THE RELATIONSHIP OF SERUM IMMUNOGLOBULIN LEVELS WITH AGE, SERUM TOTAL PROTEIN AND LIPEMIA IN THE CANINE NEONATE

by

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INTRODUCTION

The study of the immune system and how it relates to health and disease has become a very important area of study in veterinary medicine. Much of the information available concerning the interrelationship between passive immunity and diseases of neonatal animals is concerned with the bovine, equine and porcine species. Death losses due to inadequate passive immunity or insufficient immune competence of the individual in these species can result in great economic loss to the breeder. Information concerning the level of passively received immunity and early immune competence in the canine is limited.

The purpose of this investigation was to gain information concerning serum immunoglobulin levels and their relationship to age in the puppy. The relationship of serum immunoglobulin levels to other factors such as serum total protein and the error in measurement caused by lipemia was also studied. The relationship of serum total protein and serum hemolysis was investigated.

Carnivores possess an endotheliochorial placenta whereby the canine fetus receives some degree of passive immunity before birth. Serum immunoglobulin levels of pups at birth consist of mainly IgG. The transmission of passive immunity by way of colostrum to the canine neonate is much greater than that transmitted across the placenta. In pups maximal absorption of colostral immunoglobulins occurs approximately 8 hours after birth. No appreciable absorption occurs 24 hours after birth.

The developing canine fetus has been shown to have some level of immune competence. The fetus is able to respond to certain antigens only at a fixed stage of gestation. It has been shown that neonates are able to respond to certain antigens within 2 hours after birth.

REVIEW OF LITERATURE

ORIGINS OF THE IMMUNE SYSTEM

The normal animal when exposed to a foreign antigen responds with the production of immunoglobulins and specific effector cells. Immunoglobulin production is characteristic of the humoral immune system while specific effector cell production is characteristic of the cell mediated immune system.[43]

Mounting an immune response is a function of lymphocytes. Lymphoid stem cells initially arise from the yolk sac in the young fetus and later from the fetal liver. In the near term fetus and adult, the lymphoid stem cells arise from the bone marrow. These lymphoid stem cells are carried in the blood to central lymphoid organs, which are sites of production and differentiation of lymphocytes. The primary lymphoid organs are the thymus, found in mammals and birds, and the bursa of Fabricius, found only in birds. It has been suggested that the bone marrow and/or gut associated lymphoid tissue (GALT) have taken over the functions of the bursa in mammals.[43]

Lymphocytes released by the thymus are called thymus-dependent lymphocytes or T-cells. T-cells make up most of the circulating lymphocytes although some find their way to secondary lymphoid organs. T-cells mediate the cell mediated immune response.[43]

Lymphocytes released by the bursa of Fabricius or bone marrow are called bursa-dependent lymphocytes or B-cells. B-cells also seed the secondary lymphoid organs and are responsible for the humoral immune response.[43]

Immunoglobulins, or antibodies, are proteins produced by plasma cells in response to interaction between antigen-sensitive B-cells and specific antigen. Immunoglobulins are capable of specifically binding to antigen resulting in the enhanced clearance of antigen from the body.[43]

Following initial exposure to an antigen there is a lag period of several days where this is no detectable antibody response. After approximately 1 week, antibody levels start appearing and reach a peak at 10-14 days. This is known as the primary immune response. IgM is the first immunoglobulin class seen. IgG follows and reaches higher levels than IgM. Following the peak period these immunoglobulin levels start to decline rapidly.[43]

After the second exposure to the antigen there is a shorter lag period before a higher and more pronounced antibody response. This is called the secondary immune response. Again IgM is seen first followed quickly by a much greater IgG response.[43]

The structure of the IgG molecule has been extensively investigated and serves as a model for other immunoglobulins. The IgG molecule can be split into 3 equal sized fragments by papain. Two of these fragments correspond to the "arms" of the Y-shaped molecule and possess the ability to bind antigen. These are termed the Fab fragments. The third fragment corresponds to the "tail" of the Y-shaped molecule and cannot bind antigens. This portion of the molecule is called the Fc fragment.[43]

The regions on the immunoglobulin molecule responsible for antigen binding are called variable regions and are found on the N-terminal portion of the Fab fragment. Other regions on the immunoglobulin molecule responsible for such functions as: initiating the complement cascade, binding of immune complexes to phagocytic cells, control of the fractional catabolic

rate of the immunoglobulin molecule, placental transfer, and antibody-mediated cell mediated cytotoxicity are termed constant regions and are found on the C-terminal half of the Fab fragment and throughout the Fc fragment. Constant regions are made up of subunits called C_L, C_H1, C_H2, C_H3. IgM and IgE possess an additional subunit called C_H4.[43]

Immunoglobulins are classified according to their solubility in strong salt solutions, their electrostatic charge, their molecular weight, and their molecular structure. There are four major immunoglobulin classes found in domestic animals: IgG, IgM, IgA and IgE. In addition, man has an immunoglobulin class, IgD.[43]

Immunoglobulin classes are further divided into subclasses based on antigenicity, electrophoretic mobility and biologic activity. Subclasses vary in functional characteristics among the domestic species.[43]

ACQUISITION OF HUMORAL IMMUNITY

Humoral immunity is acquired in 2 ways: passively and actively. Passive immunity includes maternal immunoglobulins passed to the offspring by way of placental transfer and colostral and milk secretions. Passive immunity may also be acquired through the administration of antiserum. Active immunity is acquired when the host mounts an immune response after coming in contact with an antigen it recognizes as foreign. Although the neonate acquires the majority of its humoral immunity from the passive transfer of maternal immunoglobulin, it has been shown that the fetus and newborn are capable of mounting an active humoral response.

Immune Competence in the Fetus and Neonate

There is an apparent inability of the developing fetus to respond to certain antigens until a fixed and critical stage of gestation is reached. This critical age of development appears to be different for each antigen in the given species. Prior to this age, the fetus fails to recognize the substance as foreign and does not respond to it.[39] One reason for the differing immunologic responses to different antigens may be attributed to the fact that the fetuses may have received passive immunity by way of the placenta against some of the antigens tested.

Canine fetuses from the 40th day of gestation to adults produced antibody after stimulation with bacteriophage ØX-174 and responded promptly to challenge. Serum antibody activity increased in proportion to the age of the dog at the time of stimulation.[19]

Slight reactivity to sheep RBC's (SRBC) developed in the canine fetus at around the 48th day of gestation.[19]

A higher dose of bovine gamma globulin (BGG) was required to elicit an immune response in the newborn pup than the dose required by adult dogs.[24] None of the neonates responded significantly to a primary or challenge dose of bovine serum albumin (BSA) although adult dogs produced antibody after the first inoculation and had good responses after challenge. The neonates did, however, respond after a single dose of BSA-adjuvant emulsion.[19]

In the adult dog, the primary immune response to SRBC and BGG was characterized by the production of equal amounts of IgG and IgM. The secondary immune response consisted mainly of IgG.[21] In the newborn pup, the primary immune response to SRBC and BGG was characterized by the production of IgM, whereas the secondary immune response yielded both IgM and IgG, with IgG making up the major portion of the immunoglobulins produced. Although the newborn pup is immunoresponsive at birth, full immunologic maturity is not achieved until the 2nd or 3rd week of life where the primary and secondary immune responses match those seen in adult dogs.[24]

Passive Transfer of Immunity

In animals there are 3 routes of passive transfer of immunity from mother to offspring: 1) absorption of immunoglobulins through the endodermal cells of the yolk sac, 2) absorption of maternal immunoglobulins through the placenta, and 3) postnatal absorption through the intestine.[20,49]

Prenatal Transfer. Absorption through the yolk sac occurs in rabbits, birds and to some extent in rodents. IgG is the predominant immunoglobulin transferred. It has been shown that there are specific receptor sites on the yolk sac endothelium for IgG. These receptor sites recognize the Fc region, more specifically the C_H2 domain of the heavy chain on the IgG molecule.[44,49]

Placental transfer of maternal immunity is the major route of immunoglobulin transfer in primates and occurs during the last 1/2 to 2/3 of gestation.[20] This route of transfer occurs to a much lesser degree in carnivores, such as dogs and cats.[44,49]

Man and monkey possess a hemochorial placenta where the maternal blood bathes the trophoblast directly.[36] In the newborn infant, serum IgG levels are variable sometimes reaching a level twice that found in the mother. IgA and IgM levels in the newborn infant are always extremely low.[48] There appear to be placental receptors for only one class of immunoglobulins in the human: IgG. These receptor sites have a varying affinity for the IgG subclasses. The affinity for the subclass IgG_1 is greatest.[28] Binding to the placental Fc receptor takes place through interaction of the C_H2 and C_H3 regions on the IgG molecule.[28,47]

In 1970, Brambell[4] proposed his hypothesis on how proteins, including immunoglobulins, are transported across the human placenta to the fetus. He stated that proteins are thought to enter the endodermal cell of the placenta non-selectively by endocytosis. Within the vesicles thus formed, proteins which are ultimately transported from the cell attach themselves to receptors lining the vesicles. These receptors may be present on the surface of endodermal cell microvilli before the vesicles are formed. It was thought that such receptors provide protection from digestion by proteolytic enzymes when fusion of vesicles with lysosomes occurs and if the receptors are saturated, any free protein is broken down. The amount of IgG transmitted is dependent upon how well the attachment site on the Fc region fits the receptor. After movement of the phagolysosome through the cell, the attached proteins leave the receptor when the vesicle

contents are discharged from the cell into the intercellular space. In 1974, Gitlin and Gitlin[13] supported Brambell's hypothesis and stated that the rate of immunoglobulin transport is dependent upon the degree of binding to specific receptors.

The absorptive cells of the yolk sac and intestine are morphologically similar to those of the placenta in that they have microvilli on their surfaces and pinocytosis can be demonstrated on the villi.[49]

Carnivores possess an endotheliochorial placenta. In this type of placenta the fetal chorion is in contact with the maternal capillary endothelium.[4,36,43] There is transmission of passive immunity before birth by way of the placenta but the transmission after birth by way of the colostrum is greater.[4,43] Precolostral immunoglobulin content in serum of puppies and kittens consists mainly of IgG.[14,15]

Ungulates have an epitheliochorial placenta where the chorion is separated from the maternal blood by 3 maternal tissues: the maternal cpaillary endothelium, connective tissue and the uterine surface epithelium.[36] Virtually no placental transfer of maternal immunoglobulins occurs in these species, therefore, passive immunity is acquired through the ingestion of colostrum and milk.

In all species except ungulates, materno-fetal and materno-neonatal transfer of IgG is an Fc region-governed specific process. In ungulates, the uptake of immunoglobulins through the intestinal wall is a non-specific process.[44] IgG from species with prenatal transport will bind to the Fc receptor of human placental tissue. This is not so of IgG from animals with no prenatal transfer.[47]

Postnatal Transfer. The transfer of IgG from maternal circulation to the colostrum is a peculiar feature of early lactation in those species

which do not transfer IgG to the fetus through the placenta. Species which are able to transfer IgG to the fetus through the placenta have colostrum containing mostly locally produced secretory IgA.[25]

Although very little absorption of immunoglobulins occurs from the gut of the human infant, colostrum and milk play an important role in protecting the newborn from certain disease conditions. Human mammary secretions contain, along with immunoglobulins, T and B lymphocytes, macrophages and neutrophils that possess phagocytic activity, lysozyme, lactoferrin and certain resistance factors all of which have antimicrobial properties.[15,30] These consitituents of mammary secretion help to prevent infection in the maternal mammary gland as well as in the infant's gastrointestinal tract.[15]

Human mammary secretions contain immunoglobulins of all classes in appreciable amounts although secretory IgA is the predmoniant type.[15,30] It has been shown that small amounts of colostral immunoglobulins are absorbed from the neonatal intestine during the first 18-24 hours after birth, however, the precise mechanism of intestinal absorption is not known.[18,30]

Secretory IgA found in mammary secretions is more resistant to pH changes and proteolytic enzyme digestion than are the other immunoglobulin classes, including serum IgA, found in colostrum and milk. Therefore, it is thought that secretory IgA plays an important role in conferring resistance to infection to the infant's gastrointestinal tract.[15,41] With the decreased incidence of breast feeding, some have reported an increased frequency of colonization and infection caused by gram negative bacteria in the infant's gastrointestinal tract. A rapid improvement of diarrhea caused by Escherichia coli was observed after the feeding of breast milk.[5,15,41] As well as protecting the infant from the hazards of microbial infections, IgA may limit the absorption of dietary antigens, thereby reducing the risk of allergic reactions mediated immunologically.[5]

Rodents, carnivores and ungulates primarily depend on post-natal gut absorption of colostral and milk immunoglobulins for their acquisition of passive immunity.

In rodents, such as mice and rats, postnatal absorption of immunoglobulins occurs in the proximal small intestine[14,21,29,38] for about 2-3 weeks.[2,21,29] During this time, IgG and IgA from the maternal milk bind selectively to specific separate receptors on the villi. Receptor-bound IgG is transferred through the enterocytes into the blood stream. Receptor-bound IgA is not absorbed but remains on the cell surfaces.[29] Binding occurs on the apical surfaces of the villi and is virtually absent in the cryptal regions.[2] Binding is specific for the Fc region of the immunoglobulin.[14,32] The mechanism of transport of IgG from the gut epithelium to the circulation is presumed to be the same as that theorized for the transport of immunoglobulins across the placenta.[4] Since serum IgA and IgM levels are present in the neonatal rat, it is likely that the small amounts of IgA and IgM transported represent a background level of non-specific absorption not involving the immunoglobulin receptor system.[14]

In the distal half of the small intestine, during the first 3 weeks, IgA and IgG are non-specifically absorbed into larger vesicles. Unlike the smaller vesicles found in the proximal small intestine, the function of these large vesicles is to digest the endocytosed immunoglobulins.[29,38]

Precolostral piglet serum is deficient of immunoglobulins, however, traces of serum protein antigenically related to IgG, have been shown.[9,32]

In the sow, colostral immunoglobulin levels exceed those found in maternal serum. Sow serum IgG levels decrease 10-24 days prepartum, are minimal at parturition, and increase postpartum.[10]

All colostral IgG and a high proportion of IgM are transported from maternal serum. Forty per cent of colostral IgA is from the serum. The majority of colostral IgA is in the form of secretory IgA (sIgA) produced locally in the mammary gland. sIgA is characterized by a secretory component attached to the immunoglobulin molecule as it is produced in the secretory gland.[3,37]

IgG is the predominant colostral immunoglobulin. As colostrum turns to milk, IgA becomes the predominant immunoglobulin. IgM makes up a very small percentage of the colostral immunoglobulins and increases slightly in milk.[9] In milk, 90% of IgA and IgM and 70% of IgG is locally produced.[3]

At 24 hours of age, IgG makes up the majority of piglet serum immunoglobulins. IgG then begins to fall slowly reaching a minimum at approximately 38 days. Evidence has been presented indicating that there is selective intestinal absorption of IgA from colostrum and that intestinal absorption in the neonatal piglet seems to differentiate against sIgA.[33] After the first 24 hours, serum IgA concentrations in the piglet serum begin to fall rapidly reaching a minimum in about 20 days. The same phenomenon is seen with IgM with this immunoglobulin reaching a minimum level at approximately 11 days. The decrease in immunoglobulin levels in serum at such an early age is a function of protein catabolism and dilution due to increased body size and therefore increased blood volume.[9] After the piglet serum immunoglobulins bottom out, they begin to slowly rise due to acquired immunoglobulin production.

Calves receive no precolostral immunoglobulins from the cow. At parturition colostral levels of IgG_1, IgA and IgM are higher than those levels found in the maternal serum indicating a selective transfer. However, as colostrum turns to milk, IgG_1 and IgM levels are below those found in serum. Milk IgA has been found to occur in both greater or lesser concentrations than serum IgA.[6,34]

It has been shown that IgG_1 in the cow is selectively transported from the maternal serum into lacteal secretions before and after parturition.[6,8.10,25] Maximum transfer of IgG_1 occurs 1-3 days before calving[10] and is the most predominant immunoglobulin of bovine colostrum.[34] The control of such transport may be regulated by the estrogen and progesterone levels of the cow.[8,40]

While immunoglobulin levels are high in colostrum, they start falling rapidly within 24 hours so that only low levels of immunoglobulins are assayed on the 3rd day.[34] After calving, the selective transfer of serum proteins into the mammary gland decreases while the local synthesis of milk proteins increases.[10] The mammary gland of the cow shows mainly IgG producing cells although IgA and IgM producing cells are present as well.[7,8,50] IgG_1 remains to be the most predominant milk immunoglobulin. At no time does IgA play a dominant secretory role in bovine milk although the levels in early lactation may exceed the levels found in maternal serum suggesting a selective secretory process.[34] As compared to other species, bovine milk contains low levels of immunoglobulins.[7,34,35]

There seems to be a lack of selectivity in the calf's intestinal absorption process. Immunoglobulin profiles of calf serum resemble very closely the immunoglobulin profiles of bovine colostrum.[34] Uptake of colostral protein occurs primarily in the ileum of the calf.[9]

Foals are born with a virtual absence of circulating immunoglobulins. Mare's serum and pre-suckle colostrum has quantitatively and qualitatively similar immunoglobulin composition.[26] After suckling there is a marked decrease in total colostral immunoglobulin values.[20] IgG_2 is the predominant immunoglobulin.[20,26,27]

In the foal, uptake of colostral protein appears to take place throughout the small intestine.[20] Absorption seems to be maximum during the first 24 hours after birth and then declines rapidly until there is no demonstrable absorption at 5 days of age.[26] Immunoglobulin absorption is less selective than that of the rat, however, IgG seems to be absorbed in disproportionately greater amounts than are other immunoglobulin classes.[20,26]

Both total serum immunoglobulin concentrations and specific antibody titers found in foals approximate those found in mare's serum within 24 hours after the foal is born and permitted to suckle.[26] Foal serum immunoglobulins begin to decrease by 16 days of age reaching a minimum value at 1-2 months of age.[20,21,24,25,26] At this time, immunoglobulin synthesis is just beginning. In foals that receive little passive immunity, IgG, IgA, and IgG(T) production begins at 16-24 days of age. It appears that IgM production in these foals begins much earlier since serum IgM levels continued to climb after 3 days of age when the first serum sample was tested.[24,37]

Several days before whelping, the bitch has a sharp decrease in serum IgG with a concurrent decrease in total serum immunoglobulin concentrations.[16] This trend follows that seen in cows and sows which have selective transfer of immunoglobulins from maternal plasma to colostrum prior to parturition.

Canine colostrum is rich in IgG and IgA with IgG being the predominant immunoglobulin. Low levels of IgM and IgE are also present.[43] Colostral IgG is slightly higher in concentration than that found in the bitch's serum. Colostrum IgA is significantly higher than that of dam's serum. Colostral IgM is lower than that of dam's serum.[37,46]

As canine colostrum changes to milk, the concentration of all immunoglobulin classes decreases, with the exception of IgM. IgA becomes the predominant immunoglobulin of milk. Milk IgA values are higher than maternal serum levels throughout lactation. IgG levels are initially higher but with time drop to below maternal serum values. IgM in milk remains relatively constant and is always less than maternal serum levels.[37,46]

The majority of milk IgA is produced in the mammary gland as is milk IgM. These two immunoglobulins, as they appear in colostrum and milk, are designated sIgA and sIgM.[42,46]

In pups, maximum absorption of immunoglobulins occurs when they are ingested approximately 8 hours after birth. Absorption is complete 15 hours after feeding. No detectable immunoglobulin absorption occurs 24 hours after birth.[11]

Closure of the Gut

Closure of the gut to absorption of immunoglobulins is not clearly understood especially since closure occurs at different times in different species. Closure doesn't seem to be due to the cessation of pinocytosis by the intestinal epithelial cell, but seems to be either a failure of intracellular processing or a failure of release from the cell.[20]

Many factors seem to be able to influence closure of the neonatal gut. There is good evidence that certain endocrine secretions can change the permeability of the small intestinal cells after birth.[5] Premature closure occurred in rats after giving certain doses of exogenous corticosteroids.[27,31] Pups from bitches that were treated prepartum with ACTH or hydrocortisone had significantly lesser immunoglobulin absorption than pups from untreated

bitches, suggesting that steroids may influence immunoglobulin absorption in pups.[11] It may be that there is a change of adrenal output in neonates that induces closure.

There seem to be factors present in milk that can induce closure. These factors are heat stable, non-protein and non-fat components of low molecular weight.[20,23]

In piglets, but not in pups or calves, starvation can postpone closure for several hours. Consumption of food may be necessary before closure can occur.[27,31]

NEONATAL PASSIVE IMMUNITY AND SUSCEPTIBILITY TO DISEASE

An individual's immune system is the mechanism whereby one fights off the threat of disease caused by foreign agents. The acquisition of passive immunity is important for protection of the newborn until the newborn's own immune system is mature enough to take over the role of protection. For this reason the mechanisms concerning the acquisition of humoral immunity in the fetus and neonate should be understood. With failure of these mechanisms the neonate is susceptible to various diseases.

In the dog, high levels of antibody to the canine distemper virus is maternal plasma results in placental transfer of anti-distemper antibodies to the offspring.[22] Gillespie et. al.[12] state that approximately 3 per cent of the dam's titer is transferred placentally to the fetus. Thus a dam with a high titer transfers more antibodies in utero. Pups receiving this immunity but not colostrum, become susceptible to challenge at approximately 2 weeks of age.

Although placentally derived antibodies play an important role in the canine neonate, colostrum and milk in various domestic species provide most, if not all, of the passively acquired humoral immunity in the newborn. The colostrum and milk of these animals contain antibacterial and antiviral immunoglobulins.[1,15,17,26]

Pups and kittens deficient in maternal antibodies are susceptible to various viral diseases such as canine distemper, infectious canine hepatitis, herpes virus infection, and feline panleukopenia. The combined placental and colostral transfer of anti-distemper antibodies to the newborn pup results in a neonatal serum titer that averages approximately 77 per cent of the dam's titer. At around 1½ weeks of age the pup's titer is reduced by 50 per cent. At 5-6 weeks of age approximately 50 per cent of the pups

are susceptible to challenge of the virulent virus (their titers have dropped to below 20-30). Pups cannot respond properly to a distemper vaccine until they have lost colostral protection and become susceptible to the disease.[12]

Piglets not receiving colostrum rich in anti-corona virus igA are susceptible to TGE. Calves failing to receive or absorb colostrum are prone to certain bacterial and viral diarrheas with Escherichia coli being an important pathogen. Foals with reduced immunoglobulin levels in serum may develop bacterial septicemias.[45]

MATERIALS AND METHODS

RESEARCH ANIMALS

Four pregnant beagle bitches were obtained from Theracon, Inc., Topeka, Kansas. The bitches were in the last 1-2 weeks of gestation at the time of purchase. They had been vaccinated for distemper, hepatitis, parainfluenze, leptospirosis and rabies several months prior to breeding.

The pregnant bitches were held in individual runs where they were observed through a closed circuit television monitor. As the bitch showed signs of impending parturition she was taken to a separate cage where whelping could be personally observed.

At birth each pup was identified and 2 ml of blood collected from a jugular vein. The pup was then returned to its dam where nursing was observed for the first 24 hours. Nursing time for each pup was recorded. At the end of the first 24 hours of life, 2 ml of blood were again drawn from each pup.

The bitch and her litter of pups were returned to the holding runs 24 hours after parturition where they were maintained for approximately 3 weeks. The bitches were fed a mixture of canned and dry food[a,b] twice a day. The pups were allowed to nurse ad libitum.

At 3 weeks of age the pups, along with their dams, were moved to runs in an area where other research animals were kept. The pups nourishment consisted of bitches milk. The bitches were fed a dry food[c] free choice and were given one multiple vitamin tablet[d] each day.

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[a] Kennel Ration meat flavored canned food. The Quaker Oats Co., Chicago, IL 60654.
[b] Wayne's Dry dog food[R]. Allied Mills, Inc., Chicago, IL 60606.
[c] M-260. Grain Science & Industry, Kansas State University.
[d] Visorbits[R]. Norden, Lincoln, NE 68501.

At four weeks of age the pups were introduced to a mixture of dry puppy food[e] mixed with warm water and were allowed to eat this ad libitum. During this time the pups still were nursing from the bitch.

At 6 weeks of age the pups were weaned and divided into groups of 2 or 3 per run.

From 8 weeks of age until the end of the experiment the pups were maintained on the same dry puppy food. Each pup was weighed periodically and the weights recorded.

At 4 and 6 weeks of age each pup was given a dose of 2.5mg/lb. body wt. pyrantel pamoate for treatment of Ancylostoma and Toxacara species. Each bitch was treated at the same time as the pups.

Each pup was vaccinated at 8, 10 and 12 weeks of age for distemper, hepatitis and parainfluenza.[f] Vaccination was considered necessary since new dogs coming into the research facility could be a source of infection for these diseases.

METHODS

Following the collection of blood samples at birth and at 24 hours of age, 2 ml of blood were drawn from each pup at weekly intervals throughout the experiment. The blood was collected from the jugular vein into individual 4 ml volume glass tubes. These blood samples were allowed to sit at room temperature for approximately 45-60 minutes to permit time for clot retraction to occur. The blood samples were then centrifuged at 500g for 5 minutes. The serum was removed using a Pasteur pipette. The amount of lipemia and/or hemolysis of each serum sample was recorded. Each serum

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[e] Purina Puppy Chow®. Ralston Purina Co., St. Louis, MO 63188.
[f] Nordens Vanguard DA_2P®. Norden, Lincoln, NE 68501.