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Fowl Immunoglobulins: Quantitation in Birds Genetically Resistant and Susceptible to Marek's Disease

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The development of serum immunoglobulins was observed in chicks genetically resistant (N line) and susceptible (P line) to Marek's disease (MD), in conditions free from infection with MD. IgG was present at hatching at about 5.0 mg/ml and decreased to about 0.5 mg/ml by 12 to 15 days. Active production of IgG was apparent at about 20 days of age and reached levels of 2.0 to 3.0 mg/ml by 67 days. Low levels of IgM were present at hatching, and a gradual increase was seen to 1.0 to 2.0 mg/ml by 67 days. IgA was not detectable at hatching; it appeared in N-line birds at 5 days and in P-line birds at 13 days, and by 67 days was about 0.10 to 0.13 mg/ml. After 30 to 40 days, immunoglobulin levels were generally higher in N-line than in P-line birds. However, it was concluded that no primary immunoglobin deficiency existed sufficient to explain the susceptibility of the P-line birds to MD

Marek's disease (MD) is a lymphoproliferative disease of fowl (*Gallus domesticus*), and the causative agent is a cell-associated herpesvirus (5, 21, 27). Fowls have been bred for high levels of susceptibility or resistance to MD (6, 12). However, the mechanism of genetic resistance or susceptibility is unknown. Both genetic types become infected (3, 4, 25), and in vitro studies have shown similar infection rates of cells from resistant and susceptible birds (24, 28). Resistant birds often manifest a better antibody response than susceptible birds (2, 25), but this is probably secondary to the widespread lymphoid tissue damage in the susceptible birds.

A primary immunodeficiency in the susceptible birds is a possible explanation of their inability to survive. It seemed reasonable, therefore, to examine the development of serum immunoglobulins (Ig) in resistant and susceptible birds in the absence of exposure to MD virus as an assessment of primary functions in the humoral immune system.

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MATERIALS AND METHODS

Birds. N-line (MD-resistant) and P-line (MD-susceptible) birds were obtained from the Department of Avian Diseases' pathogen-free flock and reared in conditions free from exposure to MD or other avian pathogens. The origin of the N and P lines has been described (6).

Antisera. Antisera to fowl IgM, IgG, IgA, and albumin were prepared by injection of rabbits with immunoprecipitates obtained by immunoelectrophoresis of semipurified immunoglobulins against rabbit antisera to fowl serum or Na_2SO_4-precipitated immunoglobulins. Rabbit antisera to IgM, IgG, and IgA were made specific to heavy-chain antigens by absorption with glutaraldehyde-cross-linked (1) preparations of hen egg yolk (for absorption of anti-IgM and anti-IgA sera) or the IgG-free first elution peak from Sephadex G-200 gel filtration of fowl serum (for absorption of anti-IgG serum). Specificity of antisera was assessed by microimmunodiffusion analysis (20) against appropriate antigens. Detailed methods will be described elsewhere.

Standard solutions. Purified IgM and IgG were obtained by gel filtration of Na_2SO_4-precipitated serum immunoglobulins and recycling of first and second peaks, respectively; adult serum was used for IgM production, and day-old chick serum was used for IgG production. IgA was purified from bile by ion exchange chromatography on Sephadex diethyaminoethyl A-50, using the buffers described by Leslie and Clem (16), followed by Sephadex G-200 gel filtration of fractions containing IgA. Assessment of purity of standard solutions of immunoglobulins was based on the observation of single precipitin lines in microimmunodiffusion analysis against rabbit antisera to fowl serum and immunoglobulins. Fowl albumin was a commercial preparation (Chicken Albumin, Fraction V Powder, Sigma Chemical Co.).

Quantitation of proteins. The protein content of reference solutions of IgM, IgG, and IgA was determined by reading optical densities at 280 nm in a Beckman DB-GT spectrophotometer. The following extinction coefficients were assumed: 12.8 for IgM (16), 13.2 for IgG (16), and 13.0 for IgA (17). Standard solutions of fowl albumin were made from weighed amounts of protein.

The radial immunodiffusion test (8) was used to measure IgG, IgM, IgA, and albumin in serum sam-

ples. Plexiglas templates were layered with 1.5% agar (Noble agar, Difco) in barbital buffer, pH 8.6, T/2 = 0.05, containing appropriate amounts of antisera. Twenty-four sample wells were made on each plate; 6 were used for standard solutions, and 18 were used for test sera. Two measurements were made of each sample. Plates were incubated, immersed in paraffin oil containing some thymol crystals, at room temperature for 2 days (for IgG and albumin determinations) or 4 days (for IgM and IgA determinations). Plates were then rinsed in absolute ethyl alcohol to remove excess paraffin oil. Where necessary, precipitin rings were intensified by washing the plates in 4% aqueous tannic acid. Precipitin rings were viewed by means of a projection microscope at a fixed distance from a screen, effecting a 30-fold magnification, and the diameter of each magnified ring was measured to the nearest nanometer. Protein concentrations of samples were obtained from a straight-line graph constructed from the diameters of the precipitin rings formed by the standard solutions plotted against the protein concentrations of the standards on a logarithmic scale.

Experimental design. One hundred and thirty chicks of each line were hatched. Blood was taken from five N-line and five P-line chicks daily from the day of hatching (day 1 of experiment) for 7 days, then every 2 days until 36 days of age, and then twice weekly until 67 days of age. From day 1 until day 36 the chicks were bled by cardiac puncture and killed. Thereafter they were bled by wing vein puncture (2 ml) and kept alive, but no individual bird was bled more than once every 2 weeks. Sera were stored at -20 C, IgM, IgG, IgA, and albumin were measured by radial immunodiffusion.

Statistical analysis. Immunoglobulin levels on selected days were examined by analysis of variance (26).

RESULTS

IgM (Fig. 1A). At hatching, only 4 of 10 chicks had measurable serum IgM (0.02 to 0.05 mg/ml). By 4 to 6 days all birds had measurable amounts of IgM, which increased only slightly during the next 2 weeks. At that time the average IgM level, irrespective of genetic line, was about 0.1 mg/ml. Between 3 and 4 weeks of age there was a steep rise in IgM levels. They reached a temporary peak of about 1.3 mg/ml in P-line chicks and 1.7 mg/ml in N-line chicks between 29 and 35 days of age. A gradual decline then occurred during the next 10 to 14 days, followed by a continued rise until the end of the experiment, with IgM levels at 67 days being about 1.9 mg/ml in N-line chicks and 1.5 mg/ml in P-line chicks. From 26 days of age

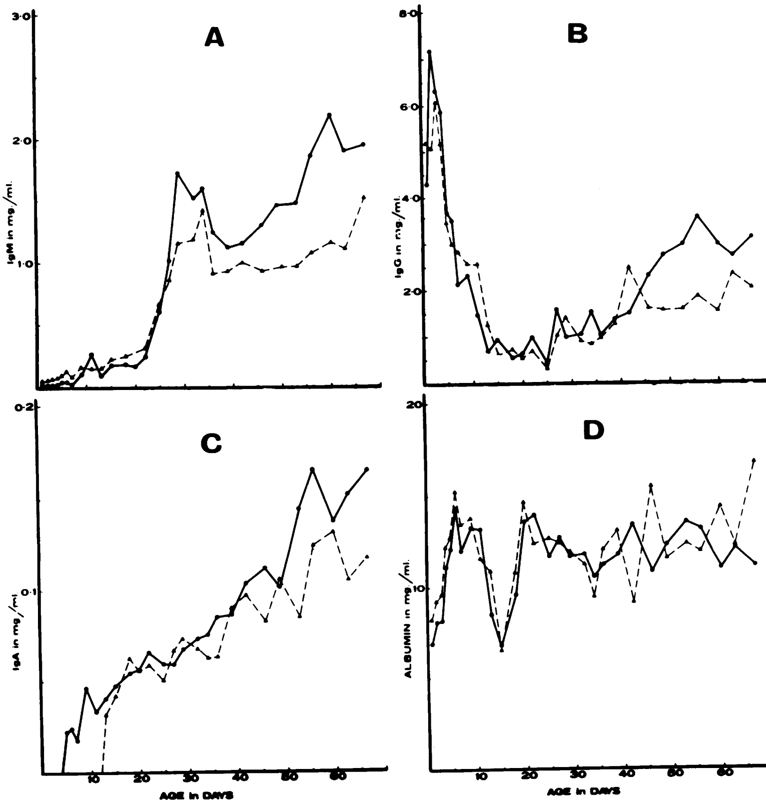

FIG. 1. *Serum proteins (in milligrams per milliliter) in N- and P-line fowls 1 to 67 days of age. (A) IgM; (B) IgG; (C) IgA; (D) albumin. Symbols: (●) N line; (▲) P line. Each point is the mean of five birds.*

throughout the remainder of the experiment, N-line birds had higher levels of IgM than did P-line birds. This difference was not statistically significant ($P > 0.05$).

IgG (Fig. 1B). Day-old chicks had mean IgG levels of 4.3 (N line) and 5.2 (P line) mg/ml. A slight increase occurred during the first 2 to 4 days, at which point IgG levels were higher than at any time during the remainder of the experiment. IgG levels fell off steeply during the next 8 to 10 days, and from 12 to 24 days were between 0.4 and 0.9 mg/ml. From 24 days on, the IgG levels rose progressively and at 67 days were about 2.0 mg/ml in P lines and 3.0 mg/ml in N lines. Between 46 and 67 days, mean serum IgG levels were consistently but not significantly ($P > 0.05$) higher in N-line than in P-line birds.

IgA (Fig. 1C). IgA was not detected in the serum of the day-old chicks. It was first seen in N-line chicks at 4 days of age and in P-line chicks at 13 days of age. Thereafter, IgA levels increased until at 67 days the mean levels were about 0.12 mg/ml in P-line birds and 0.17 mg/ml in N-line birds. From 40 to 67 days, N-line birds had consistently but not significantly ($P > 0.05$) higher mean levels of IgA than did P-line birds.

Albumin (Fig. 1D). At hatching, serum albumin levels were about 8 mg/ml. They rose to 12 to 15 mg/ml by 5 to 7 days. Thereafter, with the exception of a drop between 13 and 18 days, the levels remained between 10 and 15 mg/ml throughout the experiment. There was no difference between N-line and P-line chicks.

DISCUSSION

Quantitation of serum immunoglobulins in birds 1 to 67 days old gave results similar to those of other workers (18, 19, 31). IgG was the obvious vehicle of passive humoral immunity in the neonatal chick, and serum levels rose for a short time after hatching, presumably due to the continued passage of protein from the yolk sac to the serum. Passive transfer of IgG was so efficient that 2- to 3-day old chicks had higher levels than at any time during the remainder of the experiment. From 45 to 67 days, N-line birds had higher mean immunoglobulin levels than P-line birds. IgG at 56 days and IgM at 60 days were especially high in the N line. On these 2 days a single serum gave unusually high values for these proteins. This did not result in a statistically significant difference between the lines. Significant differences might be observed in larger groups or after 67 days of age. It seems unlikely, however, that a marked difference in susceptibility to MD would be mediated through a system where large groups were needed to observe statistically significant differences, or that susceptibility or resistance present at hatching might be expressed through a mechanism not measurably different until almost 10 weeks of age. IgA values obtained were possibly lower than actual values since the test sera were compared with a standard of secretory, polymeric IgA. Nevertheless, an observation that requires further study was the earlier development of IgA in N-line than in P-line birds. This finding is unlikely to be associated with genetically defined susceptibility to Marek's disease; such a transient difference would hardly explain the persisting susceptibility difference between the two lines.

Records of diseases susceptibility related to inherited absence of immunoglobulins are few. In cattle with lymphosarcoma, IgM is often low or absent (30), but this would seem to be secondary to the disease. A primary deficiency of IgG2 occurs in Danish breeds and predisposes these animals to gangrenous mastitis (14). In humans, antibody immunodeficiencies constitute 50 to 75% of all recorded primary deficiencies (29); a common clinical outcome is the recurrence of chronic bacterial infections, but predisposition to disease is usually associated with an absolute deficiency of one or more immunoglobulin classes or subclasses (7, 22). In the fowl, immunoglobulin levels have been related to the size of the bursa of Fabricius, which is in turn genetically controlled (9, 10). Families selected for large bursa size respond with higher antibody titers to *Vibrio foetus* (23) but not to *Salmonella pullorum* or *S. typhimurium* (13). Bursa size had no effect on the weights of thymus or spleen but had some significant effect on adrenal weight (11). A subsequent study of large and small bursa lines with regard to bursal histology and the effect of bursectomy suggested that the small-bursa chicks were at a more primitive stage of development in terms of bursal follicular structure than were large-bursa chicks; bursectomy of large-bursa chicks at time of hatching had only a transient and partial effect on their ability to respond to sheep erythrocytes, but rendered small-bursa chicks incompetent for at least 5 weeks (15). It is interesting to note that N-line chicks are often physically better developed than P-line chicks, but data on bursa development in these two lines are not available. Nevertheless, so little difference in serum immunoglobulins was observed between these two lines that a primary immunological deficiency in this system seems a most unlikely explanation for genetically defined susceptibility to MD.

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