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
Montana State University

Abstract:
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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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by

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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

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.

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Hemolysin and hemmaglutinin titres were performed on sera and some of the colostral samples. A modification of the radialimmunodiffusion technique of Masseyeff and Zisswiller was employed to measure the four immunoglobulins, IgA, IgM, IgG_1 and IgG_2, and the specific antibody concentrations in colostrum and serum.

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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

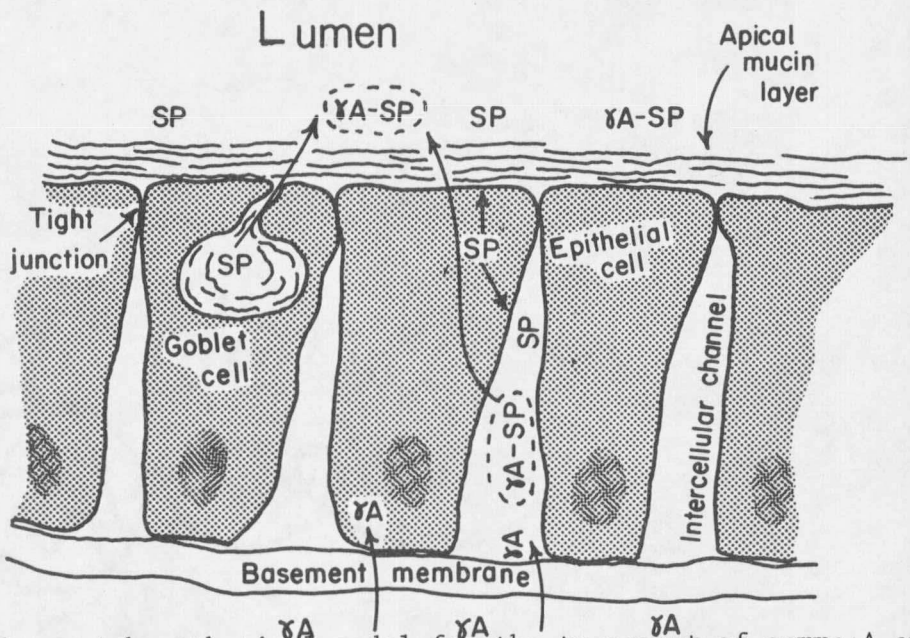


Figure 1. A hypothetical model for the transport of gamma A and SP across mucosal membrane epithelium. (From D. Tourville, R. Adler, J. Bienenstock and T.B. Tomasi, J.E.M. 129, 2, 1969.)

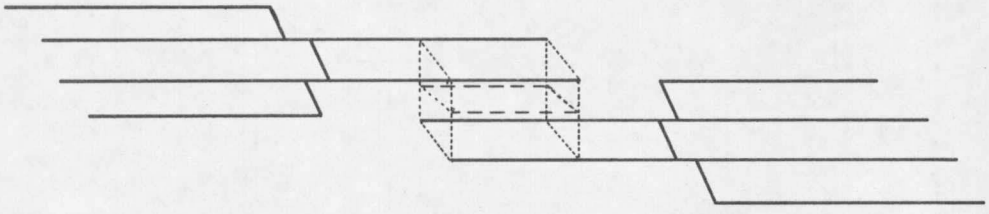


Figure 2. Proposed model of the colostral IgA. T chain is represented by a line of long dashes. (As adapted from John J. Cebra, Bact. Reviews Vol. 33, Number 2, June 1969.)

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.

At parturition and at 5 days post-partum the females were bled from the retro-orbital plexus and at the same time, one half of the litter was processed in the following manner: the neonates were bled via thoracic incision, the blood collected in non-heparinized hematocrit tubes (Clay-Adams) and the serum harvested after centrifugation in a Clay-Adams microhematocrit centrifuge. The sera were stored in the frozen state until assayed.

Colostrum was rinsed from extirpated neonatal stomachs within 24 hours of birth and mature milk was harvested at 5 days post-partum. The stomachs were rinsed with a spray of sterile water to remove residual blood before being opened along the greated curvature. The colostrum or milk of one neonate was expressed through the incision and suspended in 1 ml sterile water. The suspension was washed back and forth in a 5 ml syringe until a smooth suspension was obtained. The addition of 0.1 M HCl precipitated the casein at pH 4.8 which was subsequently cleared by centrifugation in a refrigerated International HR-2 centrifuge at a speed of 10,000 rpm and a temperature of 5°C.

The clear fluid between the upper lipid layer and the sedimented material was carefully removed and the pH adjusted to 7.2 with 0.1 M KOH. Clarified colostrum or milk was kept frozen until assayed. The pH of the colostrum at the time of collection ranged between 6.7 and 6.9. At the time of assay the colostral samples were concentrated

against dialysis tubing filled with CARBOWAX (Fisher) to a final volume of 0.2-0.3 ml.

Germfree animals were given a single injection of 260 µg of DNP_{65}-BGG in CFA and mated 10 days later. Gravid females were removed from the germfree environment zero to five days pre-partum and maintained in sterile isolets (Carworth Farms). At partuition one-half of the litter was removed and processed. The females were not bled on day zero in order to reduce the possibility of contaminating the remaining littermates. Control animals CR and GF were injected with a saline-adjuvant suspension in a manner similar to the DNP_{65}-BGG adjuvant antigenation procedure. Residual adjuvant at the injection sites was observed post-mortem in all mice.

Tissue specimens:

Adult females were bled from the retro-orbital plexus and then sacrificed by cervical dislocation five days post-partum. The skin was opened ventrally by a Y shaped incision and the skin flaps were reflected using blunt dissection.

Numbering the four pairs of mammary glands, cephalid to caudal, the right fourth and the left second mammary glands were excised, and fixed in 10% neutralized formalin. A 4 cm portion of the ileum, 5 cm proximal to the cecum, was excised and fixed in neutralized formalin.

The tissues were sectioned at 5-7 microns on an International Cryostat at 20°C and floated on a slide with a few drops of cold absolute ethyl alcohol. The slides were stored at 4°C until studied by fluorescent antibody techniques.

Fluorescent studies:

Goat anti-mouse immunoglobulins (anti-G_1, A, G_2,-M) (MELPAR) was conjugated with fluorescein isothiocyanate on celite (Calbiochem.) by preparing the following solution:

Goat anti-mouse IgG, IgG_2, IgA, or IgM	0.5 ml
Carbonate buffer, pH 10.3	0.5 ml
Fluoresceine isothiocyanate	10.0 mg
Distilled water	1.25 ml

The solution was held at 5°C for 24 hours before chromatographing in a 2.0 x 33 cm glass column packed with Sephadex G-50 (Fine) which had been adjusted to pH 7.2 with phosphate buffered saline. The first peak obtained, at the void volume, was used in the study. Five ml of fluorescein-labelled antisera were absorbed with 500 mg of dried mouse liver powder, centrifuged and diluted 1:100 before use. Antisera were stored in the frozen state.

Tissue sections were flooded with the specific antisera and incubated in a moist chamber 4-6 hours at room temperature. The

were rinsed for a total of 8 hours with a change of PBS at four hours and then mounted on 1 x 3 inch slides using Glycerol-PBS (9:1) pH 7.2 and 22 x 40 mm coverslips. The slides were observed for fluorescent cells with a Leitz fluorescent microscope equipped with the following filters: UG-38 heat filter, UG-12 exciter filter and a #530 barrier filter. The incident light source was supplied by an Osram-200 mercury vapor lamp. The number of fluorescing cells (Appendix B) observed in 50 oil immersion fields were recorded.

Quantitative Immunoglobulin Assay: (Equipment, Appendix C)

A recent modification (Masseyeff and Zisswiller, 1969) of the classic Mancini, Carbonara, Heremans (1963) procedure for single radial immunodiffusion was used to quantitate the level of specific immunoglobulins in sera and colostrum.

Ten millimeter discs were cut from 1.0% Special Noble Agar prepared in 0.2 M Vernal Buffer, pH 8.6, and poured 1.5 mm in depth in Hyland immunodiffusion plates. The discs were exposed to a drying environment of 37°C for 30 minutes. Fifty µl of antisera (Melpar, Falls Church, Virginia) diluted 1:50 with Vernol buffer, 0.02 M, pH 8.6, was applied to the discs. After 18 hours at 22-25°C, the antisera had apparently diffused into the discs and were suitable for addition of antigen. A 2 mm well was cut in the center of each disc with a blunted 13 gauge hypodermic needle.

Standard curves (Fig. 3) for each immunoglobulin were prepared using two-fold dilutions of pooled normal mouse sera obtained from retro-orbital bleedings of ten 3-6 month old male and female CR Swiss Manor mice. Serial dilutions were made in 1% BSA and in DNP_{27}-BSA. Five µl of a 1:2 and 1:16 dilution were added to the wells of the discs containing, respectively, anti-IgA and anti-IgM; 5 µl of a 1:32 and 1:64 dilution were added to wells of discs containing anti-IgG_1 and anti-IgG_2. The antigen (mouse serum) was added to the wells with a finely drawn capillary tube connected to an oil filled tygon tubing, 1.75 mm (OD) diameter, attached to an oil filled syringe, adapted to a Beckman Spinco microtitrator. The precipitin reaction progressed for 36-48 hours and the unprecipitated proteins were removed by washing with 2 changes of 5 ml normal saline for 24 hours. The discs were stained with *0.6% Buffalo Black in 7% acetic acid for 20 minutes and then decolorized with copius amounts of 10% acetic acid in methanol.

* The stain was prepared in the following manner:

Buffalo Black NBR	6 gm
Methanol	405 ml
Distilled H_2O	405 ml
Acetic Acid	90 ml

The solution was filtered through 2 layers of gauze and stored at room temperature.

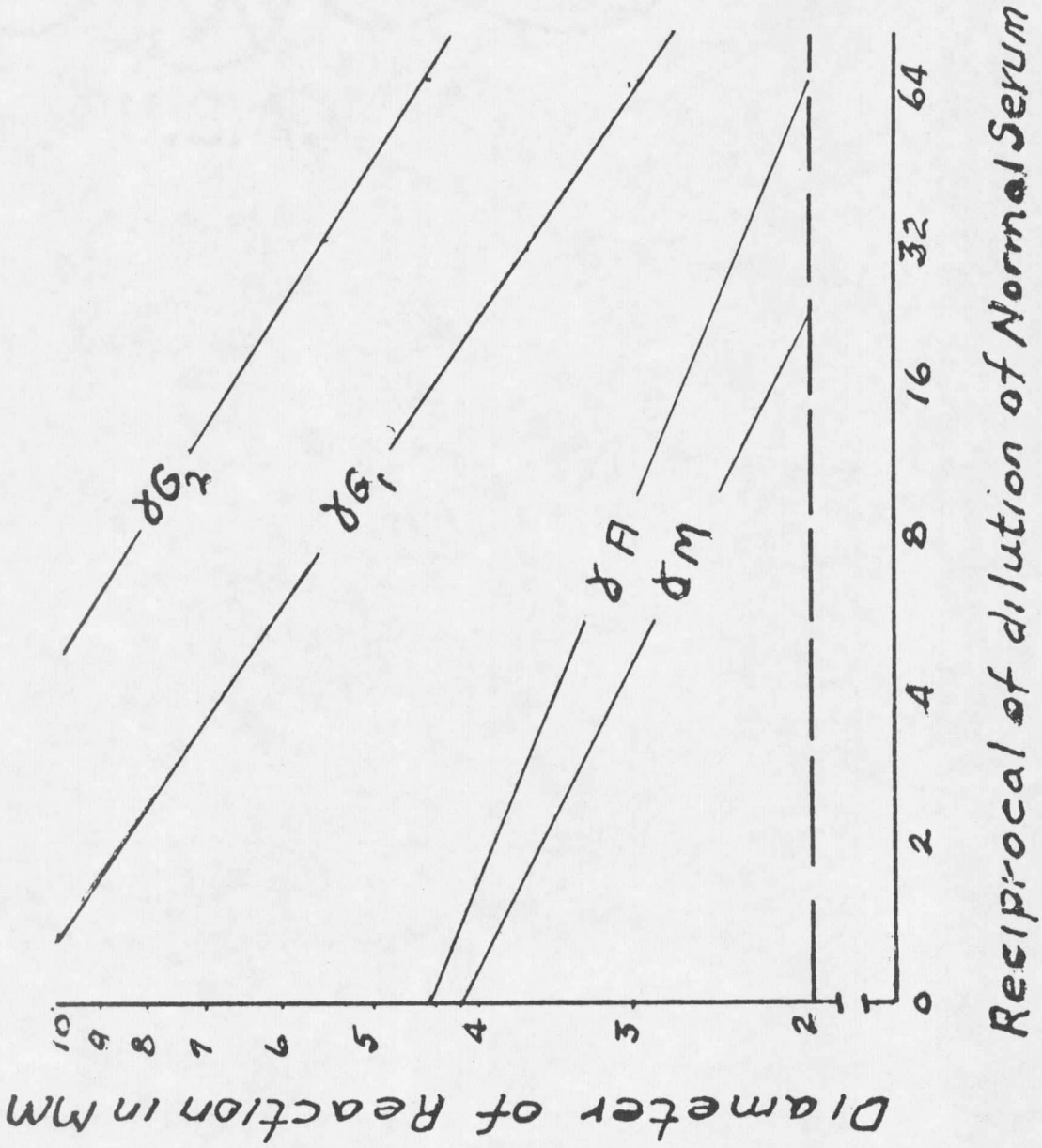


Figure 3 Standard curves developed from radial immunodiffusion reactions with various specific goat anti-mouse immunoglobulin serum and dilution of a pool of normal Swiss serum.

The diameters of the precipitin rings (Appendix C) were measured with a Gaevert measuring microscope. The diameter, in millimeters, was plotted on semi-logrithmic paper as a function of the reciprocal dilution of the antigen.

To determine the quantity of immunoglobulin reacting specifically with the DNP determinant in each preparation another set of antigen (serum or colostrum) which had been absorbed with heat denatured DNP_{27}-BSA were applied to a duplicate set of discs. The difference in the diameter of the radial diffusion pattern between nonabsorbed and absorbed serum of colostrum was established to be antibody specific for the DNP determinant.

The discs, contained in Hyland immunodiffusion plates and sealed with vaseline, were incubated in a moist environment at room temperature for 24 hours. The diameter of the precipitin rings that formed were measured in the same manner as the standard precipitin rings formed by normal mouse serum and were reported as a percent of the normal mouse serum.

Passive Hemagglutination:

A modification of the method of Bullock and Kantor, (1965) was followed using 2,4-dinitrofluorobenzene (DNFB) (Sigma Chemical Company, St. Louis, Missouri). To a solution of 20 ml of ethylenediamintetraacetic acid (EDTA) pH 8.4, plus 0.3 ml of 2% DNFB in

acetone was added 10 ml of 10% suspension of rabbit red blood cells which had been collected in sterile Alsever's solution and washed three times with physiological saline before use.

The hapten-RRBC mixture was incubated for 15 minutes at 37°C. Following incubation, the mixture was diluted in 180 ml EDTA buffer, pH 7.5, containing 1% glucose. The cells were centrifuged at a low speed, approximately 1800 rpm (500G) for 10 minutes. The cells were washed three times with EDTA-1% Dextrose buffer, pH 7.5, and diluted to a final concentration of 10%.

Mouse sera obtained from DNP_{67}-BGG antigenized Swiss Manor Mice (CR) was used to test the hapten-RRBC system in a passive hemagglutination procedure employing a doubling dilution of antisera in 0.1 ml to which was added 0.05 ml of a 2% suspension of haptenated-rabbit red blood cells. The reaction proceeded at 37°C for 3-5 hours after which the tubes were centrifuged for 1 minute and observed for agglutination. Guinea pig complement diluted 1:10 was added to the system and the tubes were returned to the water bath (37°C) for an additional hour. The last tube showing agglutination or hemolysis was considered the end point for determining the titre of hemagglutinating and hemolytic antibodies, respectively.

Mammary gland plaque forming cell assay:

Many modifications of the hemolytic plaque-in-gel technique developed by Jerne and Nordin (1963) are reported. The protocol being developed for the assay of mammary gland-derived plaque forming cells follows methods described by Ingraham and Bussard (1970) and includes materials and methods described by Daniels and Weigle (1968) and demonstrated in our laboratory by Chiller (1970).

Two excised mammary glands were forced through 80-90 mesh stainless steel or brass screen to effect a cellular suspension in Weigle's balanced salt solution (WBSS). Lipids and stroma were separated from the tissue fragments and cells after a 15 minute incubation in an icebath. The cells suspended between the lipid layer and the sedimented stroma were collected and washed three times with WBSS.

Final cell suspensions were adjusted to 10^4 to 10^6 cells per ml and determined by hemocytometer counts.

Mammary gland cell suspensions were mixed with 0.05 ml of a 1:15 suspension of DNP_{27}-BSA-Goat RBC in 0.5 ml of 1% agarose in WBSS. The mixture was evenly distributed over a glass microscope slide previously coated with 0.1% agarose. The slides were incubated 3-4 hours at 37°C in a moist atmosphere. Direct plaques were developed by adding guinea pig complement (absorbed with goat rbc's) diluted 1:10.

Indirect plaques were developed by addition of goat anti-mouse antiserum at a final concentration of 1:100 in complement (1:10) absorbed with goat red blood cells. Plaques were observed with a 40X magnifying glass and indirect illumination.

RESULTS

Immunoglobulin levels in CR dams following a single injection of 230 µgs DNP_{67}-BGG in CFA.

Female mice given a single dose of DNP-BGG were bled 1 and 5 days post-partum for serum assays of immunoglobulin concentration and specific antibody titres. These data are presented in Table I. Data obtained from female experimental animals appears in graphic form in the bar graph in Figure 4 a. Control data are represented in Figure 4 b. The values are averages of day 1 and day 5 post-partum sera levels, however, not included in these values are negative serum levels which may have occurred as a result of technical errors. For example: a negative finding in a dam sera obtained one day post-partum is not included in the computation of the average if a sample from the same animal, taken five days later demonstrated detectable levels of immunoglobulins.

IgA. Two of 5 mice tested 1 day post-partum had concentrations of IgA equal to 171% (average) of control serum values, while 5 of the 7 animals tested 5 days post-partum (Table I) showed IgA levels equal to 117 (average) of serum control levels. Although both (2) 1 day post-partum mice had specific antibody levels which exceeded 15% of the immunoglobulin concentration, the serum of only 1 of 5 showing IgA at day 5 post-partum contained detectable levels of specific

Table I. Immunoglobulin levels at day 1 and day 5 post-partum in dams antigenized with a single injection of DNP_{67}-BGG.

Day from last injection	IgA		IgM		IgG_1		IgG_2	
	Day post-partum		Day post-partum		Day post-partum		Day post-partum	
	1	5	1	5	1	5	1	5
39 [b]	0(0)[a]	0(0)	119(24)	71(2)	100(25)	----	91(9)	200(17)
40	----	76(16)	----	112(10)	----	100(0)	----	133(0)
41	192(31)	132(0)	119(24)	138(34)	----	104(22)	125(25)	90(30)
44	0(0)	0(0)	----	0(0)	----	156(69)	----	119(5)
44	8(0)	133(0)	123(4)	112(12)	107(22)	90(10)	125(25)	168(18)
60	0(0)	123(0)	161(61)	138(41)	91(17)	97(13)	138(18)	105(0)
83	150(15)	125(0)	100(15)	100(25)	60(0)	150(15)	150(15)	173(5)
Mean	171(23)	117(3)	124(25)	112(20)	89(16)	116(25)	126(18)	141(13)

[a] Figures in parenthesis indicate the percent of the immunoglobulin that reacted specifically with DNP.

[b] The immunoglobulin data from one dam is recorded for each time period.

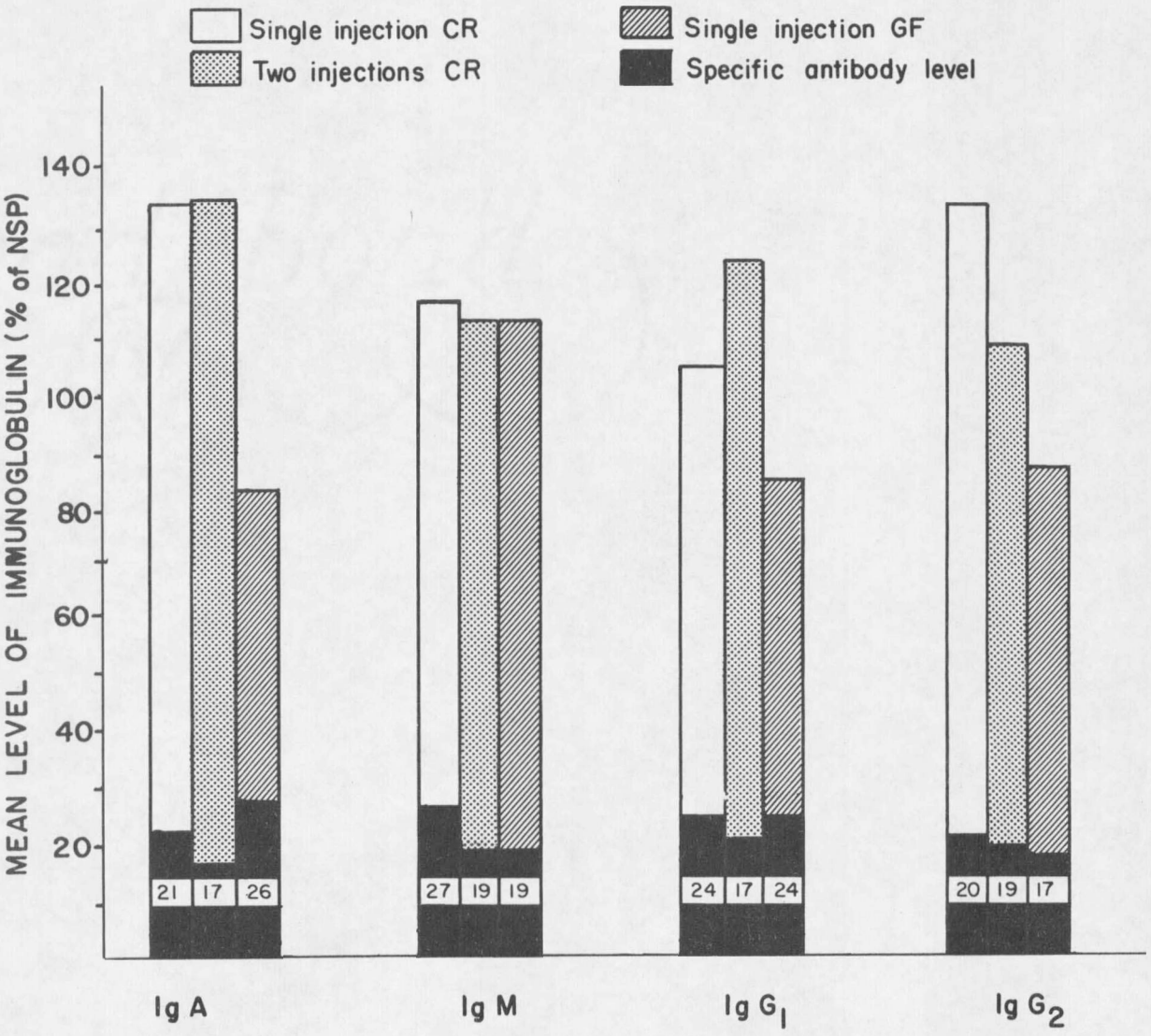


Figure 4 (a) Levels of immunoglobulin in the serum of female experimental animals.

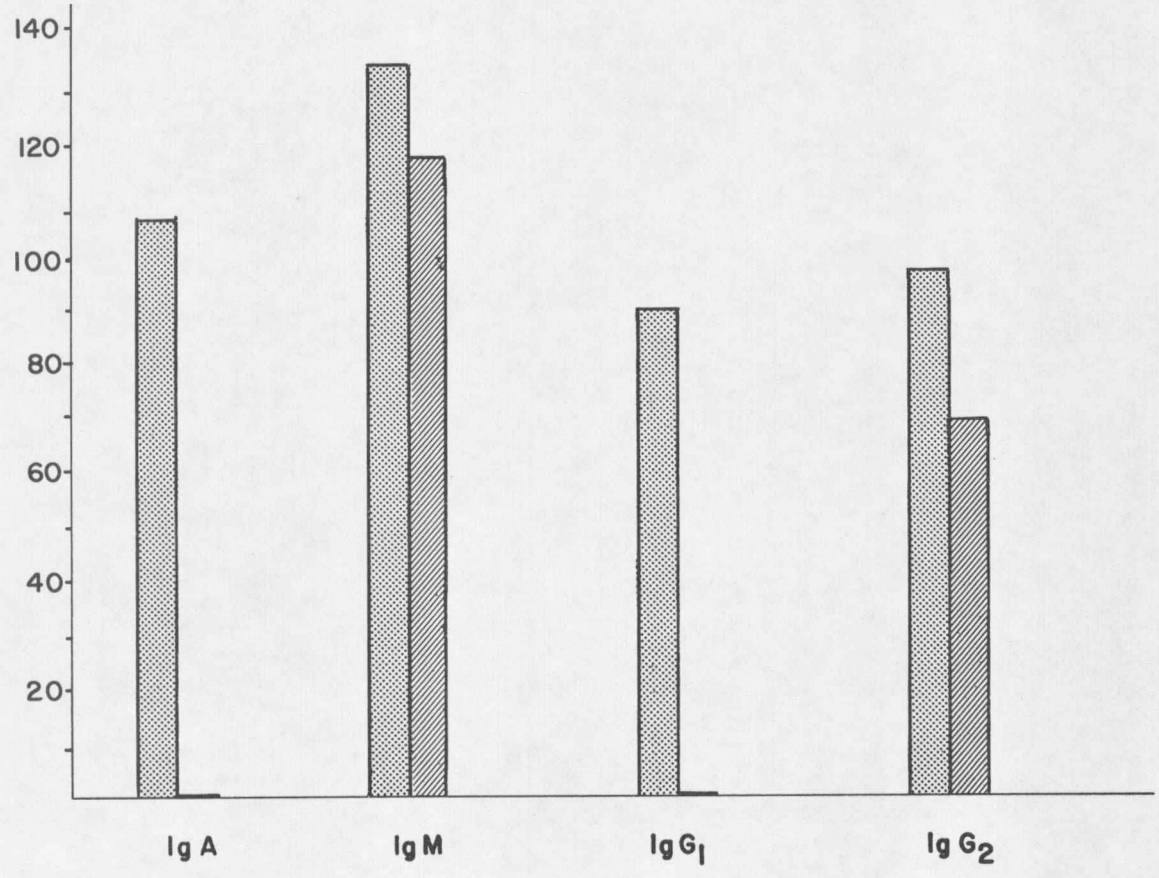


Figure 4 (b) Immunoglobulin levels in control animals.

antibody.

IgM. All of the mice tested for IgM had normal or slightly elevated levels of this immunoglobulin class averaging 104 and 96% on days 1 and 5 respectively. Eight of 11 animals (Table I) demonstrated specific antibody in excess of 12% of the total IgM concentration. There appeared to be no decline in the levels of specific antibody over an 83 day period.

IgG_1. The IgG_1 level was normal in a group of 9 of the 10 mice tested. Specific antibody levels were 10 and 71% (average) of total IgG_1 in dam serum 1 and 5 days post-partum and persisted at high levels throughout the 83 day study.

IgG_2. Serum IgG_2 levels of the 12 experimental animals were, on the average, slightly higher than in the control sera. Eight of the 12 dams had developed specific antibody titres of 10% or more of the total IgG_2 immunoglobulin levels. A decline in anti-DNP antibody was not evident at 83 days post immunization.

Immunoglobulin levels in neonates born to dams given a single injection of DNP_{67}-BGG.

Data obtained from serum of neonates appears in graphic form in Figures 5, 6 and 7. Specific antibody levels (average) and levels of immunoglobulins (average) found in neonatal serum at day 1 and day 5 post-partum are shown.

IgA. Serum levels of IgA failed to reflect the serum IgA concentration of the dams (Table II). Thus, 2 of the 7 neonates had partially adult levels, one of which possessed specific antibody 5 days post-partum. These data suggest that IgA does not contribute to circulating serum antibody levels or, if it does, only in minimal amounts. The specific antibody in the serum of 5 day old neonates born of females vaccinated 41 days previously may have been transmitted from sites of synthesis in the mammary gland and to the neonate via lacteal secretions since IgA in the serum in the dam were undetectable.

IgM. It was observed that whereas the dams showed high IgM levels and specific antibody titres the neonates exhibited low levels of IgM in serum of 6 of the 7 animals tested at 1 day post-partum. This probably indicates poor placental transmission of IgM to the neonates. Since 3 of 7 neonatal sera had adult levels of IgM with specific antibody at day 5 post-partum (55% of adult levels with 11%

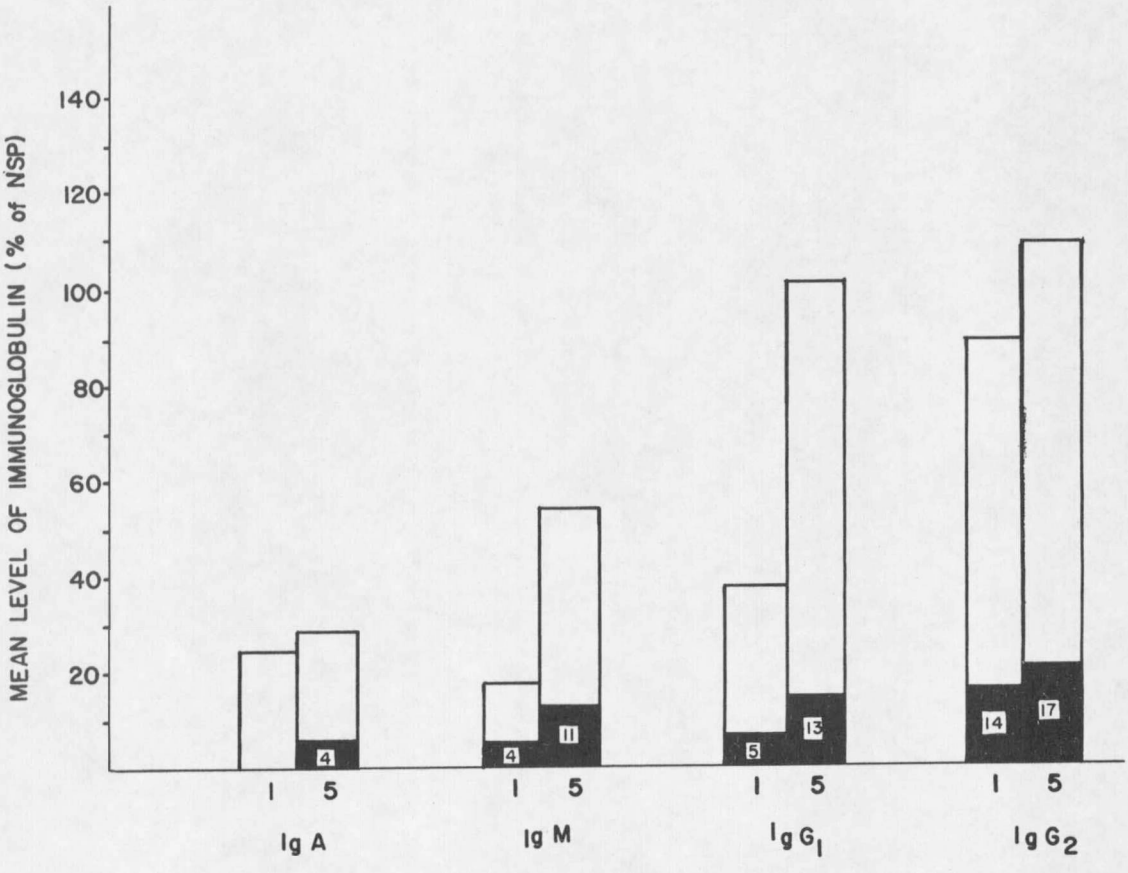


Figure 5 Immunoglobulin levels in the serum of CR neonates (Dams - single injection).

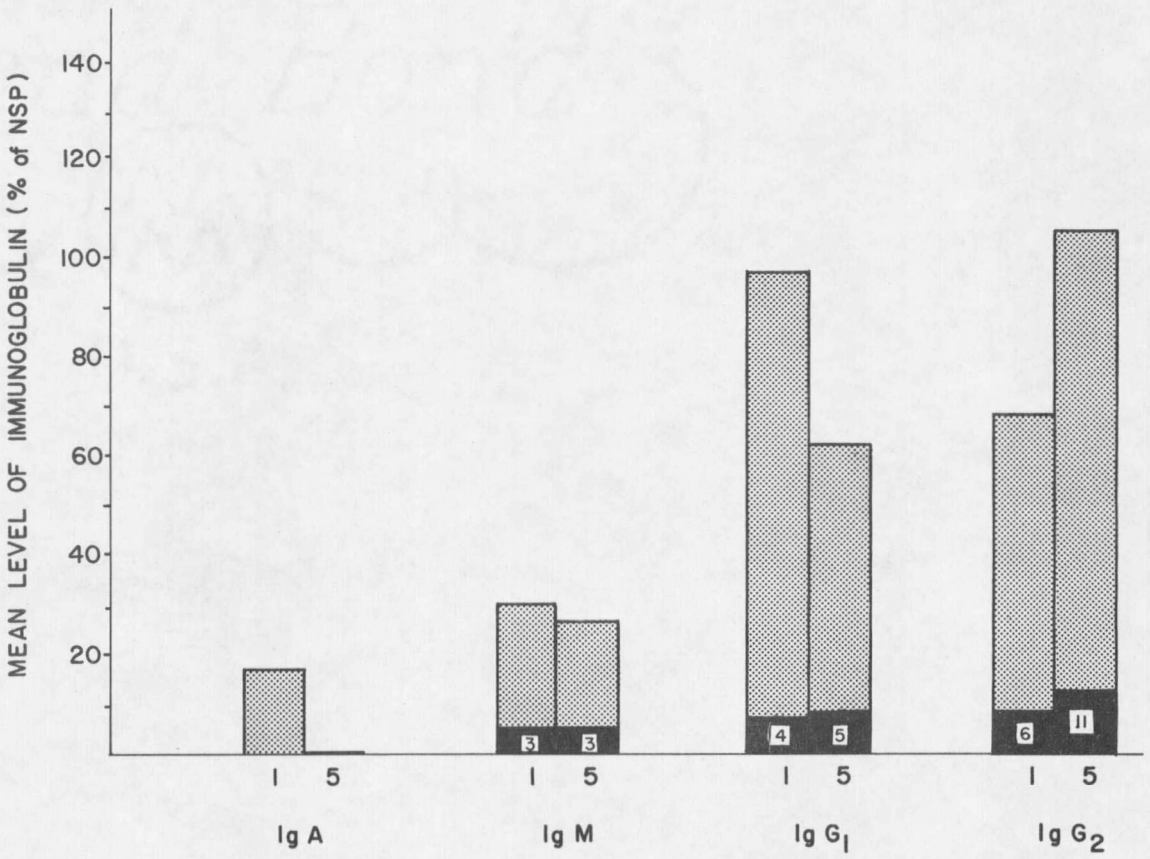


Figure 6 Immunoglobulin levels in the serum of CR neonates (Dams - two injections).

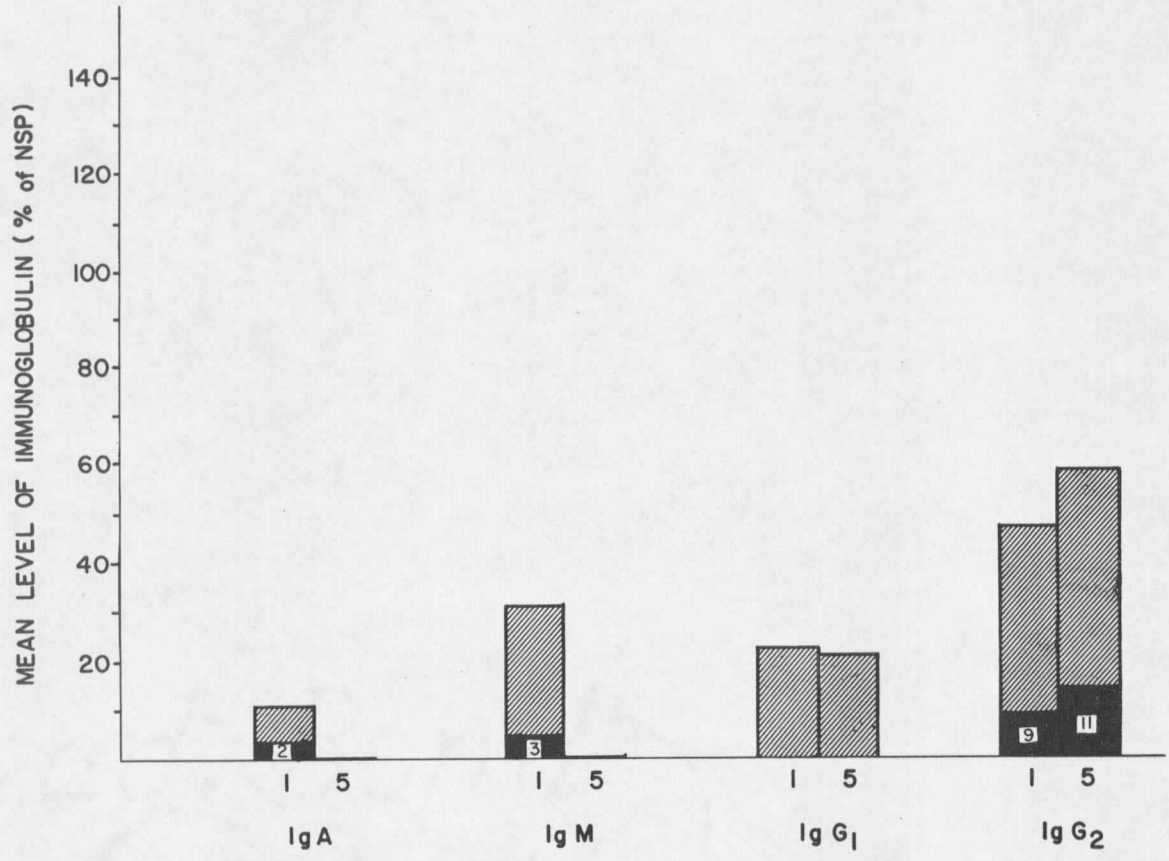


Figure 7 Immunoglobulin levels in the serum of GF neonates (Dams - single injection).

Table II. Immunoglobulin levels in the serum of neonates born to dams antigenized with a single injection of DNP_{67}-BGG.

Day from last injection	IgA		IgM		IgG_1		IgG_2	
	Day post-partum		Day post-partum		Day post-partum		Day post-partum	
	1	5	1	5	1	5	1	5
39 [a]	0(0) [b]	0(0)	0(0)	0(0)	100(13)	161(33)	91(9)	190(30)
40	----	0(0)	----	132(30)	----	0(0)	----	100(20)
41	0(0)	202(29)	0(0)	145(16)	83(0)	106(6)	95(20)	126(4)
44	68(1)	0(0)	0(0)	0(0)	0(0)	147(29)	----	120(0)
44	90(0)	0(0)	0(0)	107(36)	0(0)	----	100(0)	143(43)
60	0(0)	0(0)	111(24)	0(0)	0(0)	116(16)	80(20)	104(2)
83	0(0)	0(0)	0(0)	0(0)	50(0)	83(0)	100(23)	95(20)
Mean	29(1)	28(4)	18(4)	55(11)	40(2)	86(16)	93(14)	111(17)

[a] The immunoglobulin data for each time period was obtained from neonatal pooled sera from one half of the liter. Average litter size is 8.

[b] Figures in parenthesis indicate the percent of the immunoglobulin that reacted specifically with DNP.

specific antibody) maternal IgM was passed in lacteal secretions and in nearly 50% of the cases was transmitted through the intestinal mucosa into the blood stream of the neonate.

$\underline{IgG_1}$. As with IgM, IgG_1 was also transmitted to the neonate primarily through lacteal secretions. There were adult levels of IgG_1 in the sera of all but one neonate at day 5. The IgG_1 fraction from 5 day old neonates also contained specific antibody averaging 13% (0-33%) of the IgG_1 levels in contrast to an average of 2% specific antibody in the serum of 1 day old neonates (Table II).

$\underline{IgG_2}$. The data show that IgG_2 was acquired by the neonate both transplacentally and to come extent through lacteal secretions. All of the neonates studied had adult levels of IgG_2 and specific antibody in the serum at the one-day assay period. Hence, they must have acquired this IgG_1 from the dam. Immunoglobulin IgG_2 levels were relatively unchanged and the specific antibody titres remained stable throughout the study period.

Immunoglobulin levels in the serum of CR females following two injections of 230 µgs DNP_{67}-BGG.

The results of studies on immunoglobulin levels following 2 injections of DNP_{67}-BGG are presented in Tables III and IV.

Table III. Immunoglobulin levels in dams antigenized with two injections of DNP_{67}-BGG.

Day from last injection	IgA		IgM		IgG_1		IgG_2	
	Day post-partum		Day post-partum		Day post-partum		Day post-partum	
	1	5	1	5	1	5	1	5
47 [a]	----	100(0) [b]	----	100(35)	97(0)	89(0)	0(0)	108(8)
60	153(10)	183(16)	125(5)	110(17)	166(32)	228(0)	----	137(0)
80	127(9)	150(40)	90(0)	141(13)	150(10)	74(9)	80(20)	104(17)
115	160(20)	----	105(10)	----	150(20)	----	0(0)	----
145	83(14)	119(12)	122(12)	0(0)	83(14)	----	67(0)	76(16)
150	----	----	90(0)	141(31)	----	87(0)	133(33)	167(0)
Mean	130(15)	138(23)	106(6)	123(24)	129(15)	119(4)	93(26)	118(13)
Non-specifically immune mice.								
54	----	0(0)	----	169(3)	----	90(8)	----	116(0)
61	108(8)	----	97(0)	----	----	----	79(0)	----
99	240(40)	----	112(12)	----	----	----	0(0)	----
Mean	174(24)	0(0)	104(6)	169(3)	----	90(8)	79(0)	116(0)

[a] The immunoglobulin data from one dam is recorded for each time period.

[b] Figures in parenthesis indicate the percent of the immunoglobulin that reacted specifically with DNP.

Table IV. Immunoglobulin levels in the sera of the neonates born to dams antigenized with two injections of DNP_{67}-BGG.

Day from last injection	IgA Day post-partum 1	IgA Day post-partum 5	IgM Day post-partum 1	IgM Day post-partum 5	IgG_1 Day post-partum 1	IgG_1 Day post-partum 5	IgG_2 Day post-partum 1	IgG_2 Day post-partum 5
47 [a]	----	----	----	----	----	----	----	----
60	0(0) [b]	0(0)	0(0)	85(10)	133(0)	0(0)	100(0)	100(0)
80	0(0)	0(0)	0(0)	0(0)	97(0)	89(0)	0(0)	108(8)
115	0(0)	----	0(0)	----	90(15)	----	113(26)	----
145	72(0)	0(0)	122(12)	0(0)	70(2)	95(15)	67(0)	113(26)
Mean	18(0)	----	30(3)	28(3)	97(4)	61(5)	70(6)	107(11)
Non-specifically immune mice.								
54	----	----	----	----	----	----	----	----
61	100(0)	----	83(0)	----	0(0)	----	0(0)	----
99	----	----	----	----	----	----	----	----

[a] The immunoglobulin data from one dam is recorded for each time period.
[b] Figures in parenthesis indicate the percent of the immunoglobulin that reacted specifically with DNP.

IgA. An interesting finding was that IgA levels were higher at 1 and 5 days post-parturition, following 2 antigenations, than those found after a single injection of DNP_{67}-BGG. Moreover, 7 of the 8 female sera assayed demonstrated specific antibody at significant levels, that is, above 8% of the total immunoglobulin concentration.

IgM. All but 7 of the animals developed normal or near normal levels of IgM regardless of the lapse of time since antigenation. More than one half (55%) of these sera contained significant levels of specific antibody over a 150 day period.

IgG_1 and IgG_2. The IgG_1 was found in the sera of all animals titred. Fifty-five percent of the group had levels of specific antibody that exceeded 8% of the IgG_1 concentration. Unexpectedly, the response of IgG_2 was less dramatic than that observed following single antigenation. Specific antibody titres were not as predominant in the IgG_2 immunoglobulin species (Table III) which is in contrast to the titres following a single exposure to the antigen (Table I).

Immunoglobulins in neonates born to dams given two injection of DNP_{67}-BGG.

In spite of the elevated levels of IgA in the dams, there was not a comparable elevation of this immunoglobulin in neonatal serum at 1 day post-partum, (Tables III and IV). Moreover, little or no

IgA was detected in neonatal sera 5 days post-partum (Table IV).

IgM. Adult levels of IgM were found in 2 of 7 neonates at 1 day and 5 days post-injection. The specific antibody titres were present at low levels in both neonates.

IgG_1. Three-fourths of the neonates had adult levels of IgG_1 but had low titres of specific antibody. Four of 5 neonates had IgG_1 immunoglobulins in their sera at 1 day post-partum which indicates substantial placental passage of IgG_1 (Table IV). There was very little IgG_1 contributed to the neonatal serum pool via lateal secretion during the next 5 days.

IgG_2. IgG_2 was at adult levels in 6 of 7 of the neonates sera assayed at 1 day and 5 day post-partum. While 42% of these sera demonstrated specific antibody there was no apparent increase of IgG_2 after ingestion of colostrum or mature milk. Hence, as with animals receiving single antigenations sufficient IgG_2 was acquired passively by placental transmission.

Passive antibody acquired through lacteal and placental transmission of both IgG_1 and IgG_2 specific antibody appeared to be related to the respective concentration of IgG_1 and IgG_2 found in the dam.

Immunoglobulin levels in germfree mice after receiving a single antigenation of DNP_{67}-BGG.

Germfree mice given a single dose of sterile DNP_{67}-BGG were bled 1 and 5 days post-partum for total and specific immunoglobulin determinations. The data from these studies are presented in Tables V and VI.

IgA. Ten sera from female germfree mice were assayed for IgA immunoglobulin. Six of ten contained immunoglobulin levels (40 and 83% respectively at 1 and 5 days post-partum) similar to their conventional counterparts (Tables I and V). Specific antibody was detected in 3 of the 10 sera tested. None of the control animals displayed a IgA levels at day one or day 5 post-partum.

IgM. The levels of IgM in serum of most immune GF mice were comparable to that in immune CR mice. Four of the 14 animals displayed IgM specific antibody but most titres, on the average (8%), were lower than in the sera of CR mice. Surprisingly, control mice (GF) had normal serum levels of IgM.

IgG_1. Five of 8 experimental animals had concentrations of IgG_1 similar to immune CR mice. Also, the sera of 3 of the responding mice contained specific antibody.

Table V. Immunoglobulin levels in germfree mice antigenized with a single injection of DNP_{67}-BGG.

Day from last injection	IgA		IgM		IgG_1		IgG_2	
	Day post-partum		Day post-partum		Day post-partum		Day post-partum	
	1	5	1	5	1	5	1	5
34 [a]	0(0) [b]	----	106(4)	----	50(0)	----	90(0)	----
47	86(9)	----	120(20)	----	0(0)	----	54(0)	----
54	64(0)	----	143(24)	----	80(16)	----	100(0)	----
58	----	----	100(0)	----	107(7)	----	137(2)	----
77	58(0)	166(56)	166(0)	86(0)	75(0)	75(12)	91(25)	73(0)
78	75(13)	0(0)	0(0)	----	51(0)	117(55)	65(2)	60(10)
82	0(0)	----	0(0)	----	61(0)	116(0)	82(2)	100(0)
90	0(0)	----	71(4)	----	----	----	114(0)	----
106	50(0)	----	114(14)	----	104(14)	100(0)	100(0)	----
Mean	66(11)	166(56)	117(29)	81(0)	75(8)	102(20)	98(23)	77(3)
Non-specifically immune mice.								
33	0(0)	----	105(0)	----	0(0)	----	72(0)	----
92	0(0)	----	107(0)	----	----	----	0(0)	----
106	0(0)	----	142(2)	----	0(0)	----	67(0)	----

Table V. Continued.

Day from last injection	IgA		IgM		IgG_1		IgG_2	
	Day post-partum		Day post-partum		Day post-partum		Day post-partum	
	1	5	1	5	1	5	1	5
Mean	0(0)	----	118(1)	----	0(0)	----	70(0)	----

a/ The immunoglobulin data from one dam was recorded for each time period.

b/ Figures in parenthesis indicate the percent of the immunoglobulin that reacted specifically with the DNP.

Table VI. Immunoglobulin levels in serum of neonates born to germfree females antigenized with a single injection of DNP_{67}-BGG.

Day from last injection	IgA		IgM		IgG_1		IgG_2	
	Day post-partum		Day post-partum		Day post-partum		Day post-partum	
	1	5	1	5	1	5	1	5
34	0(0)	----	0(0)	----	0(0)	----	0(0)	----
47	0(0)	----	0(0)	----	0(0)	----	125(36)	----
58	100(18)	----	117(17)	----	----	----	----	----
77	0(0)	----	0(0)	0(0)	75(0)	67(0)	71(8)	88(28)
78	0(0)	----	0(0)	----	0(0)	----	0(0)	0(0)
82	0(0)	----	132(7)	----	112(0)	----	60(0)	----
90	0(0)	----	0(0)	----	0(0)	----	127(37)	----
98	0(0)	----	0(0)	0(0)	0(0)	0(0)	73(0)	90(5)
106	0(0)	0(0)	----	----	0(0)	----	0(0)	----
Mean	11(2)	----	31(3)	0(0)	23(0)	22(0)	48(9)	59(11)
33	0(0)	----	0(0)	----	0(0)	----	0(0)	----
92	0(0)	----	0(0)	----	0(0)	----	25(0)	----

a/ The immunoglobulin data for each time period was obtained from neonatal pooled sera from one half of the litter. Average litter size was 8.

b/ Figures in parenthesis indicate the percent of the immunoglobulin that reacted specifically with DNP.

IgG$_2$. IgG$_2$ was present in all mice, including the control animals, at levels slightly below those obtained in normal conventionally reared pooled sera. Specific antibody appeared in 2 animals at levels equal to or exceeding 8% of the serum concentrations. One control animal did not display a measurable quantity of IgG$_2$ while the remaining 2 had moderate levels of this immunoglobulin species.

Immunoglobulin levels in the neonates born to GF dams given a single injection of DNP$_{67}$-BGG with CFA.

IgA. One of 8 neonates (Table VI) had IgA as well as specific antibody in detectable quantities in the serum. The neonatal sera with IgA was obtained from young born 58 days following antigenation of the dam. Sera of 2 neonatal control litters were without detectable quantities of IgA immunoglobulin.

IgM. Only 2 of 8 neonatal groups displayed IgM or specific antibody at 1 day post-partum. Specific antibody present at 1 day averaged 2% of the total IgM. None of the control sera displayed IgM.

IgG$_1$. Three of 8 neonatal sera had IgG$_1$ in nearly normal concentrations. The 1 neonatal group which was assayed at day 1 and at day 5 post-partum for IgG$_1$ had near adult levels at day 1, but showed an 11% decrease during the following 4 days. There was no specific antibody detected in the IgG$_1$ class in these neonatal sera.

$\underline{IgG_2}$. Four of the 8 experimental neonatal groups demonstrated IgG_2 equal to moderate levels in adult levels (average 48%) compared to an average level of 58% in the nonimmune GF control groups. Four sera contained specific antibody greater than 8%. Nearly a 4-fold increase was observed in the specific antibody concentration in one group of neonatal sera. This increase occurred in the group born 77 days after the dam had been antigenized (Table VI). One of 3 control sera had adult (90%) levels of IgG_2 while specific antibodies were not detectable in the sera of nonimmune GF neonates.

IgG_2 was the predominate class of immunoglobulins in germfree neonatal serum. Also, specific antibody was found most frequently in IgG_2 (Figure 10) class and at higher levels than in IgA, IgM, or IgG_1 classes of immunoglobulin.

A synopsis of the specific antibody response (Figures 8, 9 and 10).

These figures illustrate the number of animals that responded to the hapten as well as the number of neonatal sera that contained specific antibody to DNP. Reacting the sera with heat-treated DNP_{27}-BSA was presumed to decrease the concentration of the immunoglobulin proportion 1 to the concentration of specific antibody. Since antibody avidity will influence the results of precipitin assays, the concentration of specific antibody determined is a relative measurement and should not be misinterpreted as an absolute value.

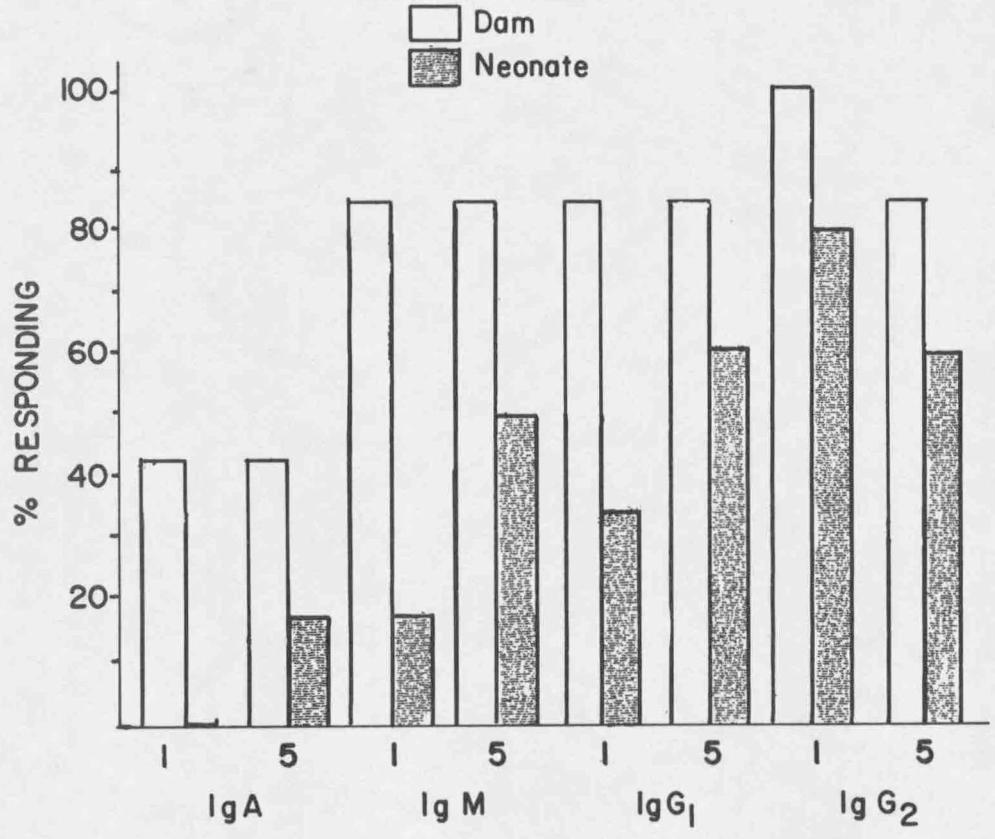


Figure 8 Frequency of specific antibody in the sera of CR female and neonates following a single injection of DNP_{67}-BGG.

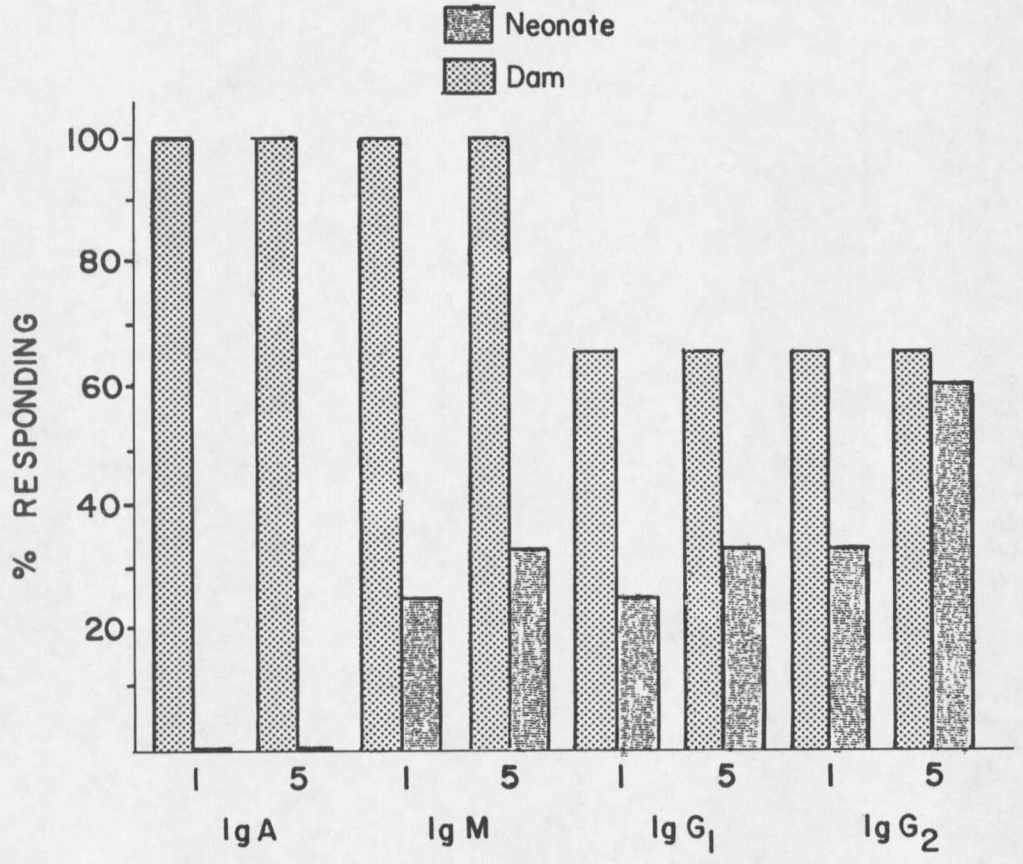


Figure 9 Frequency of specific antibody in the sera of CR females and neonates following two injections of DNP_{67}-BGG.

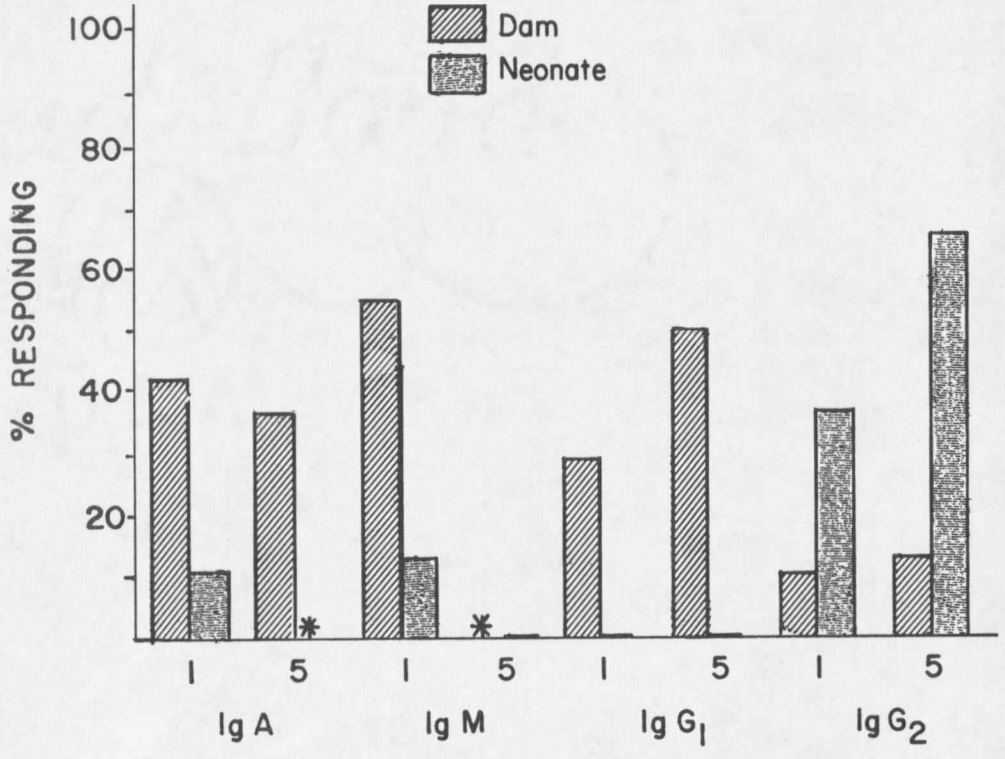


Figure 10 Frequency of specific antibody in the sera of GF females and neonates following a single injection of DNP_{67}-BGG.

As seen in Figure 8 more animals responded to the haptenic antigen with antibody production in classes IgM, IgG_1 and IgG_2 species. Many neonates acquired maternal antibody to DNP via placental transmission which was manifested by high levels of specific antibody at day 1. The specifically immune IgM and IgG_1 were found in few neonates at day 1 but at day 5 nearly half of the animals possess specific antibody. This can be interpreted as passive transfer of immunoglobulin via the colostrum of the dam.

Interestingly, the number of dam sera with specifically immune IgG_2 decreased at day 5 which is in accord with the observations of other workers. Following 2 injections of antigen there was a heightened response on the IgA and IgM fractions but a depressed response in IgG_1 and IgG_2 as the second dose seemed to suppress these immunoglobulins. Again, IgA was not transmitted to the offspring. IgM was transmitted weakly via placental and lacteal routes; IgG_1 was transmitted strongly via the lacteal route and IgG_2 strongly via placental route. The predominant immunoglobulin in the GF dam sera was IgM, followed by IgG_1 and then IgG_2. Lacteal secretions were responsible in part for donating IgG_1 to neonatal circulation. IgA and IgG_2 were infrequently transferred placentally which was indicated by a low incidence of these species at day 1 post-partum.

Passive hemagglutination titrations.(Table VII)

A total of 32 serum specimens from immunized dams and neonates were tested for hemagglutinating or hemolytic antibody activity. Both adult (female) and neonatal sera were tested. Of the 32 specimens analyzed, 12 gave positive reactions, all of which were sera from the dams. There were no titres found in the neonatal serum of in the few colostral samples tested.

Three of 7 females from the experimental group that had received 2 antigenations demonstrated hemagglutination titres ranging from 1:8 to 1:32. A questionable hemolysin titre was observed in only 1 of the sera.

One out of 5 sera from females receiving a single antigenation demonstrated a positive reaction. Hemagglutinating and hemolytic titres were 1:128 and 1:16 respectively.

Of particular interest was the response to the hapten elicited by the germfree females. Eight of the 11 sera tested gave strong reactions with the PHA technique. The titres were higher (range 1:128 to 1:512) in the sera obtained from females sacrificed less than 55 days post-injection. The titres of sera following this period were lower (1:4 to 1:64) which included specimens from 55 to 106 days post-injection. Hemolysin titres were of low magnitude and were found in serum collected after 77 days.

Table VII. Hemmagglutinating and hemolysin titres [a/] in serum obtained from females injected one or two times intradermally with DNP_{67}-BGG.

Day from last injection	Code number [b/]	Hemagglutinatin titre	Hemolysin titre
60	1A-5	1:8	----
69	1D-5	1:32	(1:8?)
82	1E-1	1:32	----
60	2H-5	1:128	1:16
33	GFB-5	1:512	----
47	GFC-1	1:128	----
54	GFD-1	1:128	----
77	GFH-1	1:64	1:8
78	GFJ-1	1:4	1:4
82	GFK-1	1:64	----
90	GFL-1	1:4	1:8
106	GFQ-1	1:64	1:8

[a/] Nine neonatal sera were assayed and none of the sera demonstrated detectable activity therefore they are not included in the table.

[b/] Definition of code: The number preceding the letter indicates the number of injections given to the dam, 1 = 2 injections. The letter indicates the sequence in which the animal was processed. The last number indicates the day post-partum.

Detection of immunoglobulin species in the colostrum.

Specific antibody levels in colostrum ranged from 0-2% of the total immunoglobulin concentrations. This is below the limits of error, at these levels, hence the specific antibody concentrations are not included in the tables.

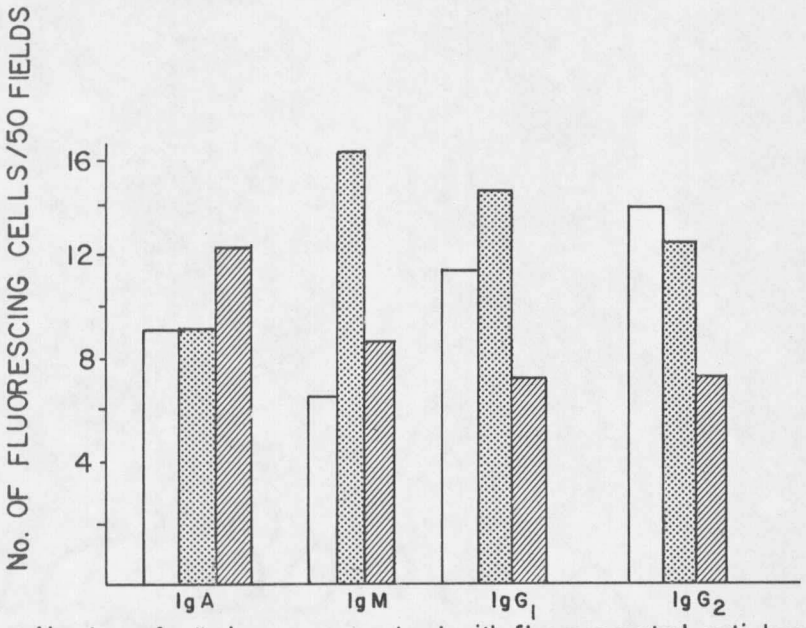
High levels of IgA (average 42%) were found in 3 of the 7 colostral samples from stomachs of neonates born to dams given a single dose of DNP_{67}-BGG. Of significance, IgM was not detected in the colostral samples of this group. IgG_1 was present at moderately high levels (average 25%) in 6 of the colostral samples. IgG_2 was also found in moderate levels but in only 4 of the 7 colostral samples assayed.

After 2 injections the colostral specimens from neonatal stomachs demonstrated high levels of IgA, averaging 45%, and with a high (83%) incidence. Again, IgM was not detected in any colostral samples. IgG_1 was present in 1 of 6 samples at an average level of 23%. IgG_2 was not as high as IgA but was detected with nearly the same frequency (66%). The IgA and IgG_1 levels correlate well with the serum levels in the dams (Table III). The frequency of IgG_2 in colostrum relates well to the increasing frequency of IgG_2 in neonatal serums from day 1 to day 5 post-partum (Figure 10).

Colostral specimens from neonates born to germfree females following a single injection displayed comparable levels of IgA (average 45%) but at a lesser frequency (37%) than those seen in the conventional reared counterparts. Again, the singular feature of these studies was a lack of detectable levels of IgM.

The concentration of IgG_1 was somewhat lower (20%) than levels in the CR counterparts and was found in only 37% of the specimens. This compares to an average level of 25% and a frequency of 85% in the conventional group. IgG_2 levels in colostrum in the germfree group averaged 24% of normal serum concentrations and were found in only 2 of the 6 neonatal groups examined. The occurrence of IgG_2 in colostrum and the moderately high levels reflect the noted incidence and decreasing concentration found in the sera of the dam (Table VI).

The correlation between the numbers of cells in mammary glands of dams associated with immunoglobulins with the levels of the immunoglobulins measured in colostrum from neonatal stomachs is presented in Figure 11 (a bar graph derived from data shown in Tables VIII and IX). A direct relationship is apparent for IgG_1 and IgG_2 and to a lesser extent to the IgA concentration.



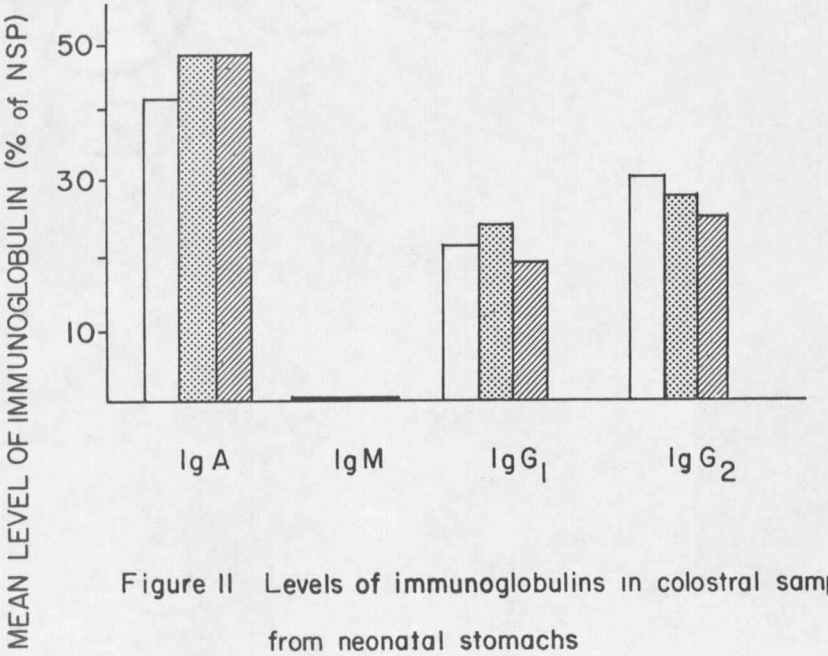


Figure II Levels of immunoglobulins in colostral samples from neonatal stomachs

Table VIII. The number of cells per 50 microscopic fields (oil immersion) exhibiting fluorescence in mammary tissue sections treated with fluorescent anti-heavy chain sera (IgA, IgM, IgG_1, IgG_2).

Days from last injection	IgA	IgM	IgG_1	IgG_2
(a) CR females that received a single antigenation.				
39	18 [a/]	5	17	12
40	3	3	5	18
41	9	5	14	17
44	4	8	19	9
60	12	8	10	3
83	13	8	4	20
Mean value	9	6	11	13
(b) CR females that received two injections.				
47	17	4	12	13
80	0	12	0	18
115	16	8	24	14
145	4	40	5	4
Mean value	9	16	13	12
Non-specifically immune mice.				
54	16	25	33	14
61	9	10	56	14
Mean	12	17	44	14
(c) GF females that received a single injection of DNP.				
34	16	13	14	19
47	2	9	4	4
54	4	0	25	13
58	1	0	1	0
77	nt	nt	nt	nt

Table VIII. Continued.

Days from last injection	IgA	IgM	IgG_1	IgG_2
78	4	2	3	0
87	60	20	2	11
99	1	0	0	0
106	4	0	3	0
Mean value	12	8	6	6
Negative control [b]	7	3	5	2
Experimental control [c]	6	0	3	0

[a] The figures represent the total number of cells/two mammary glands.

[b] Negative control animal was a non-injected animal from the GF unit.

[c] Experimental control animal had been injected with saline and CFA.

nt = no tissue specimen

Note: Table VII (b) - Both control animals cannabilized a portion of their litters.

Table IX. Immunoglobulin levels in colostrum harvested from stomachs of neonates.

Day from last injection	Code number [a]	Concentration expressed as % of normal serum pool			
		IgA	IgM	IgG_1	IgG_2
(a)	Immunoglobulin levels in colostrum harvested from stomachs of neonates born to CR dams given a single dose of DNP_{67}-BGG.				
39	2B-1	0	0	22	0
39	2B-5	39	0	17	0
40	2C-5	43	0	25	31
44	2E-5	0	0	0	31
44	2F-1	43	0	17	25
44	2F-5	0	0	28	28
60	2H-5	0	0	19	0
(b)	Immunoglobulin levels in colostrum harvested from stomachs of neonates born to CR dams given two injections of DNP_{67}-BGG.				
59	1E-1	41	0	23	19
60	1A-1	43	0	0	22
60	1A-5	43	0	0	24
80	1D-1	41	0	0	28
115	1E-1	57	0	0	0
	Non-specifically immune mice.				
61	1C-5	0	0	0	0
(c)	Immunoglobulin levels in colostrum harvested from stomachs of neonates born to germfree dams given a single antigenation with DNP_{67}-BGG.				
33	GF2B-1	42	0	0	0
33	GF2B-5	0	0	22	25
44	GF2D-1	43	0	0	0
58	GF2E-1	0	0	0	0
77	GF2H-1	0	0	0	0

Table IX... Continued.

Day from last injection	Code number	Concentration expressed as % of normal serum pool			
		IgA	IgM	IgG_1	IgG_2
78	GF2I-1	51	0	19	0
92	GF2L-1	0	0	19	23
	Non-specifically immune mice.				
33	GF2A-1	0	0	0	0

a/ Definition of code: The number preceding the letter indicates the number of injections given to the dam, 1 = 2 injections. The letter indicates the sequence in which the animal was processed. The last number indicates the day post-partum.

DISCUSSION

The results of these studies indicate that the immunoglobulin levels in sera of the dams were influenced by the antigenic regimens used in the various experimental groups. In CR mice injected with a single dose of DNP_{67}-BGG, IgA and IgG_2 were found in higher concentration than IgM or IgG_1. On the other hand, higher specific antibody levels were predominantly found in the IgM and IgG_1 classes o.. than in IgA and IgG_2 immunoglobulins. The levels of all four immunoglobulin classes were in excess of 100% of the normal serum pool and were maintained throughout the 83 day study.

Following two injections in CR females, IgA and IgG_1 immunoglobulins, obtained the highest serum concentrations. There were no appreciable differences observed in specific antibody levels in the four immunoglobulins.

Of particular interest were the noticeable lower levels of three (IgA, IgG_1 and IgG_2) of four immunoglobulins in germfree female sera following a single injection of DNP_{67}-BGG. Finding normal levels of IgM was not surprising since similar levels for this immunoglobulin in the serum of GF animals have been reported in the literature by Asofsky, Hylton and Ikara (1968) and more recently by Nash et al (1969). Specific antibody levels were found to vary within the immunoglobulin classes as evidenced by the predominance of specific antibody in IgA followed by lower levels of specific antibody in

IgG_1, IgG_2, and IgM immunoglobulins. The levels of specific antibody observed in GF females were similar in magnitude to the serum levels of specific antibody seen in the CR females. In fact, the level of specific IgA antibody in GF females was on the average the highest observed in any adult female serum. An interesting finding was the lack of a correlation between the total immunoglobulin concentration and the specific antibody levels in most of the adult female sera.

Neonates born to dams that had received a single injection demonstrated adult levels of IgG_1 at birth. This indicates adequate placental transmission to constitute the neonatal serum with this class of immunoglobulin at birth. There was good evidence that the neonates were able to supplement their levels of circulating IgG_2, from lacteal secretions and, hence, presumably obtained by transplacental passage possessed adult levels by day five post-partum. Although generous quantities of IgM appeared to be acquired lacteally (or perhaps from de novo synthesis in the neonate) the amounts were not sufficient to establish adult serum levels. In general, neonatal acquisition of IgA lagged behind the other three immunoglobulin classes in neonatal serum. The levels of specific antibody and the increases which occurred from day 1 to day 5 post-partum mimic the rise seen in the total immunoglobulin concentrations. The predominant

levels of specific antibodies at both day 1 and day 5 were observed in the IgG_2 class. A hierarchy in placental and lacteal transmission of certain immunoglobulins species was seen. The hierarchy appears to be related to the immunoglobulin class rather than to the concentration of immunoglobulins in sera of the dams. For example, the low IgA concentration and low specific antibody levels in neonatal serum at day 1 and day 5 did not reflect the elevated levels observed in the dam sera.

In the experimental group receiving two injections the hierarchy is further supported by the fact that two immunoglobulin classes passed well placentally, in fact in copious amounts, since only IgG_1 and IgG_2 are present at day 1 and day 5 post-partum. Also, very little IgG_1 was passaged lacteally, especially specific IgG_1 antibody. This is in contrast to IgG_2 which was acquired from lacteal secretions during post-partum feedings. After five days of feeding, IgA did not appear in the sera of the neonates even though the dams had high IgA levels in their sera. Curiously, IgA serum levels in the dams were noticeably higher than the other immunoglobulins in this and other experimental groups. Although the hierarchy for lacteal transmission of immunoglobulins to neonatal sera is indicated it was not as clearly defined in this experimental group.

Of particular interest was the low levels of three of the four immunoglobulin classes in GF neonates. Not unlike the CR counterparts, IgG_2 was the predominant immunoglobulin at day 1. Levels of IgG_2 were increased following feedings during the 5 day post-partum period. Of single importance was a complete lack of specific IgG_1 antibody in neonatal serum though the dams had responded well to antigenation and had normal levels of IgG_1 were observed in serums of the dam. There was a conspicuous lack of IgA and IgM in the sera of the GF neonates at day 1 post-partum neither of which increased with lacteal ingestion.

Levels of immunoglobulins were measured in colostrum obtained from the stomachs of the neonates. Interestingly, there was very little difference in the concentration of the various immunoglobulin classes from one neonatal group to the other. The average values for the various immunoglobulins in colostrum were as follows: IgA 46%, IgM not detected, IgG_1 23%, IgG_2 27%. It is apparent from this data that IgA was the predominate immunoglobulin found in colostrum obtained from the stomachs of the neonates. Except for the lack of IgM in colostrum the levels of the other immunoglobulins were in general related to the levels found in the sera of the dams. It is probable that the lack of IgM could be attributed to the procedure. IgM may have been bound with lipid material during the processing

steps and would have been removed from the colostrum with the lipid fraction. A similar finding, a lack of IgM in colostrum, has been reported by Stechschulte and Austin (1970) in the colostrum of rats. The procedures they employed in processing colostrum were very similar to those used in this study.

In GF animals the relationship of colostral levels to the levels of immunoglobulins in the sera of the dams was not as direct as that observed in the CR females. Colostral levels of IgG_1 and IgG_2, although not too different in magnitude, were in reverse order of the levels seen in the sera of the dams. These data better correlate with the data obtained from the experimental group receiving two injections than to the CR group that had receive a single antigenation.

The immunoglobulin involved in gut immunity was identified in these studies. When colostral levels of immunoglobulin compare favorably with those found in the serum of the dam but not the neonate the intestinal mucosa and its absorptive capabilities undoubtedly contribute to selective screening and retention of certain immunoglobulins to promote the so called gut immunity. Morris (1965) demonstrated a selective mechanism exists in the intestinal tract of the mouse which was also found in rats (Halliday, 1954, 1957) (Brambell et al 1958). A recent study by Porter (1969) indicated

that precolostral piglet serum was almost entirely dificient of immunoglobulins, however, post-colostral serum concentrations of IgG and IgM were similar to adult levels while IgA generally exceeded adult levels 3-4 times. Therefore, the immunoglobulins passed via colostrum to neonatal sera, and some more readily than others. Thus, a discriminating mechanism has been elucidated in certain rodents and in large animals. Those immunoglobulin classes that are not found in the serum of the neonates may be retained by the intestinal tract for "in situ" protection. Thus, it is misleading to measure the acquired immunity of the neonate and the immunological status of newborns by immunoglobulin assays performed only on neonatal serum. IgA is found very infrequently in serum of newborns as illustrated in these studies even though IgA may be the predominant immunoglobulin in colostrum.

The frequency of the occurrence of specific antibody in neonatal serum does not necessarily relate to the frequency of the specific antibody levels in the serum of the dam, nor is lacteal transmission influenced by the frequency of specific antibody found in dam sera. The immunoglobulin classes most frequently passed placentally as indicated by high incidence of specific antibody at day one in neonatal sera are as follows: IgG_2, IgG_1, then IgM, in neonates born to dams that had received a single injection of antigen; IgG_2, IgG_1

followed by IgM appear at the same frequency in neonates born to dams that had received two injections which is in contrast to the findings in the GF neonates where the order of occurrence was IgG_2, IgM and then IgA followed by a complete lack of IgG_1 specific antibodies. There is a similarity in the occurrence of the IgG_2 class in both groups. The GF neonates differ from the CR counterparts only in that they did not receive IgG_1 specific antibodies with the same frequency at day 1.

The increased frequency of specific antibody from day 1 to day 5 post-partum are compared. In neonates born to dams that received a single injection the most demonstrable increase is seen in the IgM class followed by increasing frequency of IgG_2 then IgA immunoglobulins. In the neonates born to dams injected twice with antigen the greatest increase in frequency was seen in IgG_2 followed by IgG_1 then IgM. GF neonates demonstrated lacteal transfer only in one class of immunoglobulins, the IgG_2 class.

IgG_2 appears to be the specific antibody class passing lacteally with the greatest frequency which is not unlike the frequency patterns seen for placental transmission.

Therefore, the most efficient immunoglobulin for systemic passive immunity of neonatal mice would be IgG_2 followed by IgG_1, whereas, the immunoglobulins promoting local gut immunity would be IgA and IgM.

SUMMARY

Following intradermal injections of DNP-BGG in conventionally reared and germfree Swiss Manor mice, adult serum and neonatal serum as well as colostrum from neonatal stomachs were assayed for DNP-BGG antibody and levels of IgA, IgM, IgG_1 and IgG_2.

After a single antigenation, CR females demonstrated serum immunoglobulin levels in the following descending order of magnitude: IgG_2, IgA, IgM followed by IgG_1. Specific antibodies were predominant in IgM followed by IgG_1, IgA, and IgG_2. In sera of females that had received two antigenations, immunoglobulin concentrations were highest in the IgA fraction followed by IgG_1, IgM and IgG_2 while specific antibody levels were highest in IgM and IgG_1 followed by IgA and IgG_2.

Germfree female sera displayed a higher concentration of IgM immunoglobulin than IgA, IgG_1 or IgG_2. Specific antibody was found at highest levels in IgA with decreasing levels in the following order, IgG_1, IgM and IgG_2.

Placental transfer of IgG_2 and IgG_1 was indicated when near normal levels of these immunoglobulins were found in neonatal serum at day 1 post-partum. Increased levels of IgG_2 and IgG_1 at day 5 post-partum indicated acquisition from lacteal secretions. In only one experimental group, those neonates born to dams that had received a single injection, did the serum levels of IgM increase during the

5 day post-partum suckling period.

Except for IgM there was a direct correlation between the number of cells in mammary gland tissue reacting with fluoresceinated anti-heavy chain sera and the concentration of the immunoglobulins in colostrum.

Assays of colostral samples demonstrated a lack of IgM and a predominance of IgA. Interestingly, IgA did not appear in neonatal serum except in very low concentrations at either day 1 or day 5 post-partum. Therefore, IgA is relegated to "local" gut immunity since it does not readily pass the neonatal intestine.

A hierarchy for the transmission of immunoglobulins, related to the species rather than to serum or colostral immunoglobulin concentrations, was observed. IgA and IgM were poorly transmitted across the placenta while IgA was poorly transmitted across the intestinal mucosa of the neonate. IgG_1 and IgG_2 readily pass through both the placental and the hypothetical mammary gland barriers.

APPENDIX

Appendix A

The diameter of precipitin rings in the modified Masseyeff-Zisswiller technique of immunoradialdiffusion using known anti-heavy chain and pooled normal mouse serum are recorded in the table below. The serum dilutions that gave the maximum reactions are shown in parenthesis. Mean values and standard deviations for the various immunoglobulin measurements were calculated and recorded at the bottom of the table. Figure 3, in the text, demonstrates the linearity of the reaction using serially diluted normal pooled serum and an appropriate single dilution of anti-heavy chain sera.

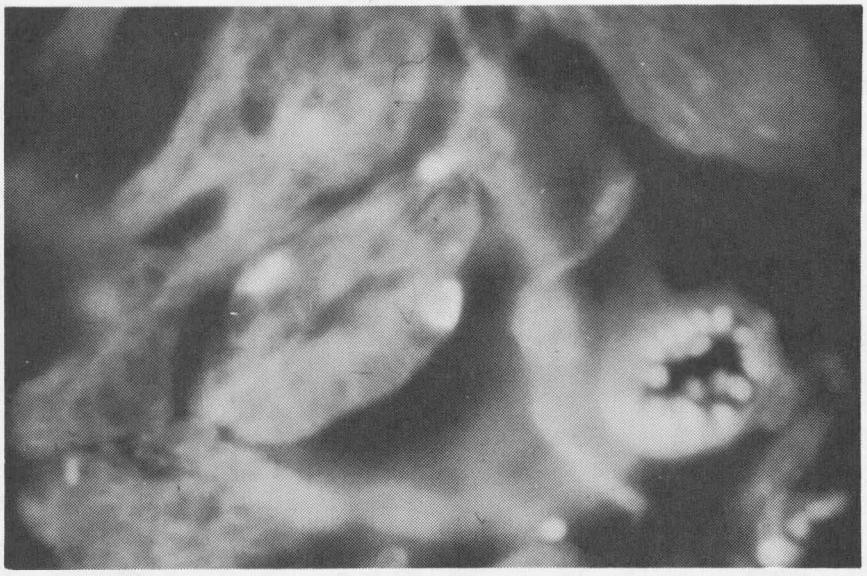
IgA		IgM		IgG_1		IgG_2	
UNAB[a]	ABS[a]	UNAB[a]	ABS[a]	UNAB[b]	ABS[b]	UNAB[b]	ABS[b]
3.9	3.9	3.4	3.3	6.6	6.1	---	4.5
4.0	4.0	3.2	3.2	6.6	6.1	4.5	4.5
3.5	3.5	2.8	2.7	6.0	---	4.0	---
4.0	---	4.0	4.0	3.0	3.4	4.0	4.4
3.2	3.2	4.6	---	6.0	---	---	5.1
4.3	---	3.2	3.2	3.5	4.0	5.0	---
3.9	4.0	3.3	3.1	4.8	---	6.0	6.0
5.5	5.4	4.0	---	5.0	---	4.0	3.6
5.2	4.7	4.2	4.2	4.0	---	5.5	5.2
5.0	4.9	4.6	4.7	---	---	---	---
4.2	4.1	4.2	4.2	4.6	---	4.0	4.2
$\bar{X}$ 4.0	4.2	3.8	3.6	5.0	4.4	4.0	4.6
=0.9	0.6	0.8	0.7	0.5	0.5	0.3	0.6
250=1.8	1.2	1.6	1.4	1.0	1.0	0.9	1.2

UNAB = unabsorbed pooled normal serum
ABS = absorbed pooled normal serum

a/ Final dilution of serum is 1:4.
b/ Final dilution of serum is 1:32.

Note: The antisera were obtained from Mel-Par (Falls Church, Virginia) and diluted in saline in the following manner: Anti-IgA 1/40, Anti-IgM 1/40, Anti-IgG_1 1/50, Anti-IgG_2 1/50.

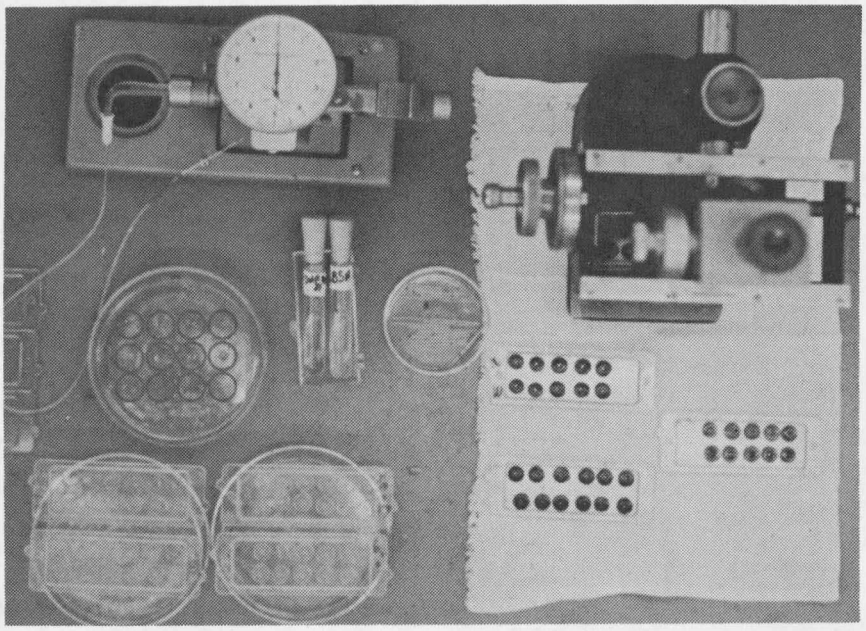
Appendix B



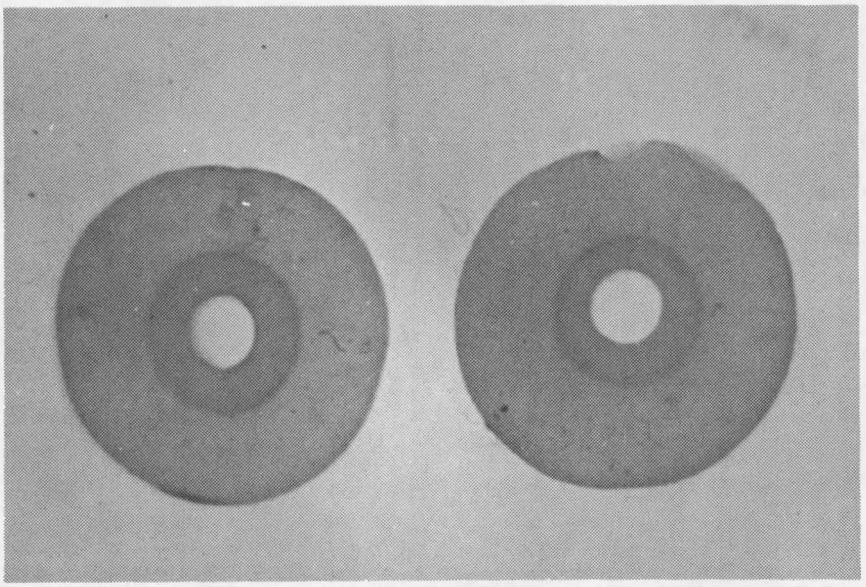
(a) A single fluorescing cell is seen in a tissue section reacted with fluorescinated goat anti-mouse IgA.

(b) A group of fluorescing cells is observed in a tissue section reacted with fluoresceinated goat anti-mouse IgA.

Appendix C



(a) Equipment layout.



(b) Precipitin rings in agar discs.

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