The nature of immunoglobulin species in passive immunity of neonatal mice
by Richard Adam Wilson

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE in Microbiology
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Abstract:
Passive immunity in neonates was studied in conventionally reared and germfree mice, using three assay methods to determine the immunoglobulin concentrations in neonatal and dam sera and in colostrum. Female mice were antigenized with one or two injections of DNP-BGG in complete Freunds adjuvant given by intradermal injection near the mammary chain. The female mice were mated 10 days after the last injection.

At day one and day five post-partum, blood samples were obtained from the females and one half of their litters were sacrificed. Colostrum was flushed from the neonatal stomachs and neonatal serum was collected. All specimens were stored in the frozen state until assayed. Mammary glands were excised from the females after they had been sacrificed on day five post-partum.

Hemolysin and hemmaglutinin titres were performed on sera and some of the polostral samples. A modification of the radialimmunodiffusion technique of Masseyeff and Zisswiller was employed to measure the four immunoglobulins, IgA, IgM, IgG1 and IgG2, and the specific antibody concentrations in colostrum and serum.

The data indicates that neonates acquired IgG2 and IgG1 passively by both placental and lacteal transmission and IgA and IgM primarily by lacteal transmission. The concentration of the immunoglobulins and specific antibodies in serum or colostrum of the dams was not an apparent factor in determining transmission of the immunoglobulins to the neonate. A hierarchy directly related to the species of immunoglobulin and how well they traverse the placenta or mammary gland barrier was observed. IgA was associated with "local" gut immunity while IgG1 and IgG2 were found in greatest concentration in the sera of fhe neonates.

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Date Dec. 4, 1970

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A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree

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ABSTRACT

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INTRODUCTION

An abundant literature clearly indicates that passive immunity affords early protection against infection for many mammalian neonates (Brambell, 1968). Immunoglobulins enter the fetal bloodstream by placental passage and/or the neonatal bloodstream via absorption of colostrum through the intestine. Rabbits, guinea pigs and human neonates aquire a majority of their passive antibodies almost exclusively in the colostrum. Dogs, mice and rats obtain immunoglobulins in utero and aquire antibodies from colostrum. The placental structure as well as its permeability properties appear to dictate fetal acquisition of passive antibodies.

There have been extensive and varied studies of colostral transfer of immune gamma globulins. In this regard the vast literature dealing with colostral immunoglobulins in bovine, ovine and porcine deserves some attention but only those investigations which will emphasize a particular point are included in this discussion.

A general study of the immunoglobulin content of the serum of calves in England was reported by Smith et al. (1967). As one might expect there were great variations in immunoglobulin levels which did not relate to the serum or colostral levels of the dam. These investigators observed no relationships between immunoglobulin content of serum and when calving occurred. This latter observation is not in accord with the finding of Gray, Fischer and McEwen (1965) in a survey

of cattle in Scotland.

Smith et al. (1967) attribute the variation in immunoglobulin level in calves to methods of husbandry in the country and suggested a genetic variation amongst offspring is involved, especially that which influences the absorptive capabilities of the intestine. The immunoglobulin concentration was assayed by absorptiometric technics.

Colostral transfer of Rinderpest neutralizing antibody compares favorably between Freisen cows and water buffalo (Sighn et al., 1967). In their studies Freisen dams were vaccinated with a lapinized strain and water buffalo were vaccinated with avianized and caprianized strains of Rinderpest virus 6-7 months pre-partum. Calves suckled 2-4 hours post-partum and sequential antibody assays began 4-5 hours later. Peak titres were seen at 8 hours after suckling and persisted for 21 days. The titres slowly decreased to zero at 6-7 months of age.

The half-life was calculated to be 33 days and 29 days for the water buffalo and Freisen calves, respectively. The half-life compared favorably with the results reported by Brown (1958) who examined Rinderpest neutralizing antibody in bovine. An interesting observation was that the half-life exceeded that of maternally derived foot and mouth disease antibody as determined by Graves (1963). The possibility of dissimilar experimental protocol must be entertained, yet, this speaks to other unknown factors, such as, those

influencing the absorption and passage of immunoglobulins in the gut.

Smith (1962) reported that Escherichia coli septicemia commonly occurs in colostrum-deprived calves. In other studies, hypogammaglobulinemia was detected in colostral-fed calves resulting in colibacillosis (Fey and Margarant, 1961). These workers have reported an incidence of hypogammaglobulinemia in 10% of "normal calves" they have studied. It seems possible that hypogammaglobulinemia precedes the establishment of gut flora via ingestion of fecal material from udders or environmental fomites. In addition, those small quantities of immunoglobulins that are passed from dam to offspring may be absorbed by organisms in or of the intestine and never contribute to passive immunity of the calf.

Data pertinent to this theory are found in a report by Klauss, Bennet and Jones (1969). Three calves from a group of 10, demonstrated low IgM and IgG levels in serum beginning day 1 and lasting until day 7. One of the dams of these calves had normal serum and colostral levels of IgM and IgG. Two of the three calves were born of dams with an infectious process; one a mastitis and the other, a case of enteritis. The mastitic cow had an increased concentration of IgM in her serum which is indicative of an acute response. The etiological agents were not indicated nor were the subclasses of IgG reported. There was, however, a contribution to serum immunoglobulin

levels of the calf presumably through the colostrum of the dam in every case.

The presence of IgG, IgM, and IgA in serum and colostrum of the dam and its gradual accumulation in the intestinal contents and neonatal serum indicate a transfer function of the mammary gland and the intestine.

Zuffa (1964) vaccinated brood sows with modified virus of Aujesky's disease in a study of placental transfer of virus and colostral transmission of antibody. Neither virus nor antibody were transmitted diaplacentally. However, 24 hours post-partum the piglet serum titre was equally high as in maternal serum. The half-life of these antibodies was calculated to be 10.8 days.

Passive immunity to Ascaris suum was transferred in colostrum from sows to their offspring (Kelley and Nayah, 1965). The humoral antibodies directed against ascaris larvae conferred immunity by impeding the migration of the larvae to the lungs. Sprint (1949) found that migration of Ascaris larvae was stopped in the liver of young immune mice. Soulsby (1961) observed a cellular reaction about the 3rd stage larvae given intraperitoneally to immune animals. The reaction in the liver and intestine included a leucocyte response which could possibly have blocked migration.

The presence of absorbed colostral antibody will effect a response to Escherichia coli (6 species) in porcine neonates. Sharpe (1966) compared antibody response in normally reared piglets partially deprived of colostrum and noted that the deprived piglets produced antibody at an earlier age and were still able to absorb, but to a lesser degree, antibodies when returned to a sow 26 hours post-partum. Both anti-"O" and anti-"K" antibody titres varied in a similar fashion. Titres were negative at birth as determined by the techniques employed.

Pierce and Smith (1967) investigated homologous and heterologous colostral immunoglobulin transfer in porcine neonates. The colostral immunoglobulins were absorbed similarily if fed separately in high concentration. Intestinal absorption in piglets fed heterologous colostrum of both were absorbed readily without apparent competition. Internal degradation of immunoglobulins, however, made definitive interpretation difficult. Porter's (1969) work supports the findings of Pierce and Feinstein (1965) and earlier workers that the intestinal tract of neonatal bovine will absorb proteins indiscriminately. On the other hand, in vitro studies (Pierce and Smith, 1967b) indicate a selective mechanism with the most pronounced immunoglobulin passage in the middle third (ileum) of the porcine small intestine. Also, Halliday and Keckwith (1960),

Locke et al. (1964) have shown that IgG immunoglobulins are absorbed more effectively than IgM in the new born rat and pig.

A sieve-like function has been postulated for the mammary gland of the rat (Lowell and Morgan, 1965). The dominant proteins demonstrated in rat milk are serum albumin, transferrin and alpha-globulins. Lipo-proteins were not found in the milk which attests to the selectivity of this gland. Also, the heavier molecular weight fraction of alpha globulins were not transferred.

Dixon, et al. (1961) have shown large amounts of immunoglobulin are transferred from serum to colostrum in procine due to a concentrating mechanism in alveolar cells of the mammary gland. Immunoflourescent studies of the udder showed a few specifically staining cells.

Pierce and Feinstein (1965), Tomasi et al. (1969) have shown IgG_1 "fast moving fraction" predominates in bovine colostrum. It is distinct from the 11S secretory IgA reported in rabbits (Cebra and Small, 1964) and humans (Tourville et al., 1969) and in mice (Asofsky and Hylton, 1968).

Mach, Pahud and Isliker (1969) report finding IgA with "secretory piece" in bovine colostrum and salina. Free "S" piece was demonstrated in bovine colostrum and milk but not in serum.

A study to detect porcine colostral antibody to Escherichia coli 08 and Escherichia coli 0141 was conducted by Parker (1960). He was unable to detect IgA in sow serum or post-colostral piglet serum; IgM was found to be the main component with activity in the sow serum or post-colostral piglet serum; IgM was found to be the main component with activity in the sow serum. On the other hand, the agglutinating activity found in colostral samples was lowered when treated with rabbit anti-porcine IgA antibody. The conclusion was made the IgA does not participate in passive immunity of the piglet because it was not found in the serum. Sephadex fractionation, hexose assay (10%) and immunochemical determinations indicate the immunoglobulin found by Mach and co-workers (1969) may be IgA. These workers postulated that synthesis of "T piece" takes place in secretory epithelial cells and IgA synthesis in localized plasmocytes.

Asofsky and Small (1967) incorporated radio-labelled amino acids in "in vitro" studies and indicated "T piece" is synthesized in the rabbit mammary glands.

These studies strongly support the earlier proposal by Tomasi et al. (1958) that "T piece" is synthesized by a cell clone distinct from the IgA clone, moreover, it has been suggested both may be stimulated effectively by local antigenation.

A hypothetical model for the transport of IgA and "T piece" across mucous membranes has been postulated by Tourville, Adler, Bienenstock and Tomasi (1969), Figure 1.

The structure for secretory IgA has been proposed by Cebra (1969), Figure 2.

Secretory IgA is not effected in the usual manner by the proteolytic enzymes, papain or pepsin, and is more refractive to intestinal proteolytic activities than IgG and IgM. This indicates a protective role for "T piece" which may increase the effective half-life of these antibodies in the intestine or other sites on the mucous membrane, (Tomasi, Calvanico, 1968). "T piece" will not bind to IgG or IgM. There are no known antigenic similarities between "T piece" and light or heavy chains determinants. There is evidence, (Brandtzaeg, 1969) that "T piece" obtained by reductive treatment of secretory IgA has antigenic determinants that are not recognized by antisera raised to "T piece" stimulated by the 11S secretory IgA molecule.

Complement fixation does not occur because secretory IgA is unable to activate C_1. Recent studies by Vaerman and Heremans (1969) have shown that scialic acid removed by neuaminidase from the secretory IgA molecule, does not allow complement activation. In the presence of lysozyme and complement (Adinolfi, 1966) bacteriolytic

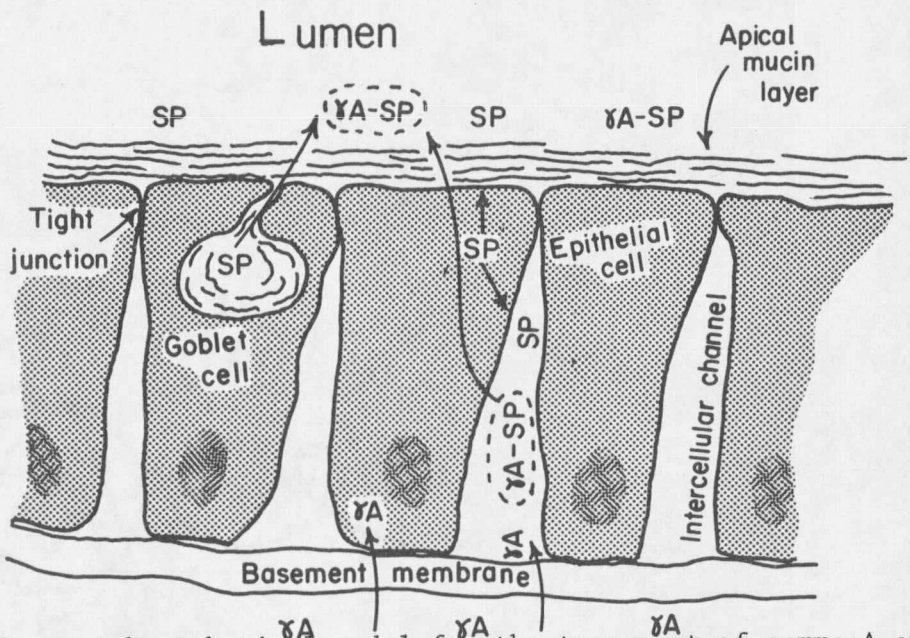

Figure 1. A hypothetical model for the transport of gamma A and SP across mucosal membrane epithelium. (From D. Tourville, R. Adler, J. Bienenstock and T.B. Tomasi, J.E.M. 129, 2, 1969.)

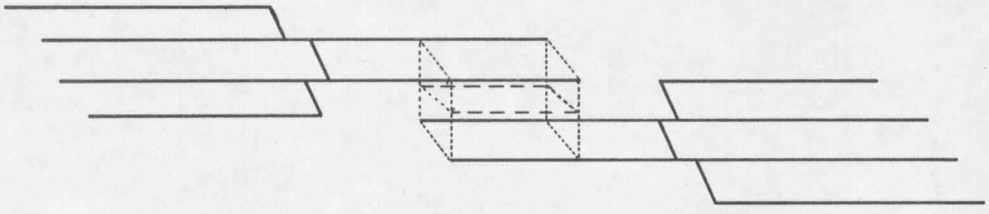

Figure 2. Proposed model of the colostral IgA. T chain is represented by a line of long dashes. (As adapted from John J. Cebra, Bact. Reviews Vol. 33, Number 2, June 1969.)

reactions with Escherichia coli have been demonstrated. This activity has not been observed in other systems nor confirmed by other workers.

Although the exact mechanism of "T piece" has not been fully elucidated and 11S secretory IgA was not specifically assayed for in many of the afore mentioned studies it is evident that secretory IgA should not be considered in humoral immunity but primarily in local intestinal immunity. "T piece" performs three basic functions in the intestine; (1) attachment and absorptive discrimination at the epithelial membrane level, (2) immobilization against flushing actions in the gut and (3) to protect the immunoglobulin from proteolytic enzymes in the intestine.

Introduction to the Thesis

Secretory IgA has been studied in rabbits by Cebra, Robbins, Small, Feinstein and others, in man by Patton and Pigman, Tomasi, Zeigleman et al., Lehner et al., Crabbe, Heremans, Gelzayd, Rosen et al., Brandtzaeg, Jeffries, Chordirker and others and in mice by Mandel and Asofsky and others. It is possible that IgA has been studied or discovered in other species but not encountered during this literature search.

The existence of secretory IgA and its relevance in localized immunity and in passive intestinal immunity in neonates makes it a

pertinent study both of economic as well as academic importance. Realizing further, that large animals and humans are not amenable for intensive research, the mouse becomes the model of choice. Conventional reared (CR) and germfree (GF) mice were selected for this study, especially since IgA has not been (unequivocably) demonstrated in the germfree mouse, hence, it serves a useful control system.

There are obvious shortcomings in interpolating and extrapolating data from one mammalian system into another, hence, this study is undertaken to "demonstrate the classes of immunoglobulins synthesized in CR and GF mice stimulated by a well defined antigen." Emphasis will be directed toward the examination of the secretory immune system and to relate findings to the studies of other mammalian systems.

The "modus operandi" of experiments dealing with secretory IgA greatly influences the findings. Thus, Genco and Taubmann (1969) elicited a secretory IgA response by locally antigenizing rabbit mammary glands while Ogra et al. (1960) demonstrated a significant difference in secretory IgA titres if human subjects were antigenized with live polio virus rather than dead virus; the route of injection and nature of the antigen were deemed to be of signal importance. Panse and Dutta (1964) observed no protection of neonatal rabbits if

the mother had been antigenized with formalinized or inactivated vaccine even though maternal serum titres indicated a good response. Significant protection was conferred if the mother was antigenized with live vibrio vaccine. Panse et al. (1969) demonstrated a similar phenomenon with parenteral administration of passive antibodies from serum of antigenized females. Intramammary instillation of myxovirus and adenovirus elicited neutralizing antibodies in goats (immunoglobulins not speciated) (Guerin, Mitchell and Pasieka, 1969). In a similar experiment propagation of Herpes simplex failed when installed in goat mammary gland in 2 of 3 test animals, therefore, a significant titre was not elicited.

Studies concerning the effect of the serum concentration of IgA circulating or infused, on the secretory IgA concentration in secretory substances are not conclusive. The data available is limited and does not conclusively indicate a positive effect.

To relegate secretory immunoglobulins to the IgA class is understandable, especially with the finding of "T piece". In mammals which have not shown secretory IgA in saliva, colostrum and intestinal washings, a different local immune mechanism requires attentions or, perhaps a more intense search for "T piece" utilizing more sensitive methods and greater concern for the techniques employed for assaying IgA activity. Especially important is the antigenation protocol which

is obviated in the introductory remarks. Localized (juxtaposed to the mammary glands) antigenation should elicit colostral IgA in the conventional and germfree mouse. IgA production in some murine strains displays disparity under various environmental conditions, i.e., the absence of IgA in the serum and colostrum of germfree mice in contrast to the presence of IgA in the serum and colostrum of conventionally reared, (Asofsky and Hylton, 1968). The germfree mice in this study produced a protein material that coprecipitated with IgA and had a sedimentation coefficient of 4S. There was good evidence this material was "T" piece since, in the presence of IgA myeloma protein and radio-labelled amino acids which were added to a tissue culture of germfree mammary gland, and 11S radio-labelled protein was formed, (Murine species not specified).

Four immunoglobulin species, IgG_1, IgG_2, IgM and IgA have been shown to increase in the serum and/or colostrum of many strains of mice following antigenation with a variety of antigens. Their absolute role in passive immunity and origin in the serum of the neonate is strongly debated. Thus, these studies sought to identify specific immunoglobulin species in the dam and neonates using immunization procedures, serologic assays, and, animals reared under antigen free environments in order to trace the origin and activity of passively acquired immunoglobulins in neonates.

MATERIALS AND METHODS

Animals:

Randomly bred conventionally reared (CR) and germfree (GF) Swiss Manor mice, obtained in 1964 from Manor Farms (Staatsburg, New York) were used throughout the study. The CR mice were maintained on Purina Laboratory Chow and water ad libitum with occasional feedings of Quaker Rolled Oats. The GF mice were housed in Trexler germfree units, fed sterile Purina Laboratory Chow #5010C and sterile water ad libitum with occasional feedings of sterile Quaker Rolled Oats.

The germfree techniques used in the study were those adapted in previous work in gnotobiotic systems (Reed, 1966). The germfree feed was dried for 30 minutes, autoclaved for 35 minutes at 120°C and 18 pounds pressure, then dried again for 30 minutes. The sterilized feeds were cultured in Thioglycollate broth and Brain Heart Liver broth to insure sterility before commitment to the germfree environment. Fecal samples from germfree mice were cultured at monthly intervals.

Antigen Preparation:

Dinitrophenylated bovine gamma globulin (DNP-BGG) was prepared according to the technique described by Eisen (1964). The sodium salt of 2,4-dinitrobenzenesulfonate (2,4-DNBS) was crystallized by

adding 7.5 gms of 2,4-DNBS to 500 ml of ethyl alcohol (95%). This solution was heated to 70°C in a double boiler and maintained at this temperature until the material was dissolved. One and five tenths grams of Norit powder was then added and the solution was stirred for two minutes.

Norit and impurities were removed from suspension by passing through a double layer of Whatman #3 filter paper in a D-3 Buchner funnel. Suction was applied to aid filtration flow. The filtrate was refrigerated to hasten crystal formation. The crystals were again dissolved in hot alcohol (95%) and the procedure repeated. Finally, crystals were formed in a large evaporating dish and then stored in a dark bottle until used in the substitution procedure.

To 50 ml of distilled water was added 500 mg of the sodium salt of DNBS (crystallized), 500 mg of bovine gamma globulin (BGG) (Fraction II, Armour Laboratories, Kankakee, Illinois) and 500 mg anhydrous potassium carbonate. The solution was stirred for 24 hours, then dialyzed against 16 changes of distilled water over the next 8 days. The dialysate was kept refrigerated throughout the dialysis. All of the glassware was covered with aluminum foil to protect the light sensitive 2,4-DNP.

To determine the substituting efficiency of the procedure, the dialysate was diluted 1:100 with distilled water and the optical

density measured at 360 nanometers (nm) and 278 nm in a Perkin-Elmer model 124 Spectrophotometer. The Molar Extinction Coefficient for 2,4-DNP used in all calculations was 17,400 and the $E^{1\%}_{278}$ for BGG was 14.8 (as determined in our laboratory). Calculations of the amount of haptencarrier synthesized indicated that 65 residues of DNP reacted with each BGG molecule. Hence, the antigen used in this study is referred to as DNP_{65}-BGG.

Preparation of antisera and colostrum:

Sixteen female CR Swiss Manor mice were injected intradermally (I.D.) in 2 sites; (1) the midline between the most posterior mammary glands and (2) the midline between the second most anterior mammary glands.

The antigen injected consisted of 230 µg. DNP_{65}-BGG emulsified as a saline solution in an equal volume of Freunds complete adjuvent (CFA). Prior to injection the DNP_{65}-BGG was passed through a 0.45 µ millipore membrane contained in a Swinney adapter. The antigen was handled asceptically throughout the study. Control animals received 0.2 ml of sterile saline in CFA administered in the same fashion. Ten days later, eight experimental animals received a second injection of the test antigen by the intradermal route. All mice were mated ten days after the last injection.