APPLICATION OF IN VITRO PROCEDURES IN EVALUATING THE PROGRESSION OF MAREK'S DISEASE

by

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ABSTRACT

Mitogens are plant lectins that can stimulate lymphocytes to undergo DNA synthesis and blast cell formation. The failure of lymphocytes to respond to mitogens can be indicative, in part, of a viral infection or a malignant condition. Marek's disease is a neoplastic lymphoproliferative disease of chickens.

This study was an investigation of the response of peripheral blood lymphocytes from chickens with Marek's disease to the mitogens phytohemagglutinin and Concanavalin A. In the experiments, we observed the mitogenic response as well as body weight, leukocyte count and visual signs of the disease over time as the Marek's disease progressed.

Chickens with Marek's disease had a severely depressed response to PHA at four and five weeks of age compared to noninfected chickens. Differentiation of the infected versus noninfected chickens using mitogenic descriptors in multiple discriminant analysis could be accomplished as early as three weeks of age. The depression of the PHA/Con A ratios obtained for chickens with Marek's disease could be indicative of alterations in subpopulations of T lymphocytes.

INTRODUCTION

During the past twenty years, the immune system has been differentiated into two distinct functional units: the humoral immune response and the cell-mediated immune response. In 1956, Glick, Chang and Jaap (1) reported the role of a hindgut lymphoid organ in the chicken, the bursa of Fabricius, in eliciting an antibody response to a foreign agent. Warner, Szenberg and Burnet (2) confirmed this and suggested that the thymus was involved in a second functional branch of the immune system. In 1965, Cooper et al. (3) were instrumental in establishing that 1) bursa-derived B lymphocytes were required to form 19s and 7s gamma globulins in response to antigenic stimulation, and 2) thymus-derived T lymphocytes were necessary in order to develop a delayed allergic reaction and exhibit a graft versus host reaction. In addition, since irradiated-thymectomized chickens often had lower quantitative levels of antibody, they speculated that the thymus was in some way involved in antibody production.

B lymphocytes possess surface immunoglobulin and differentiate upon antigenic stimulation to form antibody releasing plasma cells. T lymphocytes possess distinct antigenic markers such as the theta and Lyt antigens in the mouse (4). Experiments have proven that a cellular cooperation exists between T lymphocytes primed to a carrier and B lymphocytes primed to a hapten in eliciting an antibody response (5).

Cantor and Gershon (4) have shown the existence in mice of subsets of T lymphocytes based on the Lyt antigens. T cells that help in amplifying an immune response possess the Lyt 1 antigens, T cells that suppress the immune response or are cytotoxic possess the Lyt 2,3 antigens and a precursor set of cells expresses the Lyt 1,2,3 antigens. In the chicken, Moticka (6) has shown two distinct classes of thymocytes: 1) suppressor cells which are prevalent in young chickens and those with intact bursas, and 2) helper cells which are prevalent in older birds and those that have been bursectomized. Based on such characteristic properties such as density, immunoglobulin synthesis rate and staining with fluorescent antilight chain antibody, four populations of immunoglobulin-synthesizing cells have been identified in the chicken (7).

Mitogens are lectins which are plant glycoproteins that were originally discovered to be capable of agglutinating erythrocytes (8). Mitogens, such as phytohemagglutinin (PHA), an extract of the kidney bean, Phaseolus vulgaris and concanavalin A (Con A) stimulate T cells to undergo DNA synthesis and blast cell transformation (9, 10, 11). In the murine system, Stobo and Paul (12) have used PHA and Con A to describe two subpopulations of T cells. One subpopulation demonstrates approximately equal reactivity to PHA and Con A, bears a relatively high density of theta determinants, recirculates through the body, is found in the lymph node and spleen, is relatively sensitive to radiation and is involved in the mixed lymphocyte reaction as well as being

involved in the graft versus host reaction. The second subpopulation responds mainly to Con A, bears a relatively reduced density of theta determinants, is relatively sessile, is found in the spleen and bone marrow, is relatively resistant to radiation, functions as a cytotoxic cell, is an effector cell in the graft versus host reaction and is required for DNA synthesis in response to an antigen. The use of mitogens to study a disease state was first reported in 1965 by Hersh and Oppenheim (13) who found that patients with Hodgkin's disease had an impaired response of their lymphocytes to in vitro stimulation with PHA. Lymphocytes from infants three months of age or younger with congenital rubella have been shown to be less responsive to PHA as determined by PHA-induced DNA synthesis when compared to lymphocytes from normal infants (14).

PHA-induced DNA synthesis in splenocytes from Swiss-Webster mice was found to be depressed when Myxovirus, paramyxoviruses, Mengo virus, leukemic viruses, herpesviruses and vaccinia virus were co-cultured with the splenocytes at the beginning of the incubation (15).

In 1907, Marek in Austria-Hungary described a chronic neuropathic disease of chickens which became known as the classic form of Marek's disease (16). Marek's disease is often characterized by the enlargement of the sciatic and brachial nerves by two to three times, and the development of lymphoid tumors in one or more organs. In acute Marek's disease, ovarian and testicular lymphomas may be commonly seen, but other tissues frequently involved are the liver, spleen, kidneys, lungs, heart and proventriculus. Atrophy of the bursa and

thymus commonly occurs (16). Pappenheimer, in 1929, first suggested that a neoplastic mechanism was responsible for the lymphoid proliferation in fowl paralysis (Neurolymphomatosis Gallinarum) (17, 18). Although neoplasia is definitely involved in the acute form of Marek's disease, early in the pathogenesis of the disease there are degenerative and inflammatory processes (19). It appears that Marek's disease is a combination of both of these mechanisms of destruction, with factors such as virus virulence and susceptibility due to genetic background playing a dominant role (16). Recent evidence has established the LY-4 locus in chickens, which codes for antigens on lymphocytes, as significant in determining susceptibility to Marek's disease (20).

A highly pathogenic form of Marek's disease first appeared in the United States in Delaware in the 1950's and eventually spread throughout the poultry industry. Rous (21) first induced a tumor in fowl with what he termed a "filterable agent." In 1967, it was shown that a DNA virus was present in tissue culture preparations of Marek's disease and that the avian leukosis virus (RNA) was not involved (22). From this research, Marek's disease was postulated to be caused by a perpesvirus. This led to the discovery that the herpesvirus caused a number of different neoplastic diseases in different hosts (23). These include primates (Herpesvirus saimiri), frogs (Lucké Renal Carcinoma), humans (Epstein-Barr virus incriminated in cases of Burkitt's Lymphoma) and chickens (Marek's Disease Virus) (24).

Marek's disease has been equated as an animal model of Burkitt's Lymphoma, due in part to the characteristic of a highly cell-associated virus in tissue culture lines of both diseases (25).

In cultured chicken kidney cells, no evidence of infectivity was found in the supernatant of infected cultures and no infectious virus could be extracted by disruption of infected cells (26). Transmission of infectivity is maintained only by means of intact cells, and the Marek's Disease Virus from cell to cell is facilitated by cell fusion. Using electron microscopy, nonenveloped intracellular virus particles have been observed in the bursa of Fabricius, leukotic gonad and the epithelial cells that line the kidney collecting tubules (27, 28, 29). Brachial nerve plexuses from chickens with Marek's disease contained Schwann cells, immature lymphoid cells and degenerated lymphoblastoid cells with characteristic intranuclear herpesvirus particles (30, 31). The body site at which mature, cell-free virus is available for collection is from feather follicle epithelium (32, 33). The complete replication of the enveloped virus in epithelial cells of the feather follicle results in the death of the cell, whereas incomplete replication of the virus in lymphoid cells was accompanied by proliferation of these cells and formation of tumors (34). It has been reported that in a few rare thymic cells, enveloped virus was found at nuclear membranes and cytoplasmic inclusion bodies containing enveloped virus, previously only seen in feather follicle epithelium, were present (35).

Using immunofluorescent tests, Marek's disease viral antigen has been detected in the nucleus and cytoplasm of bursal, thymic and splenic cells (36, 37, 38, 39).

One of the first efforts to control or prevent Marek's disease resulted in the production of an apathogenic attenuated strain of Marek's Disease Virus (40, 41). Vaccination with this agent resulted in a significant decrease in mortality, but not a total erradication of the disease (42, 43). The use of an apathogenic herpesvirus of turkeys in vaccination has resulted in the greatest flock protection, and this is the method of choice in the poultry industry (44, 45).

The presence of maternally transferred antibody to Marek's disease results in reduced morbidity and mortality (46). Specific antibody may stop early viremia and eliminate the virally-induced lymphocyte transformation which would lead to later tumor formation (47). It is also suggested that cell-mediated immunity may be involved in protection against Marek's disease. This may be visualized as 1) a lymphocyte transformation of blast cells that may produce interferon which would hamper the Marek's Disease Virus, 2) the cytotoxic function of sensitized T cells or 3) a process of initiating macrophage activity (48). Although immunity to Marek's disease is achieved through live viral vaccines, immunity may also be conferred by the use of noninfectious materials extracted from cells infected with the attenuated strain of Marek's Disease Virus (49). A humoral immune mechanism is not believed to be primarily responsible in vaccinal immunity since one day old bursectomized agammaglobulinemic

chicks are protected by vaccination with an attenuated strain of Marek's Disease Virus (49). However, a cyclophosphamide bursectomy during the first four days after hatching eliminates protection against Marek's Disease Virus by the herpesvirus of turkeys (50). Vaccinal immunity to Marek's Disease Virus may involve two steps. Vaccination at one day of age with the attenuated herpesvirus of turkeys may cause a decreased level of virus which would result in a lower level of lymphocyte transformation and produce a population of sensitized T cells which would cause subsequent destruction of any neoplastic transformed cells (51). Rouse and Warner (52) have speculated that vaccination with an attenuated Marek's Disease Virus or heterologous turkey herpesvirus may protect by the induction of a suppressor T cell response which, on subsequent infection by the Marek's Disease Virus, would result in the absence of an aggressor neoplastic proliferation against the virally-induced antigen.

Two prominant models offer explanation for the formation of lymphomas: 1) the extrinsic model in which the stimulus is external to the proliferating cells and 2) the intrinsic model in which the lymphoma is the direct result of the neoplastic cell growth. Cell preparations from visceral tumors and nerve lesions of chickens with Marek's disease have been shown to consist primarily of T lymphocytes (53). This has led to the belief that the lymphoma is the result of a T cell-mediated immunological attack on virus-infected epithelial cells (analogous to that observed in lymphocytic choriomeningitis of

mice). An antigen-antibody complex involvement in the production of an extrinsic lymphoma has been ruled out by the finding of Marek's disease in chickens which lacked antibodies, immunoglobulins, germinal centers and plasma cells (54). This suggests that the bursa and the bursa-dependent lymphoid tissues are not essential in the pathogenesis of the disease.

The intrinsic model postulates that a virus infects a lymphocyte and this transformed lymphocyte proliferates into a lymphoma. It has been shown by nucleic acid hybridization techniques that there exist three to fifteen viral genome equivalents per tumor cell and sixty to ninety genome equivalents per cell of the MSB-1 established cell line, indicating that the virus is required in tumor cells for neoplastic proliferation (55, 56). In addition, Marek's disease tumor-associated surface antigen (MATSA) has been observed on Marek's disease tumor cells and on cells of the MSB-1 cell line offering further evidence that the cells have been genetically altered. It is possible that Marek's disease may be the result of a viral infection which leads to a transformed T cell neoplasm as well as an inflammatory response induced by T and B cells which are responding to a tumor specific antigen found on virally altered cells (57).

Chickens with Marek's disease have an enhanced graft versus host reaction, a delayed homograft rejection and a decreased antibody response, although no decrease in gammaglobulin levels was observed (58). In vitro assays with splenocytes from chickens with gross tumors have shown a lowered PHA response (59). In other studies,

the mitogenic response of splenocytes from chickens with Marek's disease to PHA was decreased while the response to Con A was relatively unaffected (11). Neoplastic proliferation may be responsible for the apparent immunosuppressive effects rather than viral immunosuppression (60).

In summary, it should be emphasized that 1) certain viral infections alter the mitogen-induced DNA synthesis of the affected lymphocyte and 2) chickens with Marek's disease apparently experience immunosuppression which is reflected by a decreased response to mitogenic stimulation. In view of these facts, a pertinent question would ask if a prognosis of Marek's disease can be made earlier by evaluating the peripheral blood lymphocyte response to mitogenic stimulation. This study was designed to evaluate a number of different features of chickens with Marek's disease and normal chickens. These include body weight, leukocyte count, clinical signs, mitogenic response of peripheral blood lymphocytes and death. The data obtained were analyzed to determine their correlation to the progression and severity of Marek's disease at weekly time periods during the course of the disease.

MATERIALS AND METHODS

Animals

Randomly selected outbred white leghorn chicks were obtained from the University of Arizona Experimental Poultry Farm at less than 24 hours after the time of hatching. The chickens were maintained in cages inside positive pressure plastic isolation units. The chicks were fed chick starter feed and tap water, ad libitum.

Preparation of Reagents

Marek's Disease Virus

A stock preparation of the GA strain of Marek's Disease Virus was obtained from Dr. E. W. Marty, Jr. (American Scientific Laboratories, Madison, Wisconsin). The stock preparation was a fifty percent suspension of heparinized chicken blood in dimethylsulfoxide which was stored in liquid nitrogen. The suspension was rapidly thawed, diluted 1:5 with Cell Balanced Salt Solution (CBSS, Appendix A) and kept on ice until use. Two-tenths ml was injected subcutaneously into the back of the neck of each chicken.

Culture Medium

Roswell Park Memorial Institute 1640 medium (RPMI 1640, Gibco, Grand Island, New York) was prepared containing HEPES buffer (Appendix A) at a concentration of 10 mM and Gentamicin sulfate (Schering

Corporation, Kenilworth, New Jersey) at a concentration of 0.0825 mg per ml. The complete medium was stored at 4C until use.

Mitogens

Phytohemagglutinin (PHA-P, Gibco) was prepared as a 10 mg per ml stock solution in water and diluted to 100, 50 and 25 µg per ml in complete medium. Concanavalin A (Con A, Sigma Chemical Company, St. Louis, Missouri) was prepared as 1.0 mg per ml stock solution in complete medium and diluted to 25, 12, 6 and 3 µg per ml in complete medium.

Preparation of Ficoll-Hypaque for Gradients

Eighteen grams of Ficoll (Pharmacia Fine Chemicals, Piscataway, New Jersey) was imbibed into 230 ml of double distilled water. To this was added 60 ml Hypaque (Wintrop Laboratories, New York). One ml volumes per tube were dispensed and the preparation was autoclaved and stored at 4C until use.

Procedure for Preparation of Peripheral Blood Lymphocytes for Assay

One ml of blood was obtained from the jugular vein into a heparinized syringe (10 IU heparin per ml). One-tenth ml of the heparinized blood was immediately removed for the leukocyte count and treated as described below. Four ml of CBSS were immediately

added to the remaining 0.9 ml of heparinized blood and the mixture was stirred in a vortex mixture. This suspension was centrifuged at 600 x g for ten minutes to pellet the cells, the supernatant removed, four ml of CBSS added, the cells resuspended, centrifuged to pellet the cells, the supernatant removed and the cells resuspended in complete medium to the volume of the original blood (0.9 ml). This suspension was diluted 1:20 and kept on ice until use in culture.

Procedure for Determining Body Weight and Leukocyte Count

Body Weight

The animals were weighed prior to bleeding to the nearest 0.1 g on an animal scale (Ohaus Scale Corporation, Union, New Jersey).

Leukocyte Count

The 0.1 ml heparinized blood was diluted in Natt-Herricks solution (Appendix A) to a final dilution of 1:100 and the total number of leukocytes, exclusive of thrombocytes, was counted on a hemocytometer under 450x magnification.

Procedure for Lymphocyte Stimulation by Mitogens

One-tenth ml volumes of the PHA and Con A concentrations listed above were added in triplicate to the wells of a 96 well flat-bottomed microtiter plate (Microtest, Falcon). One-tenth ml volumes of the 1:20 dilution of the peripheral blood lymphocytes were added. The

microtiter plates were covered and placed in a culture humidifier (Bellco Glass, Vineland, New Jersey) which contained an atmosphere of 5% CO_2 and 95% air. The cultures were incubated for 48 hours at 37C at which time 0.5 uCi of tritiated thymidine (^{3}HTdR, Amersham/ Searle Corporation, Arlington Heights, Illinois, 5 Ci per mM) in a 0.05 ml volume at room temperature was added to each well. At 72 hours, cultures were harvested using a Chap-100 semiautomated harvesting apparatus (Adaps, Inc., Dedham, Massachusetts). The culture contents of each well were transferred onto glass fiber pads (Whatman, Inc., Clifton, New Jersey), washed with approximately 5 ml of physiological saline and washed with approximately 3 ml of absolute methanol. The glass fiber pads were dried and then transferred into glass scintillation vials. Into each vial was added 5 ml of the PPO-POPOP scintillation mixture (Appendix A). The vials were counted for one minute on a Packard Tri-Carb Liquid Scintillation Spectrophotometer (Model 332, Packard Instrument Company, La Grange, Illinois) at a gain value of 9.5 and window settings of 50 and 1000.

Experimental Design and Statistical Treatment of the Data

Experiment 1: Comparison of the Efficacy of the Virus Infected Spleen and Blood Preparations

Two-tenths ml of the stock preparation of the 50% Marek's disease chicken blood was injected into each of twenty, one-day old chicks. At five weeks, the chickens were sacrificed and the blood

and spleen from animals with signs of Marek's disease (tumors on the liver and spleen, enlargement of the spleen and kidneys) were pooled. The heparinized blood was diluted 1:2 with CBSS. The spleen cells were passed through a stainless steel screen, centrifuged until pelleted, the supernatant liquid removed and the packed splenocytes resuspended in four parts CBSS. Dimethylsulfoxide was added to both preparations to a final concentration of 10% dimethylsulfoxide and the cell preparations were stored in liquid nitrogen until use. Twelve one-day old chicks were inoculated with 0.2 ml of a 1:5 dilution of the blood preparation in CBSS. Twelve one-day old chicks were inoculated with 0.2 ml of a 1:5 dilution of the spleen preparation in CBSS.

Experiment 2: Protocol Used in the Study of the Progression of Marek's Disease

The major experiment followed the progression of Marek's disease using a number of descriptors including body weight, leukocyte count, responsiveness of peripheral blood lymphocytes to the mitogens PHA and Con A, and visual signs of the disease. The experiment consisted of three trials with five chickens in each Marek's disease group and five chickens in each normal group. The Marek's disease chicks received 0.2 ml of the 1:25 dilution of the splenic suspension injected subcutaneously in the back of the neck. Both groups were assayed at weeks 1, 2, 3, 4 and 5 to obtain the data described below. The leukocyte counts, body weight, geometric mean

of the lymphocyte response to the mitogens and PHA to Con A ratios were transferred to logarithms of the base ten. The data were analyzed by analysis of variance using the Cyber 175 Computer (Control Data Corporation, Minneapolis, Minnesota) at the University of Arizona Computing Center. The multiple discriminant analysis program was supplied by Dr. Lee Kelley.

RESULTS

Clinical Signs and Mortality

Experiment 1 compared the efficacy of the virus infected spleen and blood preparations. By six weeks, ten of the twelve chicks in the blood group and eleven of the chicks in the spleen group had died of Marek's disease or showed signs of the disease. Upon examination during necropsy, the chicks inoculated with the splenic preparation appeared to have more severe and obvious signs of Marek's disease than the chicks injected with the blood preparation. The splenic preparation was used in subsequent experiments.

Experiment 2 was a study of the progress of the Marek's disease over a five week period. Of the fifteen one day old chicks inoculated with the Marek's disease splenocyte suspension, thirteen showed clinical signs of disease including emaciation, immobility, paralysis and eventually death. At post-mortem examination, all thirteen chicks had developed kidney tumors and an assortment of other signs including ovarian tumors, liver tumors and nodules on the liver and heart. Only data from these thirteen chickens that had Marek's disease were included in the Marek's disease group that was subjected to further statistical analysis. The mean survival time of the chicks was 41 days. None of the fifteen control chicks showed any clinical signs of Marek's disease, and post-mortem examination failed to show any organ abnormalities.

Figure 1 compares the body weight and the leukocyte count of the chickens infected with Marek's Disease Virus and the noninfected chickens. Chickens developing the disease were lighter than non-infected chickens at four and five weeks post infection ($p<.05$).

Sequential Study of Mitogen Response in Peripheral Blood Lymphocytes

Table 1 shows the mean values for the mitogen-induced DNA synthesis as measured by ^{3}HTdR uptake at the weekly assays. Optimum doses appear to be 10 µg per microwell for PHA and 1.2 µg per microwell for Con A. One can detect a trend towards lower ^{3}HTdR uptake in the chickens with Marek's disease compared to the normal chickens as a function of age. Further evaluation of these data is presented in Figures 2 and 3 showing the trendal changes with PHA at 10 µg per microwell and Con A at 1.2 µg per microwell.

Figure 2 shows the Con A response (1.2 µg per microwell) of the chickens with Marek's disease and the control chickens as a function of time. In general, there was no significant difference between the two groups except at the fourth week ($p<.05$).

Figure 3 shows the PHA responses (10 µg per microwell) of the chickens with Marek's disease and the control chickens as a function of time. At the fourth and fifth week of assay, the chickens with Marek's disease exhibited a severe depression in the response to PHA as compared to the noninfected birds ($p<.05$).

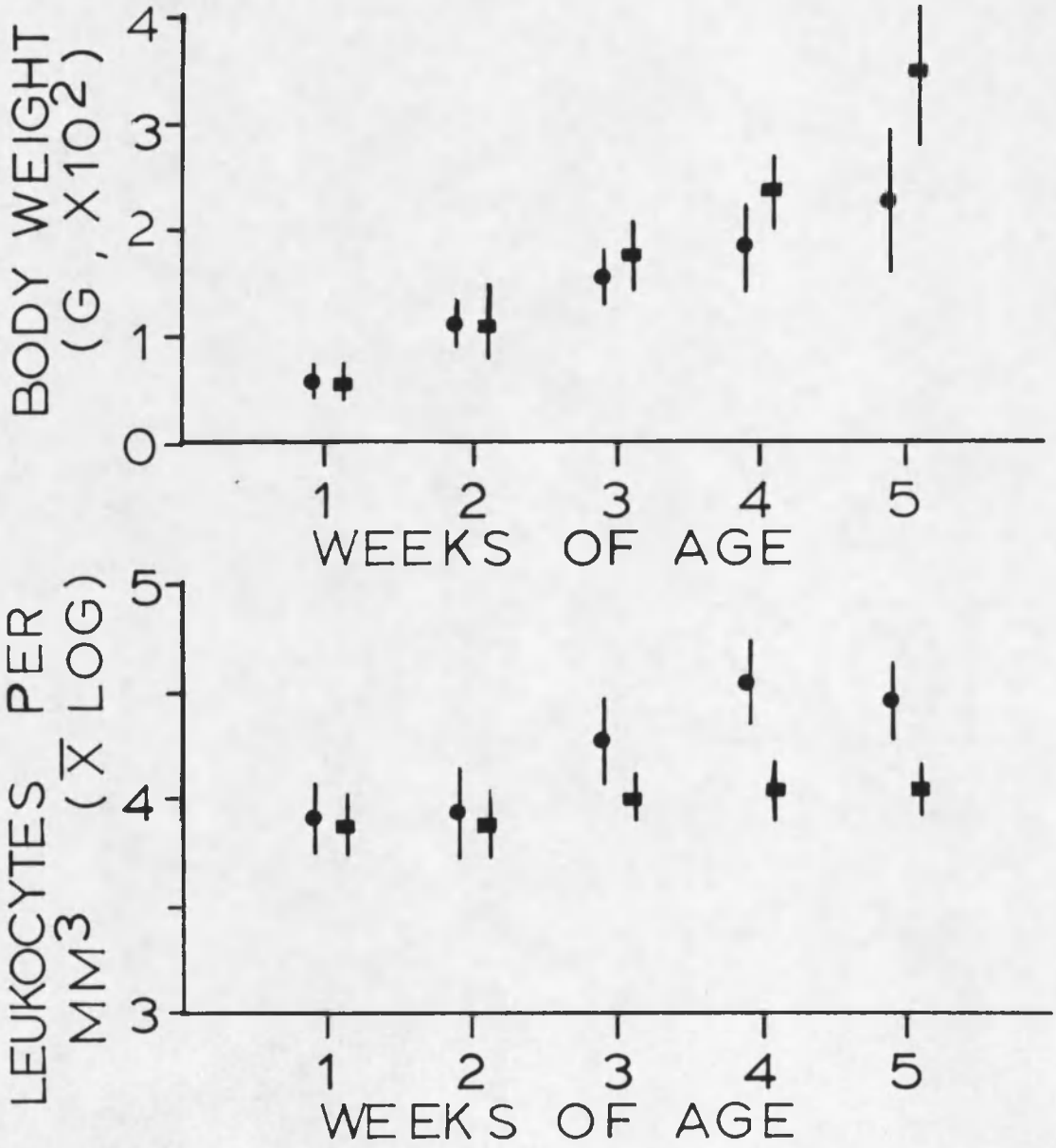

Figure 1. Changes in body weight and leukocyte count as a function of age in control white leghorn chickens and chickens infected with Marek's Disease Virus. -- ●-Mean of MDV, ■-mean of control. Vertical lines represent ± one standard deviation.

Table 1. ^{3}HTdR uptake in chickens with Marek's disease and control chickens as a function of age.

Weeks after Infection	Controls	PHA (µg/well) 2.5	5.0	10.0	Con A (µg/well) 0.3	0.6	1.2	2.5
		Noninfected chickens						
1	180[a]	132	262	523	68	426	395	319
2	123	239	474	1270	204	681	672	413
3	163	279	422	1949	458	804	712	533
4	221	291	737	2473	387	1421	1266	691
5	140	310	1232	2481	284	1351	1160	644
		Chickens with Marek's Disease						
1	192	312	302	221	145	378	337	167
2	162	363	466	1168	358	897	732	406
3	216	335	946	1473	691	1054	593	319
4	261	544	886	646	372	781	588	289
5	223	582	950	788	266	1035	835	427

[a]Geometric mean of cpm of triplicate samples

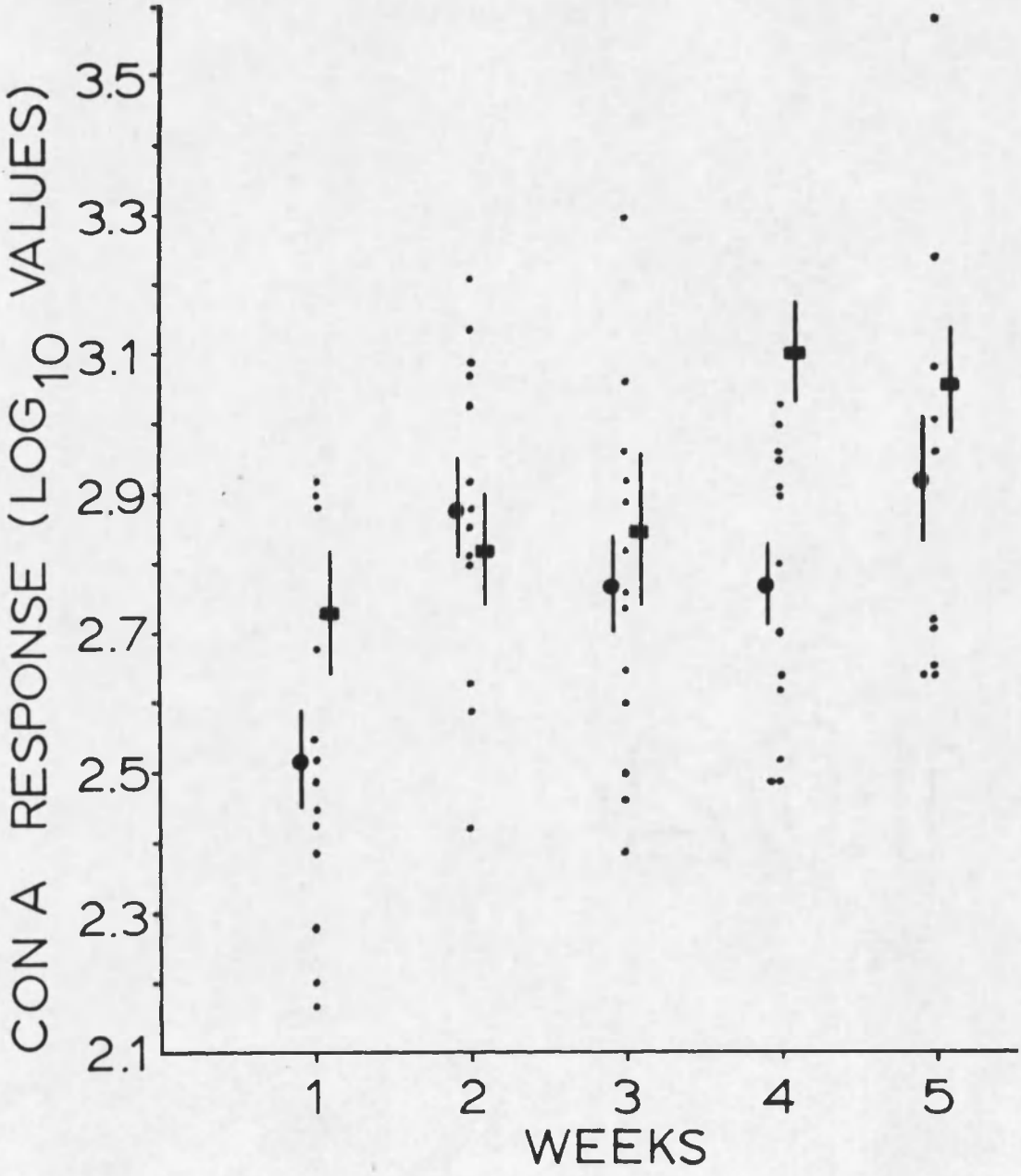

Figure 2. Changes in the uptake of ^{3}HTdR as a function of age in Con A stimulated peripheral blood lymphocyte cultures obtained from control white leghorn chickens and chickens infected with Marek's Disease Virus. -- ●-Mean of MDV, ■-mean of control, •-individual values for MDV. Vertical lines represent ± one standard error.

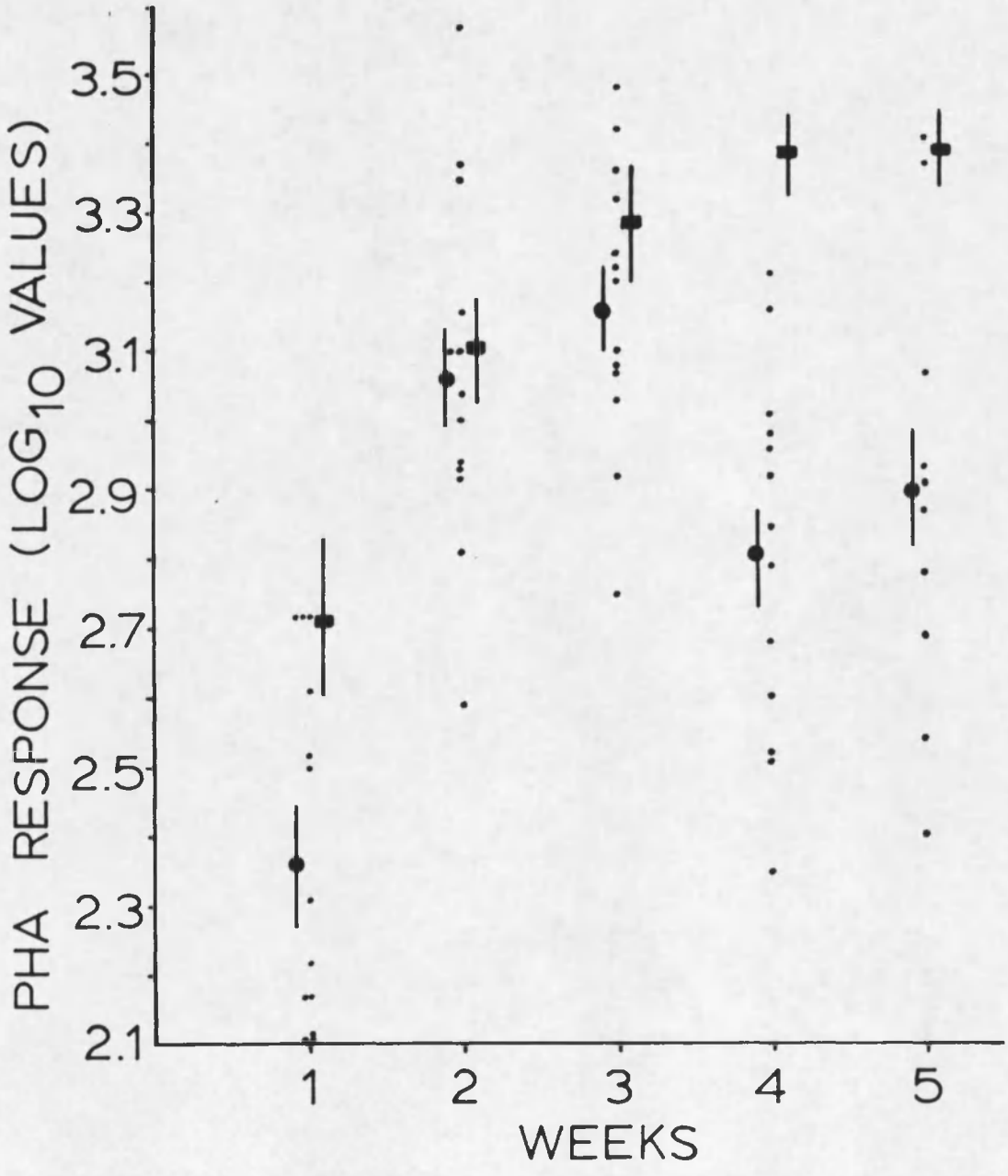

Figure 3. Changes in the uptake of ^{3}HTdR as a function of age in PHA stimulated peripheral blood lymphocyte cultures obtained from control white leghorn chickens and chickens infected with Marek's Disease Virus. -- ●-Mean of MDV, ■-mean of control, •-individual values for MDV. Vertical lines represent ± one standard error.

Figure 4 compares the PHA/Con A ratios (PHA equals 10 μg per microwell and Con A equals 1.2 μg per microwell) in noninfected and in chickens developing Marek's disease. The chickens with Marek's disease had a lower ratio at weeks four and five post infection than the noninfected chickens ($p<.05$).

Multiple Discriminate Analysis of the Data

During this experiment, data were collected pertaining to body weight, leukocyte count, mitogenic response, signs of the disease and death as a function of time after infection with Marek's Disease Virus. Multiple discriminate analysis is a statistical tool which uses descriptors such as those mentioned above to differentiate between groups of subjects. The use of multivariate statistical methods may expose relationships between the descriptors which can prove diagnostic in the evaluation of the progression of Marek's disease.

Figure 5 presents a histogram of the multiple discriminate analysis of the chickens with Marek's disease and the control chickens based on the biological descriptors of body weight, leukocyte count and visual signs of disease. The multiple discriminate analysis showed a significant difference between the diseased and normal chickens only at the fourth and fifth weeks post infection ($p<.05$).

Figure 6 shows a histogram of the multiple discriminant analysis of the chickens with Marek's disease and the control chickens based on the mitogenic stimulation response (Con A at concentrations of 0.3, 0.6,

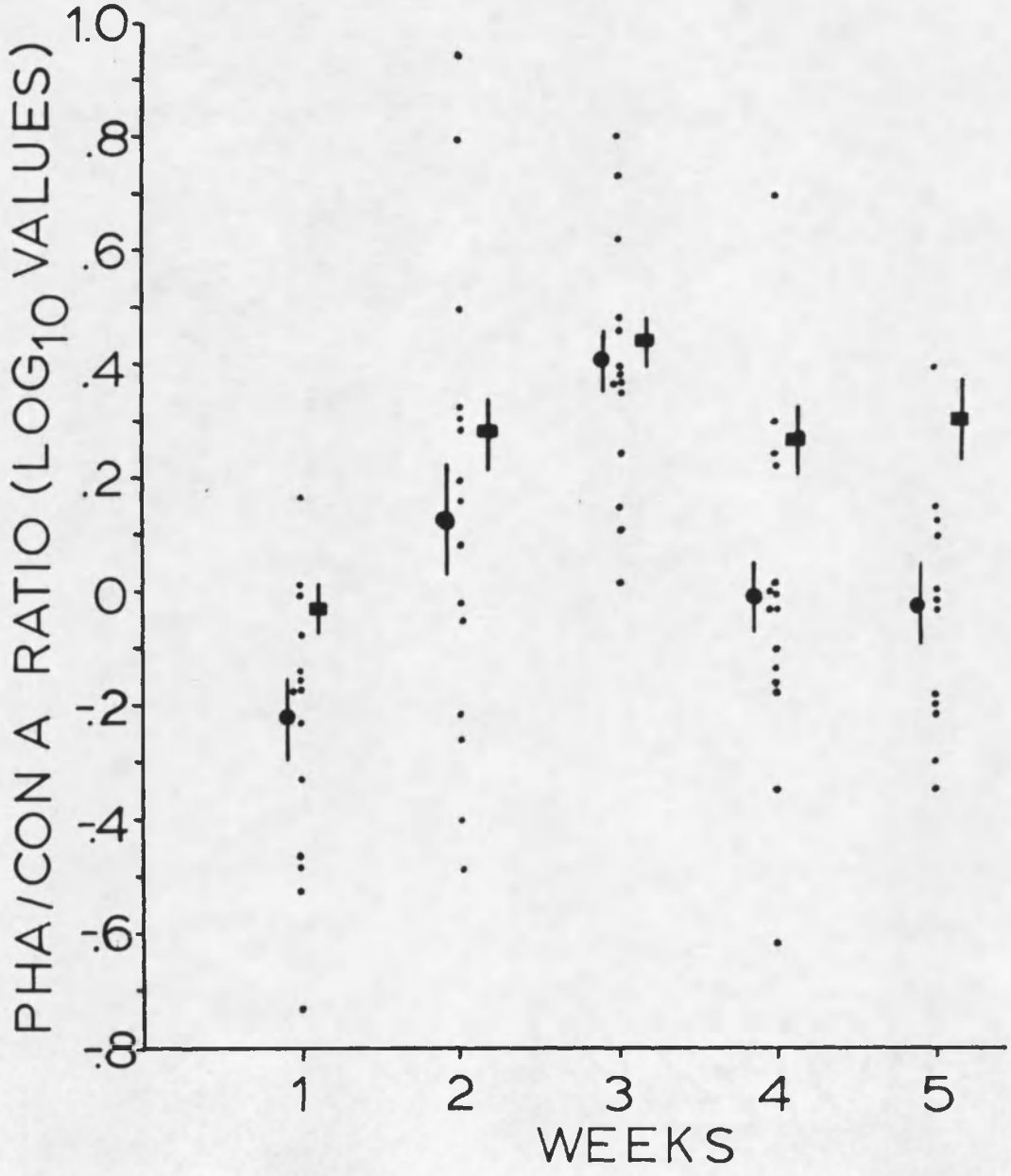

Figure 4. PHA/Con A ratios as a function of age in peripheral blood lymphocyte cultures obtained from control white leghorn chickens and chickens infected with Marek's Disease Virus. -- ● -Mean of MDV, ■ -mean of control, • -individual values for MDV. Vertical lines represent ± one standard error.

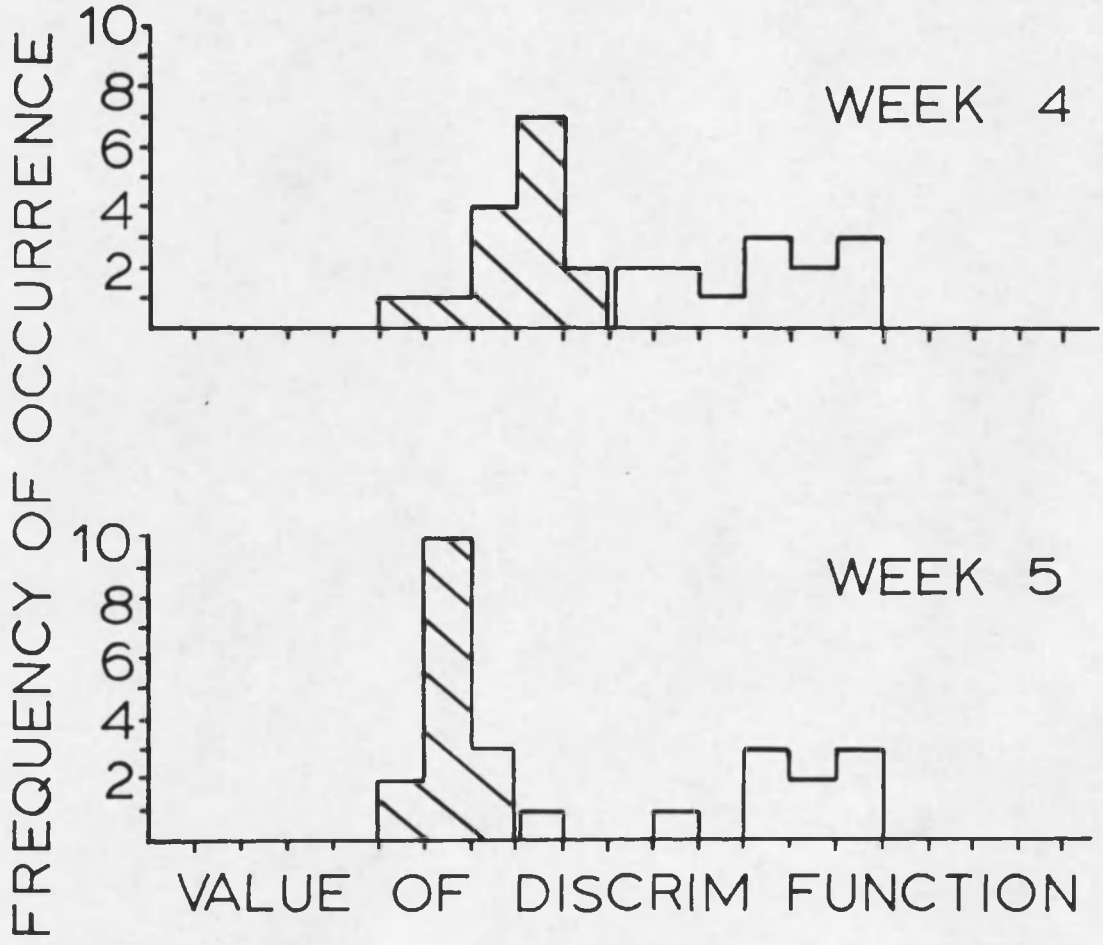

Figure 5. Histogram of the multiple discriminant analysis of the control white leghorn chickens and the chickens infected with Marek's Disease Virus based on body weight, leukocyte counts and the visual signs of the disease. -- Nonhatched areas represent the chickens infected with Marek's Disease Virus; hatched areas represent the control chickens.

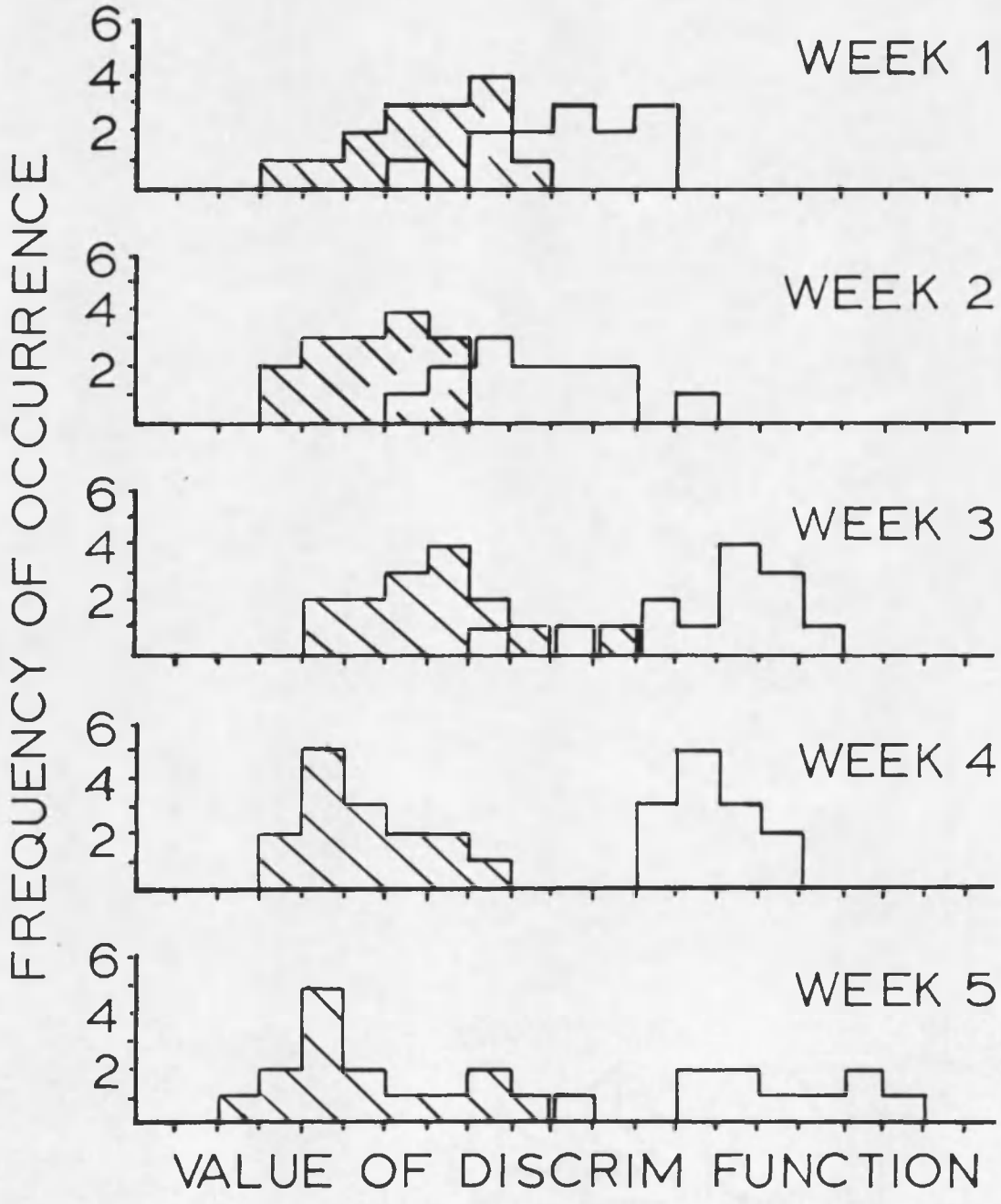

Figure 6. Histogram of the multiple discriminant analysis of the control white leghorn chickens and the chickens infected with Marek's Disease Virus based on the mitogenic responses. -- Nonhatched areas represent the chickens infected with Marek's Disease Virus; hatched areas represent the control chickens.

1.2 and 2.5 μg per microwell and PHA at concentrations of 2.5, 5.0 and 10.0 μg per microwell). The chickens with Marek's disease show a significant difference from the normal chickens at the third, fourth and fifth weeks of age ($p<.05$).

Figure 7 presents a histogram of the multiple discriminate analysis of the chickens with Marek's disease and the control chickens based on body weight, leukocyte count, visual signs of the disease and the mitogenic stimulation responses based on all of the mitogen concentrations. A significant difference was observed between the chickens with Marek's disease and the control chickens at the third, fourth and fifth weeks of age ($p<.05$).

It is of interest to investigate the ability of the multivariate analysis to differentiate between the two experimental groups using a limited number of biological and mitogenic stimulation response descriptors. Figure 8 shows a histogram of the multiple discriminate analysis of the chickens with Marek's disease and the control chickens based on the biological descriptors of body weight and leukocyte count and the clinical descriptors of PHA at 10 μg per microwell and Con A at 0.3 μg per microwell. These particular mitogen concentrations were selected because when using multiple discriminate analysis they were generally the most significant for each of the five weekly assay times. A significant difference was observed between the two experimental groups at the third, fourth and fifth weeks of age ($p<.05$).

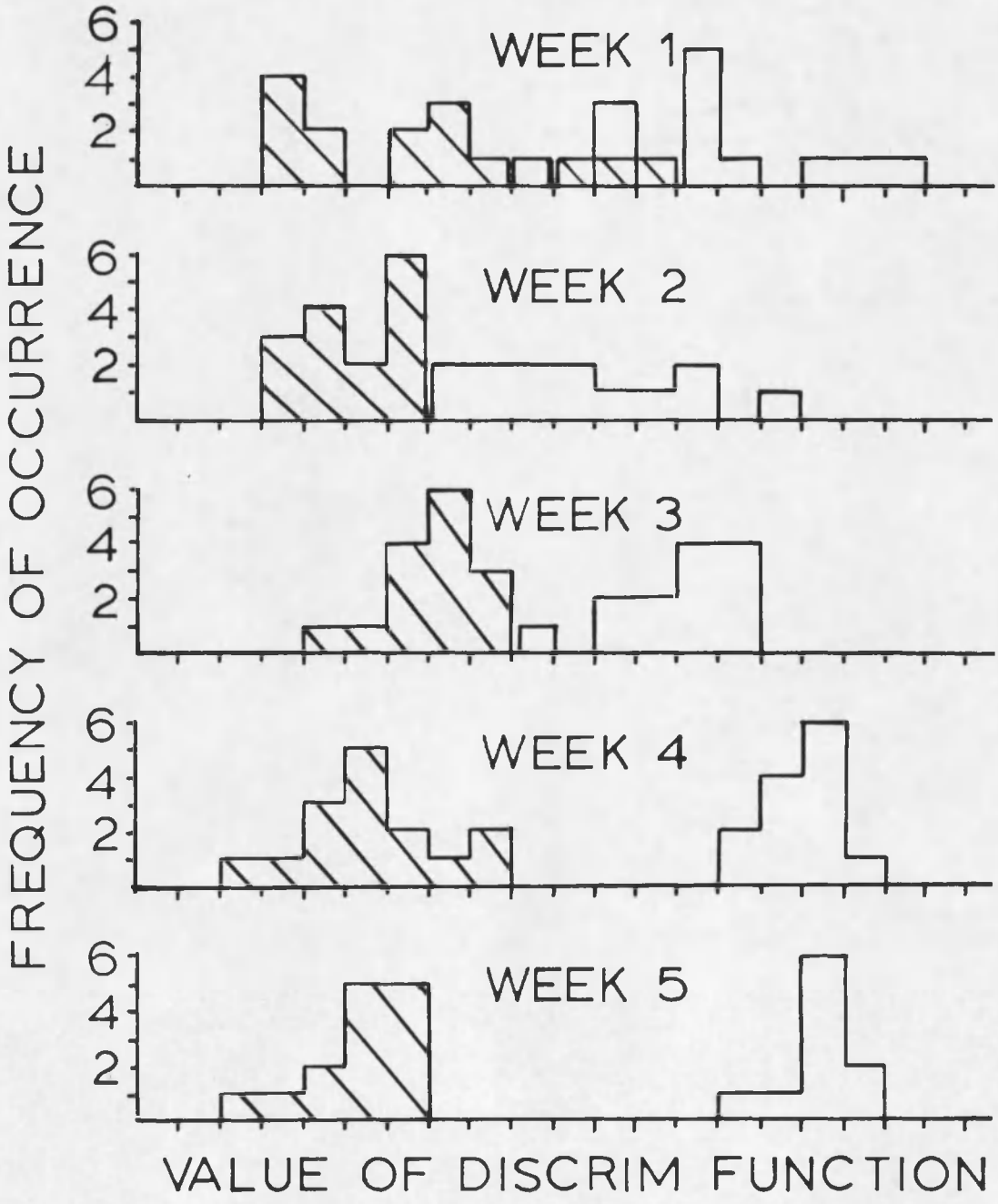

Figure 7. Histogram of the multiple discriminant analysis of the control white leghorn chickens and the chickens infected with Marek's Disease Virus based on the biological and clinical descriptors. -- Nonhatched areas represent the chickens infected with Marek's Disease Virus; hatched areas represent the control chickens.

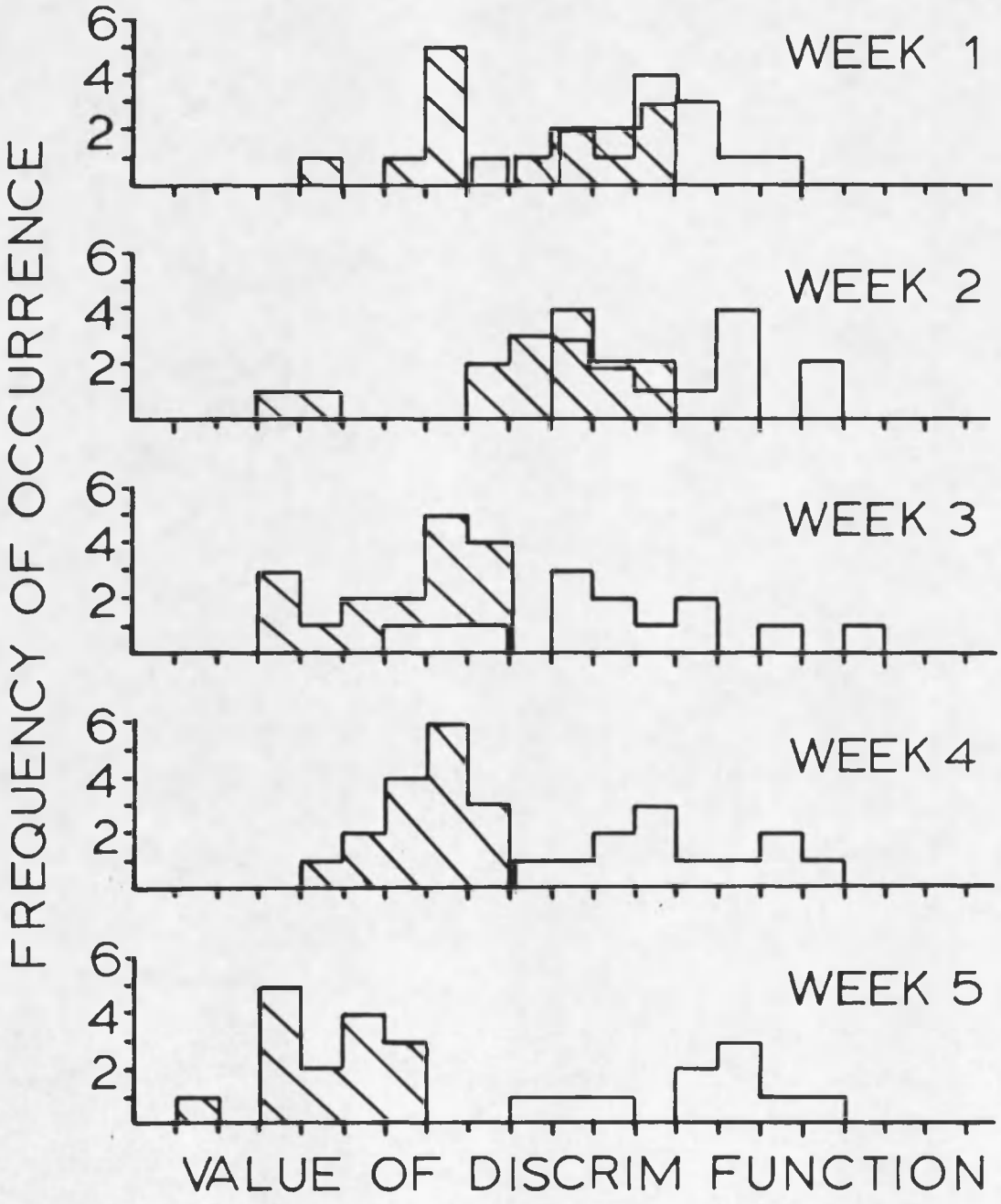

Figure 8. Histogram of the multiple discriminant analysis of the control white leghorn chickens and the chickens infected with Marek's Disease Virus based on body weight, leukocyte count and PHA and Con A mitogenic response. -- Nonhatched areas represent the chickens infected with Marek's Disease Virus; hatched areas represent the control chickens.

DISCUSSION

The clinical signs and characteristic tumors of chickens with Marek's disease have been well documented (14). This study confirms that the GA strain of Marek's Disease Virus produces similar findings such as elevated leukocyte count, decreased body weight and the formation of tumors of the kidneys, gonads, spleen and other organs which usually lead to death.

The response of cultures of peripheral blood lymphocytes to the mitogens PHA and Con A was studied at weekly intervals in chickens with Marek's disease and in control chickens. There was little difference in the response of lymphocytes from chickens with Marek's disease and control chickens to stimulation in culture by Con A. The response to Con A increased as the birds matured (61). The chickens with Marek's disease had a severe depression in the response to stimulation by PHA at weeks four and five as compared to the noninfected chickens. Consequently, the PHA/Con A ratios of the chickens with Marek's disease were significantly lower than the control chickens at weeks four and five. The lower ratio in the chickens with Marek's disease may be indicative of the failure of the development of normal subpopulations of T lymphocytes in the peripheral blood of a growing and maturing chicken.

In the murine system, subpopulations of T cells that respond better to Con A than PHA function as cytotoxic cells, suppressor cells and are effector cells in the graft versus host reaction. In chickens, an increase in cells with lower PHA/Con A ratios as Marek's disease progresses may reflect alterations in lymphocyte subpopulations during the pathogenesis of the disease. A change in the quantitative level of a subpopulation or subpopulations may impair the ability of the immune response to recognize and prevent the development of a malignant condition.

Statistical analysis is able to differentiate between groups of data and to provide discriminatory information that would not otherwise be available. Statistical analysis based on the leukocyte count, body weight and the visual signs of the disease was not able to establish a significant difference between the two groups until the fourth week of the study. The multiple discriminant analysis of mitogenic response, however, was able to show a significant difference between the chickens with Marek's disease and the control chickens at the third week of the study. The statistical evaluation of mitogenic responsiveness of lymphocytes further encourages the feasibility of using the lymphocyte response to mitogenic stimulation in the early diagnosis of the onset of certain diseases (62).

APPENDIX A

PREPARATION OF REAGENTS

Cell Balanced Salt Solution

Cell balanced salt solution is prepared by dissolving the following:

Dextrose	1.000 g
KH_2PO_4	0.060 g
$Na_2HPO_4*7H_2O$	0.358 g
$CaCl_2*2H_2O$	0.186 g
KCl	0.400 g
$MgCl_2*6H_2O$	0.200 g
$MgSO_4*7H_2O$	0.200 g
NaCl	8.000 g
Phenol Red (0.5%)	2 ml
H_2O	1000 ml

Cell balanced salt solution is filter sterilized with a 0.45 μ Millipore filter and stored at 4C until use.

HEPES Buffer

Hepes (N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic Acid) is supplied in powder form from Grand Island Biological Company and prepared in 100x concentration in the following buffer:

NaCl	0.800 g
KCl	0.040 g
Na_2HPO_4	0.010 g
Dextrose	0.100 g
HEPES	23.800 g
H_2O	100 ml

Natt-Herrick Solution

Natt-Herrick solution is prepared by dissolving the following in distilled water (in order):

NaCl	3.88 g
Na_2SO_4	2.50 g
Na_2HPO_4	1.15 g
KH_2PO_4	0.25 g
Formalin (37%)	7.50 ml
Methyl Violet 2B	0.10 g

The volume of the solution is brought up to 1,000 ml and allowed to stand overnight. The solution is then filtered through Whatman No. 2 filter paper.

PPO-POP Scintillation Cocktail

PPO-POP scintillation cocktail is prepared by mixing 1000 ml of toluene and 600 ml of 2-methoxyethanol. To this mixture 8 g of PPO (2,5 diphenyloxazole) and 0.48 g POPOP 1,4-bis[2-(5-Phenyloxazolyl)] benzene are added. The mixture is stirred for approximately two hours on a magnetic stirrer.

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