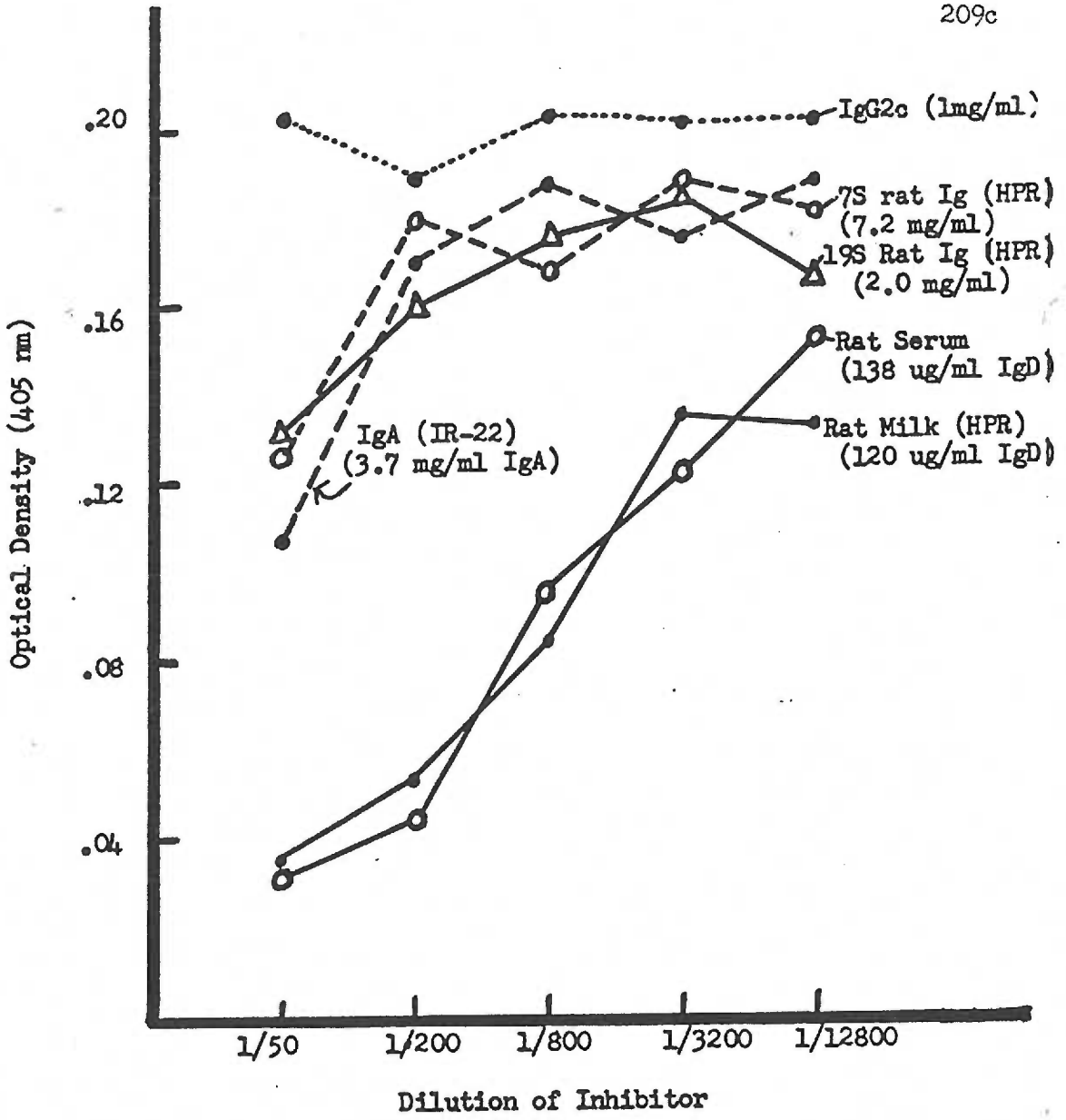


Figure 6. Binding inhibition of rabbit anti-rat IgD to plates coated with peak I milk IgD by clarified HPR rat milk (120 µg/ml IgD) (●——●), a high-IgD rat serum (138 µg/ml IgD) (○——○), rat Lou/Wsl plasmacytoma IgA (IR-22, 3.7 mg/ml) (●– –●), 40% SAS precipitated 7S HPR rat serum Ig (7.2 mg/ml total protein) (○– –○), 40% SAS precipitated 19S HPR rat serum Ig (△——△), and monoclonal rat IgG2c anti-SACHO (30.8) (1 mg/ml) (●·····●).

precipitated and concentrated from rat ascitic fluid and rat serum which has been shown to enrich for IgD in such preparations (see appendix 1) and as IgD is present in rat serum in trace quantities (Bazin et al., 1978) some inhibition of conjugate binding in these preparations is to be expected. A monoclonal rat IgG2c anti-SACHO (30.8) at 1 mg/ml specifically purified from an N-acetyl glucosamine immunoadsorbant showed no inhibition of the conjugate at any dilution.

References

Bazin, H., Beckers A., Urbain-Vansanten, G., Tilkin, A.F., Platteau, B., and Urbain, J. (1978) Transplantable IgD immunoglobulin-secreting tumors in rat. *J. Immunol.* 121: 2077.

Oi, V.T., and Herzenberg, L.A. (1980) Immunoglobulin-producing hybrid cell lines. In *Selected Methods in Cellular Immunology* (B.B. Mishell and S.M. Shiigi, eds.) W.H. Freeman Co., San Francisco, p. 351.

Steele, M.G., and Leslie, G.A. (1985) Immunoglobulin D in rat serum, saliva and milk. *Immunology* 55: 571.

Steele, M.G., and Leslie, G.A. (1986) IgD antibodies to DNP are induced in ascitic fluid in ascitic fluid after intraperitoneal immunization. *submitted.*

APPENDIX 3

The Limitations of Calibration Standards and Molecular Weight Determination Using the Sephacryl-300 Gel Filtration Column

Most of the gel filtration experiments presented in this thesis were performed at room temperature on a 2.5 x 90 cm column of superfine Sephacryl-300 (Pharmacia Uppsala, Sweden), which is a gel filtration medium consisting of allyl dextran covalently cross-linked with N,N'-methylene bisacrylamide. The elution buffer was 0.01 M tris-buffered saline pH 7.4 supplemented with 0.01 M epsilon-aminocaproic acid as a protease inhibitor. The range of molecular size species which can be resolved range from IgM (971 kDa), which elutes about 10 ml beyond the void volume of the column, to BSA and ovalbumin at 45 to 65 kDa (Fig. 1). The molecular weight standards recommended by Pharmacia for the calibration of the column (thyroglobulin, ferritin, catalase, and BSA) yield a log molecular weight versus elution volume curve (upper curve) which is distinct from that generated when the immunoglobulin standards IgM, IgA, partially reduced and alkylated IgM, rat IgG, human IgG, and bovine IgG (lower curve). The tendency of immunoglobulins to elute at a higher apparent molecular weight than indicated by the Pharmacia standards probably has to do with the oblong shape of the immunoglobulin standards, hence the limitations of the method. As rat IgD presumably is similar to other immunoglobulins in its structure and dimensions it seems more appropriate to use the lower curve in molecular weight estimates, with the necessary *caveat* that since the exact structure of the IgD species examined in Appendix 1 and paper 2 are unknown, these estimates must be referred to as "apparent"

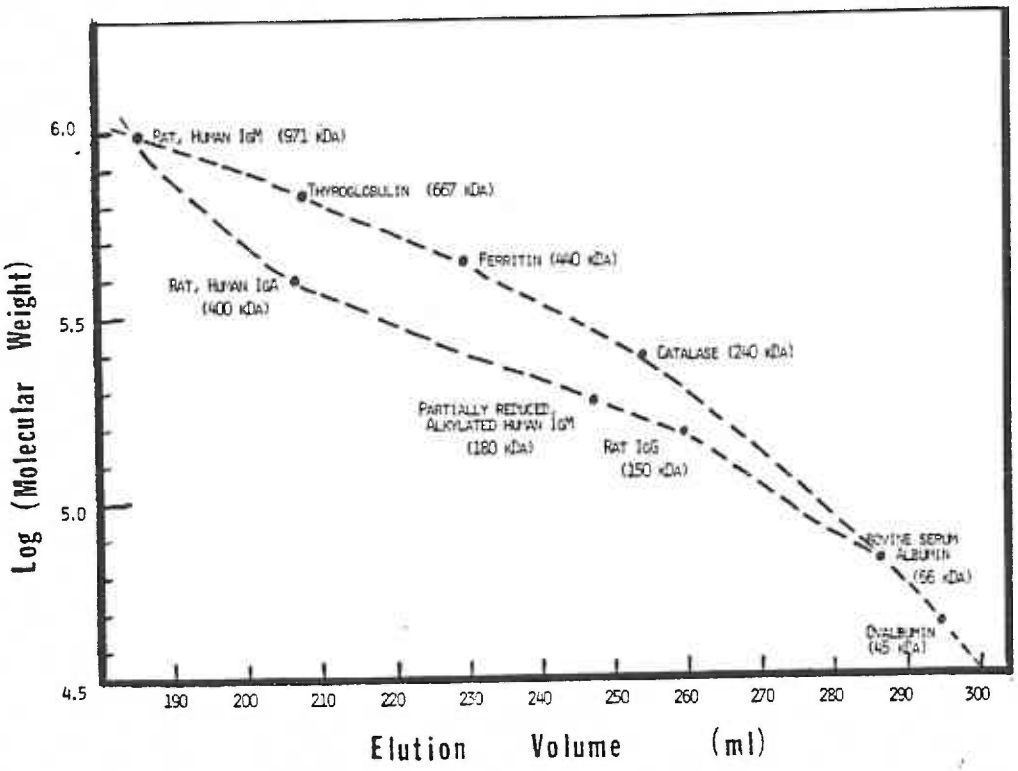


Figure 1 . Log molecular weight versus elution volume curves as defined by IgM, thyroglobulin, ferritin, catalase, and BSA (upper curve) and by IgA, partially reduced and alkylated IgM, IgG and BSA (lower curve).

molecular weights.

The precision with which molecular estimates can be made is determined primarily by the resolution of the column, and in a secondary way by the size of the fractions collected. Most of the experiments were performed using a Gelman fraction collector set to collect 150 drops per fraction which amounted to about a 4 to 5 ml volume for each fraction. Consequently at best the precision of measurement for elution volumes was between ±2 ml and ±2.5 ml.

In some cases internal molecular weight markers could be used by assaying gel filtration fractions for IgA, IgM, and IgG, however usually estimates of molecular weight were determined through prior calibration runs in which the mean elution volume for molecular weight standards were determined from 2-3 measurements.
The results of one series of calibration runs for different molecular weight standards is shown in Table 1. The standard deviation for the elution volume for human IgM is 3.2 with a mean of 187.3 (±1.7%).

TABLE 1

Elution Volume Measurements for Molecular Weight Standards on a 2.5 x 90 cm. S-300 Gel Filtration Column

Standard	MW	Elution Volume measurements
Nurse shark Ig peak I	~1000 kDa	178, 178
Human IgM	971 kDa	185, 186, 191
Rat, Human IgA	400 kDa	206, 208
Thyroglobulin	670 kDa	208, 207, 209
Ferritin	440 kDa	230, 228, 232
Nurse shark Ig peak II	180 kDa	237, 239
Partially reduced, alkylated IgM	180 kDa	247
Catalase	240 kDa	254
Rat, human bovine IgG	150 kDa	257, 260, 263
Bovine serum albumin	66 kDa	284, 286, 289
Ovalbumin	45 kDa	293, 295, 296

APPENDIX 4

The Induction of IgD Antibodies in Rat Milk and Serum by Intramammary and Enteric Immunization

Introduction

Reports indicate that IgD antibodies to antigens of likely enteric or respiratory exposure may occur in human milk (Sewell et al., 1979; Keller et al., 1985). This suggests either that (1) the presented antigens reach the precursors of IgD-producing cells in mammary tissue, that (2) IgD antibodies produced systemically are transported into the milk, or that (3) precursors of IgD-producing cells migrate from other lymphoid organs to the mammary tissue, in a manner similar to that which has been reported for IgA. Considering the lack of change in serum IgD during lactation, the high concentration of IgD in milk and the volume of milk produced, it seems very likely, though not proven, that IgD is synthesized locally in the mammary tissue (Steele and Leslie, 1985). The observation that IgD antibodies in milk can be produced by intramammary immunization reinforces the concept of local antigen-presentation and synthesis. Similar observations with respect to colostrum/plasma ratios of total IgD and IgD antibodies in human studies support the concept of local synthesis of IgD in human milk (Bahna et al, 1982, Keller et al, 1985), and histological studies of human mammary tissue have demonstrated the presence of IgD-containing cells (Brandtzaeg, 1983).

In this preliminary study the ability of enteric or intramammary immunization to induce serum and milk IgD antibodies was examined,

using dinitrophenylated ovalbumin (DNP-OVA) and dinitrophenylated group A *Streptococcal* vaccine (DNP-GASV). Samples were also tested for IgA and kIg antibodies (kIg was used as an approximation of total antibodies (Hood et al., 1968)). The results indicate that while periodic oral immunization with either dinitrophenylated antigen failed to induce a significant milk or serum IgD antibody response, DNP-OVA given in drinking water produced IgD antibodies in both serum and milk. Intramammary immunization was the most effective method for induction of IgD antibodies in milk using the protocols employed in these preliminary studies.

Materials and Methods

F344 rats were used in all experiments, and were ~100 days of age at the beginning of the experiments. DNP-OVA and DNP-GASV were prepared as described (see paper 3; Steele and Leslie, *submitted*). Five groups of 5 rats each were immunized and paired with males for breeding according to the protocol shown in Table 1. Rats were visually examined for pregnancy. When it was determined that the rats were pregnant they were removed from the breeding cages, and immunization was discontinued.

Blood or blood and milk was collected during immunization, pregnancy

TABLE 1

Oral and Intramammary Immunization Protocols

Group	Immunization	schedule of immunization
DNP-OVA/OI	3 mg DNP-OVA by oral intubation	3x/week beginning 10 days before breeding
DNP-GASV/OI	3 mg DNP-GASV by oral intubation	3x/week beginning 10 days before breeding
DNP-OVA/IM	250 µg DNP-OVA in 1 ml 1:1 FCA dist. in 8 sites in mammary gland	7 days and 26 days following breeding
DNP-GASV/IM	250 µg DNP-GASV in 1 ml 1:1 FCA dist. in 8 sites in mammary gland	7 days and 26 days following breeding
DNP-OVA/DW	0.1% DNP-OVA in drinking water	*ad libitum* beginning 10 days before breeding.

Abbreviations: OI, oral intubation; IM intramammary; dist., distributed; FCA, Freund's complete adjuvant.

and lactation. Clarified milk and serum were prepared from these fluids by centrifugation (Steele and Leslie, 1985). IgD, IgA, and kIg antibodies to DNP were assayed by ELISA (Steele and Leslie, *submitted*).

Results

Oral immunization by intubation with either DNP-OVA (DNP-OVA/OI) or DNP-GASV (DNP-GASV/OI) did not result in significant IgD or kIg antibodies in serum or milk, whereas some IgA antibodies in milk resulted from DNP-OVA/OI but not from DNP-GASV/OI immunization (Figure 1). Oral immunization with 0.1% DNP-OVA in drinking water (DNP-OVA/DW) produced substantial IgA (Figure 1) and IgD antibodies (Figure 2) in milk and some IgA and IgD antibodies in serum. Nevertheless negligible kIg antibodies were detected in either serum or milk by DNP-OVA/DW (Figure 3), which indicates that the total antibody response was relatively small. The larger amount of antigen and the more sustained exposure to it via DNP-OVA/DW immunization may have been a factor in the greater IgA and IgD response. While DNP-OVA/OI and DNP-GASV/OI immunized rats received 9 mg of antigen per week, DNP-OVA/DW immunized rats received about 160 mg of antigen per week, based on the expected water consumption of a 250 g rat (Baker, Lindsey, and Weisbroth, 1980).

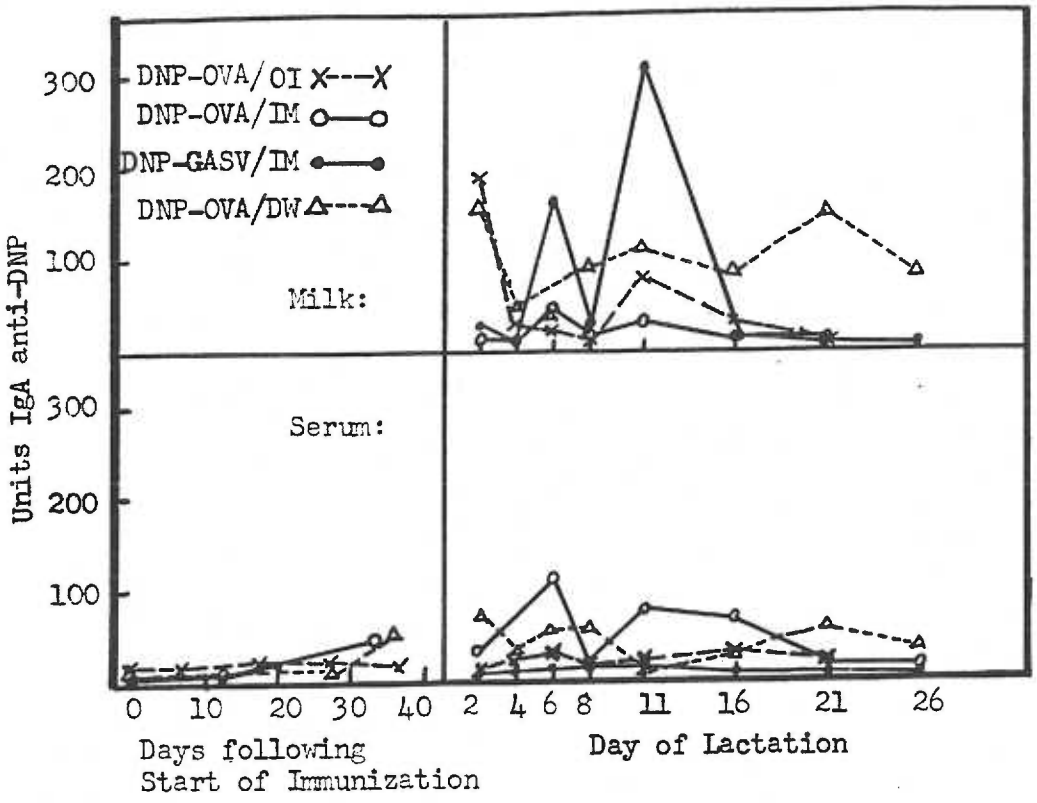


Figure 1 . IgA anti-DNP in milk and serum following intramammary and oral immunizations. IgA anti-DNP expressed in terms of standard IgA anti-DNP milk = 100 units. DNP-GASV/OI produced no significant antibodies. Abbreviations: OI, oral intubation; IM, intramammary; DW, drinking water immunization.

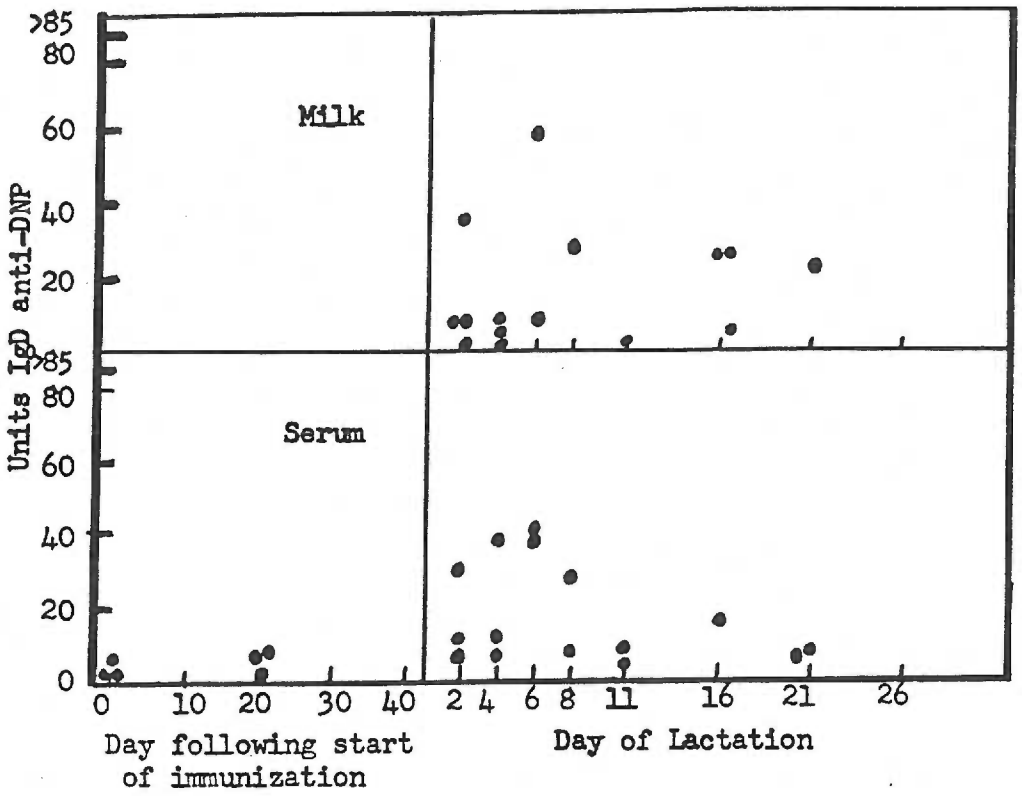


Figure 2 . IgD anti-DNP in milk and serum resulting from DNP-OVA/DW immunization. (0.1% DNP-OVA in drinking water).

IgD anti-DNP expressed in terms of standard IgD anti-DNP = 100 units.

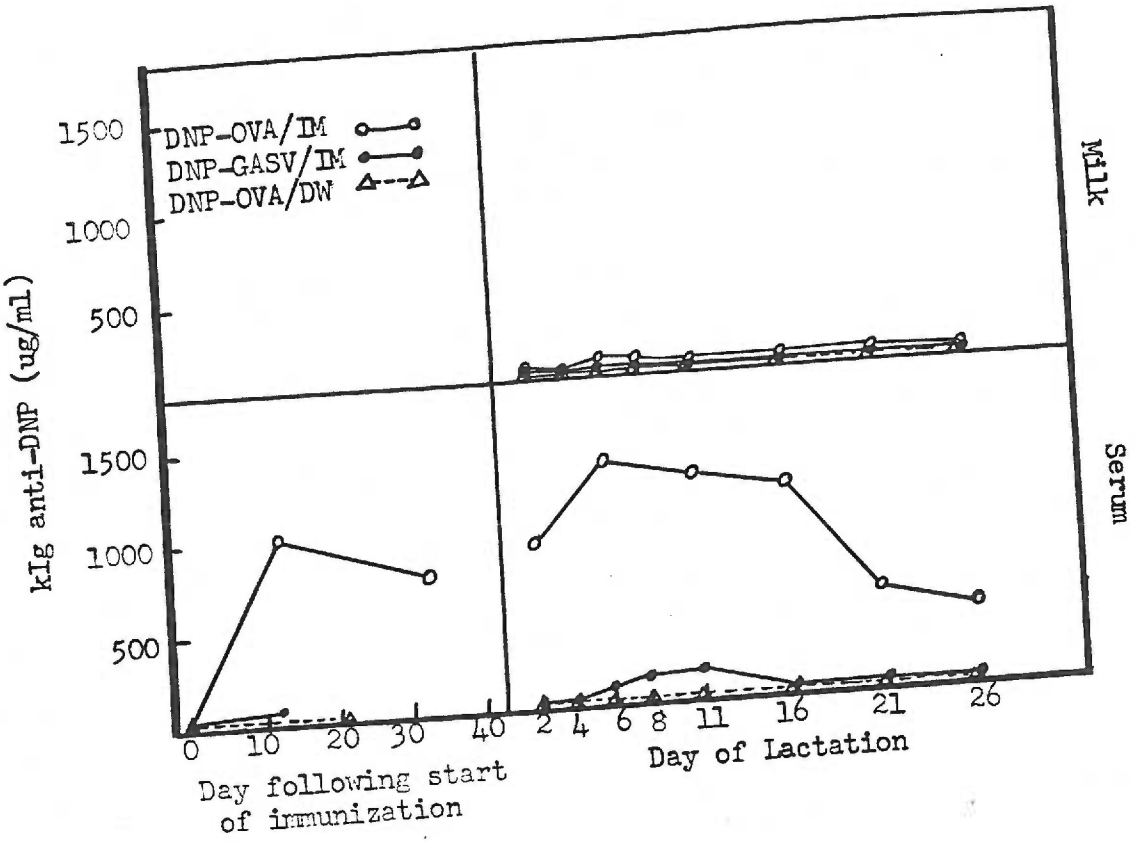


Figure 3 . kIg anti-DNP in milk and serum resulting from intramammary and oral immunizations. DNP-OVA/OI and DNP/GASV/OI did not produce significant responses in either milk or serum.

Abbreviations: IM, intramammary; DW, drinking water; OI, oral intubation.

Intrammammary immunization with DNP-OVA (DNP-OVA/IM) produced a strong serum kIg antibody response before and during lactation whereas a small serum kIg response to DNP-GASV intramammary immunization (DNP-GASV/IM) with FCA was observed during lactation but not in the pre-lactation period (Figure 3) . Neither immunization produced significant kIg antibodies in milk. In contrast, DNP-GASV/IM produced substantial IgA antibodies in milk and negligible IgA antibodies in serum, while DNP-OVA/IM produced a more modest IgA response in milk and a larger IgA response in serum (Figure 1). Thus intramammary immunization results in a largely serum kIg antibody response, and in a largely IgA milk antibody response.

DNP-OVA/IM produced a strong IgD antibody response in milk (Figure 4), while DNP-GASV/IM produced a more modest one in milk (Figure 5), with responses being highest early in lactation, as previously reported (Steele and Leslie, *in press*). Serum IgD responses were negligible for either IM immunization protocol.

Discussion

The existence of a common mucosal system characterized by the emigration of IgA-producing cell precursors from one mucosal site to another is well established. Montgomery et al., (1974) showed that both

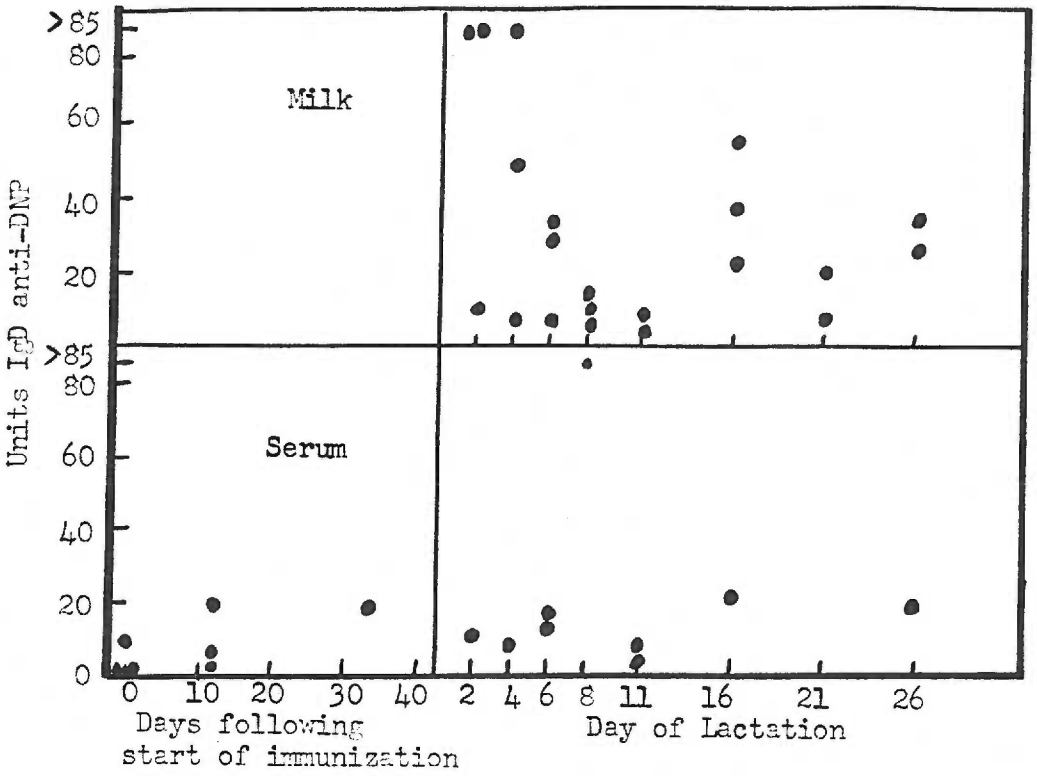


Figure 4 . IgD anti-DNP in milk and serum resulting from DNP-OVA intramammary immunization (DNP-OVA/IM).

IgD anti-DNP expressed in terms of standard IgD anti-DNP = 100 units.

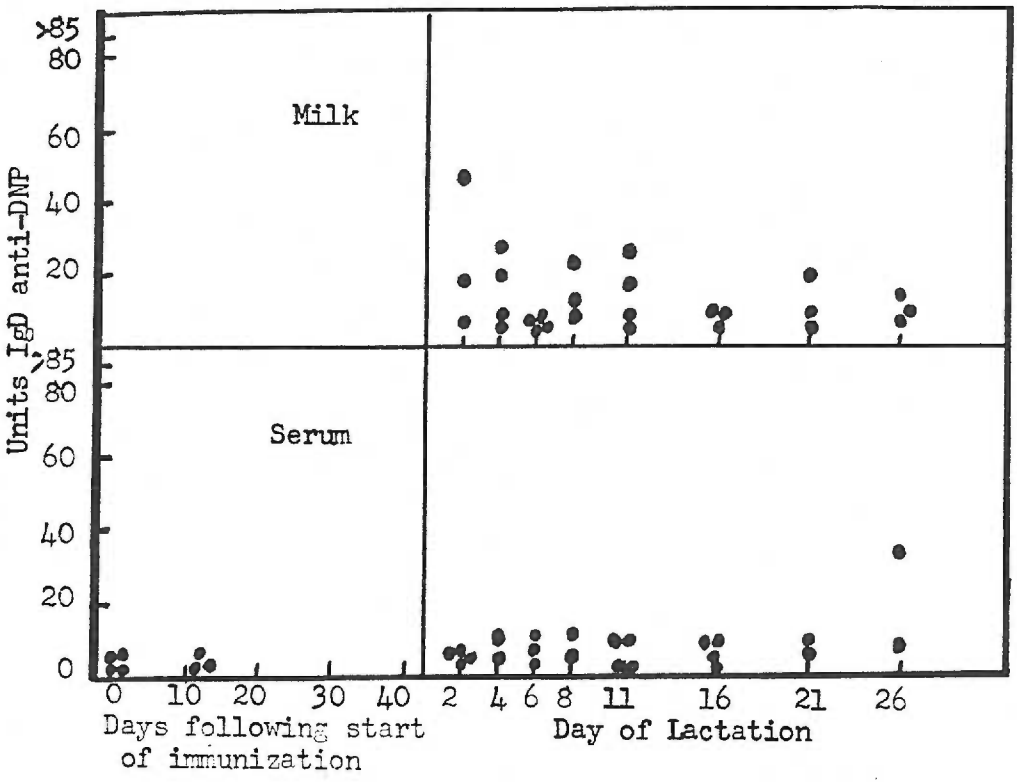


Figure 5 . IgD anti-DNP in milk and serum resulting from DNP-GASV intramammary immunization (DNP-GASV/IM).

IgD anti-DNP expressed in terms of standard IgD anti-DNP = 100 units.

intramammary (IM) immunization and oral (OI) immunization with either dinitrophenylated bovine gamma globulin (DNP-BGG) or dinitrophenylated type III pneumococcal vaccine (DNP-Pn) resulted in IgA antibodies in rabbit milk but not in serum. IM immunization with the soluble antigen (DNP-BGG) also produced IgG antibodies in serum whereas none were produced by the particulate antigen (DNP-Pn). Similarly soluble protein antigens have been shown to produce serum antibody responses via oral immunization, which suggests that the serum response was the result of antigen entering the circulation, and similarly that antigen absorbed from the gut may have directly stimulated the IgA antibody responses in the mammary gland. However Montgomery et al. could not explain the IgA responses in milk resulting from oral immunization with particulate antigen in the same way, and showed that intravenous immunization with the antigen did not similarly result in a milk IgA response. Therefore they proposed the precursors of IgA producing cells migrated from the gut lymphoid tissue to the mammary gland. Subsequently other researchers have provided more direct evidence for such a migration, showing homing of cells to mammary tissue.

The attempt made in this study to demonstrate a similar relationship between gut immunization and milk IgD antibody responses in rats was not entirely successful. While gut immunization with the particulate antigen DNP-GASV (DNP-GASV/OI) did produce an IgA response in rat milk, no similar IgD response was apparent. Similarly, DNP-OVA/OI

immunization failed to produce an IgD response in milk. The fact that DNP-OVA/DW immunization did produce an immune response in serum and milk may have merely been the result of distribution of antigen systemically rather than an emigration of cells from the gut. Alternatively, precursors of IgD secreting cells may have emigrated from the gut both to mucosal and systemic sites. Human milk IgD has been reported which has specificity for *E. coli* (Sewell et al., 1979) as well as to grass pollen antigens and BSA (Keller et al., 1985). Human serum IgD antibodies have also been reported in several cases of hypersensitivity resulting from likely gut or respiratory exposure to antigens such as milk (Galant et al., 1983), the food dye, tartrazine (Weliky et al., 1979), *Lollium perenne* Fr. C (Bringel et al., 1982), and wheat antigens (Bahna, Tateno, and Heiner, 1980). These studies indicate that gut exposure to antigen may under some circumstances result in both milk and serum antibodies. Nevertheless the hypothetical movement of antigen or responding cells responsible for IgD antibodies in serum and milk at present is unknown.

Cox et al., (1980) performed similar IM immunizations with DNP-BGG and DNP-*S. mutans*, finding that while IgA responses to both antigens in milk were comparable, IgG responses in serum were much greater with the soluble antigen, DNP-BGG, which they speculated was the result of escape of the antigen to systemic sites. The particulate antigen (DNP-*S. mutans*), did not show an IgG response in serum. The results of

this study show a similar pattern with IM immunization, with a IgA response mostly in milk and a much larger kIg antibody in serum following DNP-OVA/IM than following DNP-GASV/IM immunization. The observation that IgD antibodies are largely restricted to milk with either antigen suggests that IgD responses as well as IgA responses can be induced by local exposure to antigen in the mammary tissue.

References

Bahna, S.I., Keller, M.A., and Heiner, D.C. (1982). IgE and IgD in human colostrum and plasma. *Pediatr. Res.* 16: 604.

Bahna, S.I., Tateno, K., and Heiner, D.C. (1980). Elevated IgD antibodies to wheat in celiac disease. *Ann. Allergy* 44: 146.

Baker, H.J., Lindsey, J.R., and Weisbroth, S.J. (1980). Selected normative data. In *The Laboratory Rat, Volume II: Research Applications* (H.J. Baker, J.R. Lindsey, and S.J. Weisbroth, eds.). Academic Press, New York. p. 411.

Brandtzaeg, P. (1983). The secretory immune system of lactating human mammary glands as compared with other exocrine organs. In *The Secretory Immune System* (J.R. McGhee and J. Mestecky, eds.) *Ann. N.Y. Acad. Sci.* 409: 353.

Bringel, H., Vela, C., Urena, V., Gurbindo, D., Garcia, R., and Lahoz, C. (1982). IgD antibodies in vitro blocking activity of IgE mediated reactions. *Clin. Allergy* 12: 37.

Cox, D.S., Taubman, M.A., Ebersole, J.L., and Smith, D.J. (1980). Secretory antibody response to local injection of soluble or particulate antigens in rats. *Mol. Immunol.* 17: 1105.

Hood, L., Gray, W.R., Sanders, B.G., and Dreyer, W.J. (1968). Light chain evolution. *Cold Spring Harbor Symp. Quant. Biol.* 32: 133.

Galant, S., Nussbaum, E., Wittner, R., DeWeck, A.L., and Heiner, D.C. (1983). Increased IgD milk antibody responses in a patient with Down's syndrome, pulmonary hemosiderosis, and cor pulmonale. *Ann. Allergy* 51: 446.

Keller, M.A., Heiner, D.C., Myers, A.S., and Reisinger, D.M. (1985). IgD in human colostrum. *Pediatr. Res.* 19: 122.

Montgomery, P.C., Cohn, J., and Lally, E.T. (1974). The induction and characterization of secretory IgA antibodies. In *The Immunoglobulin A System* (J. Mestecky and A.R. Lawton, eds.) Plenum Press, New York. p. 453.

Sewell, H.F., Chambers, L., Maxwell, V., Matthews, J.B., and Jefferis, R. (1978). The natural antibody to *E. coli* includes antibodies of the IgD class. *Clin. Exp. Immunol.* 31: 104.

Steele, M.G., and Leslie, G.A. (1985). Immunglobulin D in rat serum, saliva and milk. *Immunol* 55: 571.

Steele, M.G., and Leslie, G.A. (1986). IgD and the maternal-fetal interface. In *Immunoregulation and Fetal Survival* (T. Gill and T.G. Wegmann, eds.) Oxford University Press, New York. *in press*.

Steele, M.G., and Leslie, G.A. (1986). IgD antibodies to DNP are induced in ascitic fluid after intraperitoneal immunization. *submitted*.

Weliky, N., Heiner, D.C., Tamura, H., and Anderson, S. (1979). Correlation of tartrazine hypersensitivity with specific serum IgD levels. *Immunol. Commun.* 8: 65.

ACKNOWLEDGEMENTS

I would like to thank my thesis advisor, Gerrie Leslie, for the privilege of working in his laboratory and for allowing me to pursue my research into rat IgD. His intelligence, personality, energy, and intellectual curiosity were a continual inspiration to me and for which I am deeply grateful.

I thank Joan Olson, Mary Brewer, Anwyl Cooper-Willis, Bob Riggs, Walid Tadmori, Louie Shapiro, Tim Vincent and Mimi Wing for their technical assistance, forbearance, patience, and friendship.

I thank my thesis and examining committee, Wes Bullock, Denis Burger, William Koler, Marvin Rittenberg, Peter Stenzel, and Art Vandenbark for their helpful suggestions during the completion of my project and in their review of the thesis manuscript.

I thank Audrey Moore, Ann Smith, and Judy Hoff for their patience and help in managing the paperwork and red tape involved in producing a thesis and in scheduling its defense, and Jules Hallum for finding financial support for me in the months when I was writing it.

And lastly I would like to thank the many other people in the Department and in the School who have made my time here a rewarding and unforgettable experience.