INFECTION AND IMMUNITY, Nov. 1971, p. 567-574
Vol. 4, No. 5

Printed in U.S.A.

Correlation of Immunological Responsiveness with Lymphocyte Changes in Chickens Infected with Marek's Disease

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Received for publication 4 June 1971

Several immunological, hematological, and pathological responses associated with Marek's disease were determined. Four-week-old Marek's disease-infected and control chickens were injected with *Salmonella pullorum* antigen. About one-half of all infected chickens tested were unresponsive to antigenic challenge. Antibody titers in responsive infected chickens were significantly depressed at 1 and 2 weeks post-inoculation when compared to controls. Total white blood cell counts of control and control-antigen chickens were significantly lower than counts in infected chickens. Based on response to antigenic challenge, 24% of the responsive group had leukemia compared to 54% of the unresponsive chickens. The predominant cell populations in these two groups responsible for the mononuclear cell leukemia were large lymphocytes and blast cells. These cell increases were significantly greater in unresponsive chickens. Also, transient increases in the granulocytic elements were observed in some infected chickens. Large fluctuations in hematocrit values were observed in Marek's disease-infected chickens. As many as 30% of the infected chickens were anemic throughout the testing periods. Infected chickens which did not receive antigen had lower incidences of mortality and gross lesions than similarly treated chickens which did receive antigen. In addition, those chickens which were unresponsive to antigenic challenge had a higher mortality rate and increased percentages of gross lesions when compared with responsive chickens.

Studies correlating Marek's disease (MD) with blood cell changes have shown that some abnormalities occur. Previous work has demonstrated that leukemia of the fowl caused a reduction of red blood cells (17) and an increase in total white blood cell count with increased numbers of large lymphocytes and atypical mononuclear cells. Differential counts in this study showed a lymphocytosis of 50 to 85% and the appearance of blast cells in association with the leukemia. Others (7), however, reported that leukemia was not associated with this disease, although they presented no data to support this conclusion. In a description (15) of the blood cellular changes occurring during neurolymphomatosis (MD), it was found that the disease caused increased leukocyte counts of 20 to 30%, the changes due to heterophil increases in the early stages (47%) and lymphocytes in the later stages (68% increase). Increases in basophils (5) and in total white blood cell counts, decreases in hemoglobin levels, and prolonged coagulation times have also been described. One study (1) reported slight increases in total cell counts associated with fowl paralysis (MD). In attempts to differentiate the blood picture in terms of whether paralysis was present (4), it has been demonstrated that the non-paralytic type produced large and immature lymphocytes and severe anemia; the paralytic condition was correlated with small- to medium-sized lymphocytes with no anemia.

In these studies, diseases of the avian leukosis-sarcoma complex were not differentiated from MD, and, because of inadequate isolation procedures, control chickens many times had mixed infections.

In other work (3), atypical globule-containing cells have been observed in the peripheral blood of infected chickens. More recently it was observed (8) that infected chickens demonstrate an increase in total leukocyte count (30,000 to 300,000 cells/mm^3) with the predominant cell population consisting of lymphocytes. Additional

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criteria for determination of leukemia in these chickens were based on the appearance of large numbers of blast cells, mitotic figures, and abnormal cells of the lymphocyte, monocyte, and erythrocyte series. Hematocrit values in infected chickens were decreased at 3 and 4 weeks, returning to normal or higher than normal at 6 to 7 weeks. In another study (16), hematocrits of MD-infected chickens were found to be lower than controls after 2 weeks postinoculation, returning to normal about the 8th week.

Changes in immune response mechanisms as related with these blood cell dyscrasias have not been indicated. It has been proposed (9, 11) that, in terms of the humoral responses, the primary and secondary antibody responses are decreased in Marek's disease-infected chickens with immunoglobulin (Ig) G and IgM levels remaining normal. The cellular reactions showed: homograft reaction was normal or decreased, graft versus host reaction was normal or slightly increased, delayed hypersensitivity reaction was decreased, and the tuberculin reaction was decreased.

In the present study, evidence is presented which demonstrates that, in MD, neoplastic alterations of cells involved in antibody production occur, that these transformed cells are present in large numbers in the peripheral circulation giving rise to mononuclear cell leukemia, and, like other leukemogenic virus diseases (10, 13, 14), that MD causes a suppression of immunological responsiveness.

MATERIALS AND METHODS

Approximately 250 chickens (Athens-Canadian) were used in these studies. One-half of the test chickens were injected at 1 day of age with 0.2 ml of infective whole blood (from chickens with MD UA-1 strain; *unpublished data*) and were subsequently kept in both Horsfall-Bauer type isolators and in batteries. All control chickens were housed in Horsfall-Bauer type isolators. At 4 weeks of age, all infected and one-half of all control chickens were injected intra-abdominally with 0.4 ml of a 1:50 dilution of *Salmonella pullorum* antigen (approximately 5×10^6 organisms). At 3, 7, 14, and 21 days postinoculation, chickens were bled by heart and brachial wing vein puncture and the following measurements were made.

(i) Total white blood cell counts. An indirect count was made of chicken white blood cells utilizing the modified Pilot's stain for eosinophil counts. By using this technique, counts were made of granulocytes (heterophils and eosinophils) in a hemocytometer and the total white blood cell determinations were calculated by extrapolation after the differential counts were made. This technique was utilized because it was found that direct counts with standard staining procedures that allow counting of normal cells gave highly variable results due to the many aberrant cell types present in MD-infected chickens.

(ii) Blood smears were stained with Wright's stain. Differential counts were made by dividing the total granulocyte count by the per cent of granulocytes obtained in the differential count and multiplying this value by 100.

(iii) Hematocrit determinations. Packed cell volume determinations were obtained by collecting blood from the brachial wing vein into microcapillary heparinized hematocrit tubes and centrifuging for 5 min in an International Micro-Capillary Centrifuge. Readings were made on an International Micro-Capillary Reader.

(iv) Agglutination tests. All sera collected were tested for agglutinins to *S. pullorum* antigen by using a standard tube agglutination test. Twofold dilutions were made of the sera and the antigen/antibody mixtures were then evaluated after incubation for 2 hr (37 C) and overnight refrigeration.

RESULTS

Certain cellular responses in chickens infected with MD were determined. These responses were correlated with the chickens' ability to respond to antigenic challenge.

The frequency distribution (Fig. 1) of agglutination tests of 127 infected and 117 control chicken sera at 7, 14, and 21 days postinoculation were plotted. Values represent data taken at 1, 2, and 3 weeks postinoculation. Control titers approximate a normal distribution with the largest number of chickens giving titers of 40 to 640. Mean agglutinin titers at 1, 2, and 3 weeks after primary stimulation were 442, 244, and 132, respectively. In infected chickens, however, the distribution of titers was skewed; one segment of the population did not produce detectable antibody (the unresponsive group), whereas the remaining group has agglutinin titers predominantly in the range corresponding to those associated with the control distribution. Mean

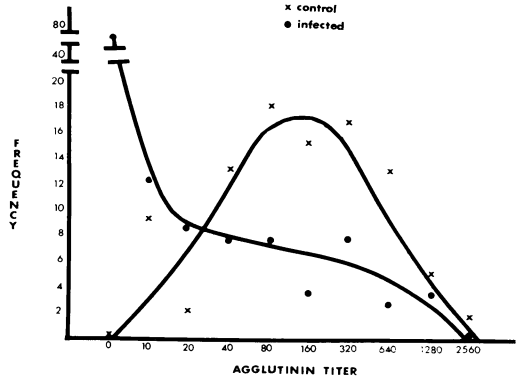

FIG. 1. *Frequency distribution of Salmonella pullorum agglutination titers.*

TABLE 1. *Agglutination titers from control and Marek's disease-infected chickens*

Treatment	No. of chickens	Reciprocal antibody titer[a] at day postinoculation		
		7	14	21
Control				
Uninjected	26	<10	<10	<10
Injected	93	442 ± 66.5[b]	244 ± 54.8[c]	123 ± 36.8[d]
Infected				
Responsive	56	285 ± 83.1	141 ± 18.4	118 ± 23.4
Unresponsive	71	<10	<10	<10

[a] Mean ± standard deviation.
[b] Significantly different from responsive group at $P = 0.001$
[c] Significantly different from responsive group at $P = 0.001$
[d] Not significantly different from responsive group.

TABLE 2. *White blood cell (WBC) and lymphocyte counts of control and infected chickens (maximum values)*

Determination	Control	Control-antigen	Responsive	Unresponsive
WBC per mm³	38,025 ±1,738[a]	40,975 ± 4,255	68,460 ± 3,678	91,319 ± 14,485
Range	33,235–43,333	25,221–60,625	18,636–182,857	44,583–247,500
Lymphocytes per mm³	30,983 ± 843	32,838 ± 1,026	55,158 ± 2,243	73,967 ± 10,114
Range	25,923–37,142	16,902–53,956	16,026–162,742	29,859–230,175

[a] Values represent mean ± standard deviation.

antibody titers of the responsive group were 285, 141, and 118 at the three testing periods (Table 1).

Forty-five per cent of all infected birds tested were responsive to primary antigenic challenge, whereas approximately 55% of infected chickens did not produce titers of 10 and were thus considered to be unresponsive. Statistical analysis (*t* test) of the mean reciprocal antibody titers of all birds tested revealed that control, noninfected chickens produced agglutinins significantly higher than the infected responsive group at 1 and 2 weeks postinoculation, but not at 3 weeks. Total white blood cell counts were taken from controls not injected with antigen, controls injected with antigen (i.e., control-antigen), and infected chickens injected with antigen. Mean white blood cell counts of control non-antigen-injected chickens were calculated at the intervals corresponding to the testing periods of antigen-injected chickens (i.e., at 3, 7, 14, and 21 days postinoculation). These values were 22,500, 23,300, 30,900, and 32,500, with the total increase over the 3-week testing period being less than 10,000 cells per mm³. Calculation of the coefficient of variation (CV) for white blood cell counts of five different chickens over the 3-week testing period revealed a 13% CV. In the control-antigen group, there was a slight increase in white blood cell counts when compared to the non-antigen-injected control group during this same testing period. Mean values of 25,400, 28,300, 34,400, and 40,400 cells per mm³ were recorded. Of the 40 chickens tested in this group, two had counts of over 50,000 cells per mm³. These two animals, however, demonstrated only a transient leukocytosis, in that their counts eventually returned to the normal range of variation. Chickens infected with MD and submitted to antigenic challenge, gave highly varied white blood cell counts. At 3, 7, 14, and 21 days postinoculation, mean values for the responsive group counts were 50,000, 38,000, 39,000, and 36,000 cells per mm³. Values for the unresponsive group for this same time period were 69,000, 58,500, 41,000, and 50,000 cells per mm³. Twenty four per cent of the white blood cell counts of the responsive chickens were greater than 50,000 cells per mm³, and 54% of the unresponsive group had counts of 50,000 or greater.

The mean maximum values for total and lymphocyte counts of all the groups were determined. This was done to account for the wide range of values in the infected groups, some of which were within the normal range. Also, this would take into consideration the small number of high counts associated with the control groups. Control group mean values of 35,025 and 40,975 cells per mm³ for the lymphocyte counts were not significantly different (ranges of 25,000 to 60,000 for white blood cell counts; Table 2). Corresponding values for infected chickens equaled

and exceeded control values. Counts of 100,000 cells per mm^3 for white blood cell counts and lymphocyte counts were encountered with relatively high frequency. Mean white blood cell counts of 68,460 and 91,319 and mean lymphocyte counts of 55,158 and 73,967 were significantly higher ($P = 0.05$) than control values.

In general, a linear relationship existed between total leukocyte and lymphocyte counts in all animals tested. Many infected chickens, however, had very large numbers of lymphocytes in their peripheral circulation. Thirty-four per cent or 10 of 29 responsive chickens had lymphocytic leukemia (i.e., lymphocyte counts greater than 50,000 cells per mm^3), with some counts exceeding 100,000 lymphocytes per mm^3.

Thirty-eight per cent or 12 of 32 unresponsive chickens had lymphocyte counts exceeding this minimum value. A larger number of chickens in this group had high counts, however, with one having 230,175 lymphocytes per mm^3.

Some chickens with MD had abnormal increases in granulocytic elements (over the normal range of 2,500 to 7,500 granulocytes per mm^3), especially the heterophils. Seven unresponsive and three responsive infected chickens had granulocytic increases amounting to 39 to 81% of the total white blood cell count, compared to the normal 20 to 30% ranges characteristic of controls. In absolute numbers, this amounted to a range of 11,649 to 61,583 cells per mm^3. Myeloid proliferation was not, however, characteristic of the blood cell picture in general.

Differential counts of control non-antigen-injected chickens revealed heterophil-lymphocyte proportions of approximately 18 and 76%, respectively. Control antigen-injected chickens exhibited a similar type of relationship.

Blood smear examination of control blood (Fig. 2) revealed that the predominant lymphocyte population consisted of small- to medium-sized cells (a and b). Control-antigen smears showed increased numbers of small- to medium-sized lymphocytes, many with cytoplasmic blebs (c). Very few blast cell types were observed in either of the control groups, and the large lymphocyte population (d) was comparatively small. Cells (e) and (f) are a heterophil and basophil, respectively.

Both responsive and unresponsive infected groups exhibited characteristic white blood cell increases (Fig. 3). Increased numbers of large lymphocytes with cytoplasmic extrusions (a and b), abnormal red blood cells, immature granulocytes (band cells), and large blast cells (c and d) with very basophilic staining cytoplasm were all found to be associated with the disease. Cells e, f, g, and h are a heterophil, basophil, small lymphocyte, and medium-sized lymphocyte, respectively.

A few blast cells were found on examination of control blood (means of 336 and 626), but very large numbers of blast cells were observed in the infected groups (Table 3). Relative blast cell counts in unresponsive infected chickens were significantly higher than in similarly treated but responsive birds.

Determination of packed cell volume revealed no distinct correlations between responsiveness to antigenic challenge, total white blood cell counts, or differential counts. However, several infected chickens were anemic throughout the testing period. Thirty per cent of the unresponsive infected chickens were anemic, compared to 18% of the responsive birds (Fig. 4). Many infected chickens had greatly increased hematocrits over those of control values. In addition, unresponsive chickens showed very wide ranges of hematocrit values of up to 61% packed cell volume as compared to control ranges of 7 to 8%.

At 7 weeks of age, all test animals were killed and examined for gross lesions (i.e., lesions in the liver, gonads, kidneys, spleen, lungs, and muscles, enlarged brachial plexis, vagus, and sciatic nerves, as well as skin lesions). Control chickens had no gross lesions, but infected chickens which were injected with antigen were more susceptible to the pathological effects of Marek's disease than similarly treated chickens which did not receive antigen (Table 4).

Mortality during the testing period and percentages of gross lesions were higher in unresponsive than responsive chickens.

DISCUSSION

The response of control and MD-infected chickens to primary stimulation with *S. pullorum* antigen was manifested by the appearance of three different groups. The first group consisted of control, noninfected chickens which had circulating agglutinins with a wide range (10 to 3,260) of normally distributed titers. In the infected groups, however, there were two different response populations which are termed responsive or unresponsive. Infected chickens either responded to primary insult with antibody titers significantly lower than those associated with the control group, or they were completely unresponsive. This suppression of antibody response mechanisms in this unresponsive group could not be considered merely a depression, because throughout the testing periods those animals which were found to be initially unresponsive remained so until termination of the experiment or death of the

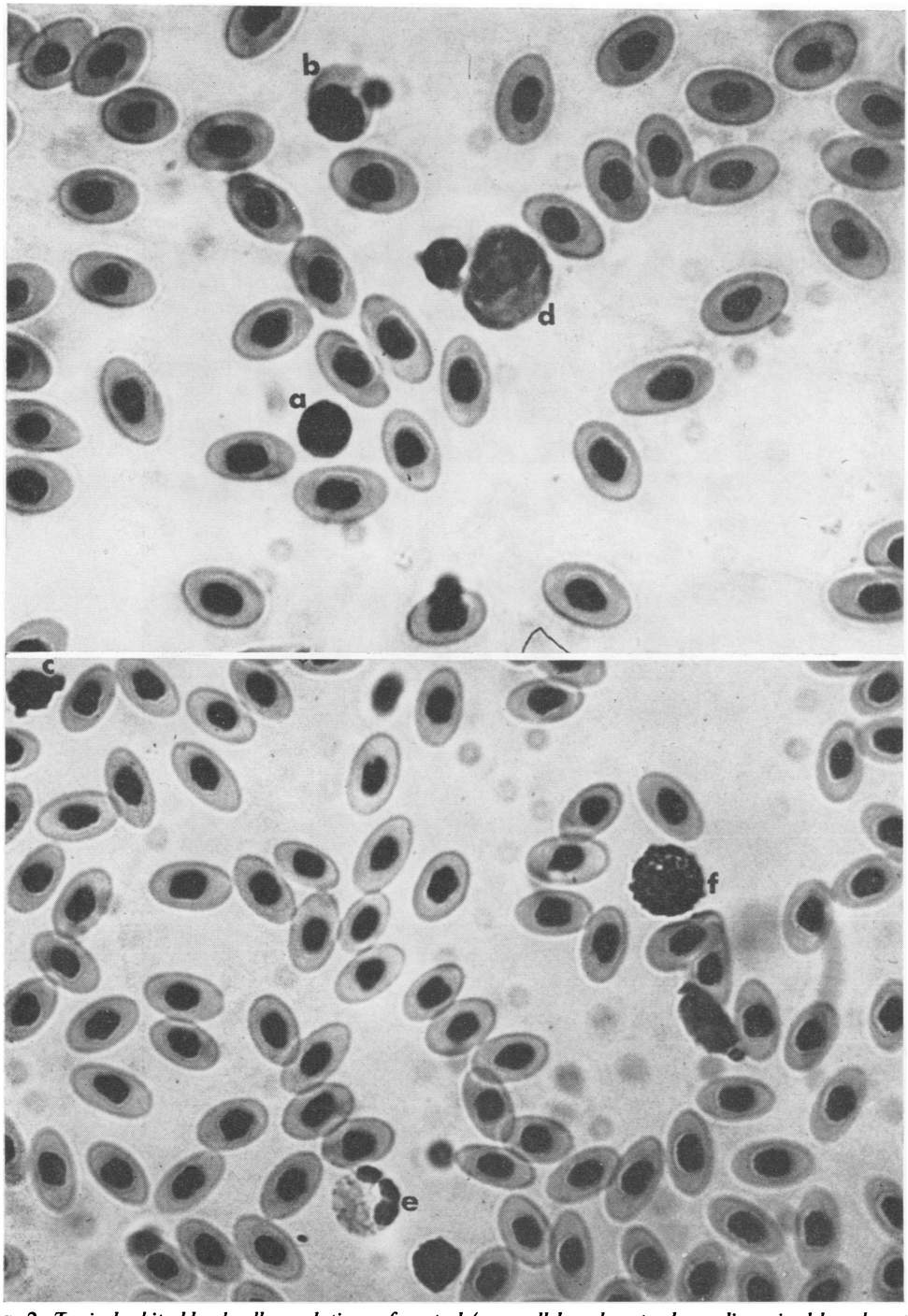

FIG. 2. *Typical white blood cell populations of control (a, small lymphocyte; b, medium-sized lymphocyte; d, large lymphocyte) and control-antigen (c, small lymphocyte with cytoplasmic blebs; e, heterophil; f, basophil) chickens. Wright's stain, ×750.*

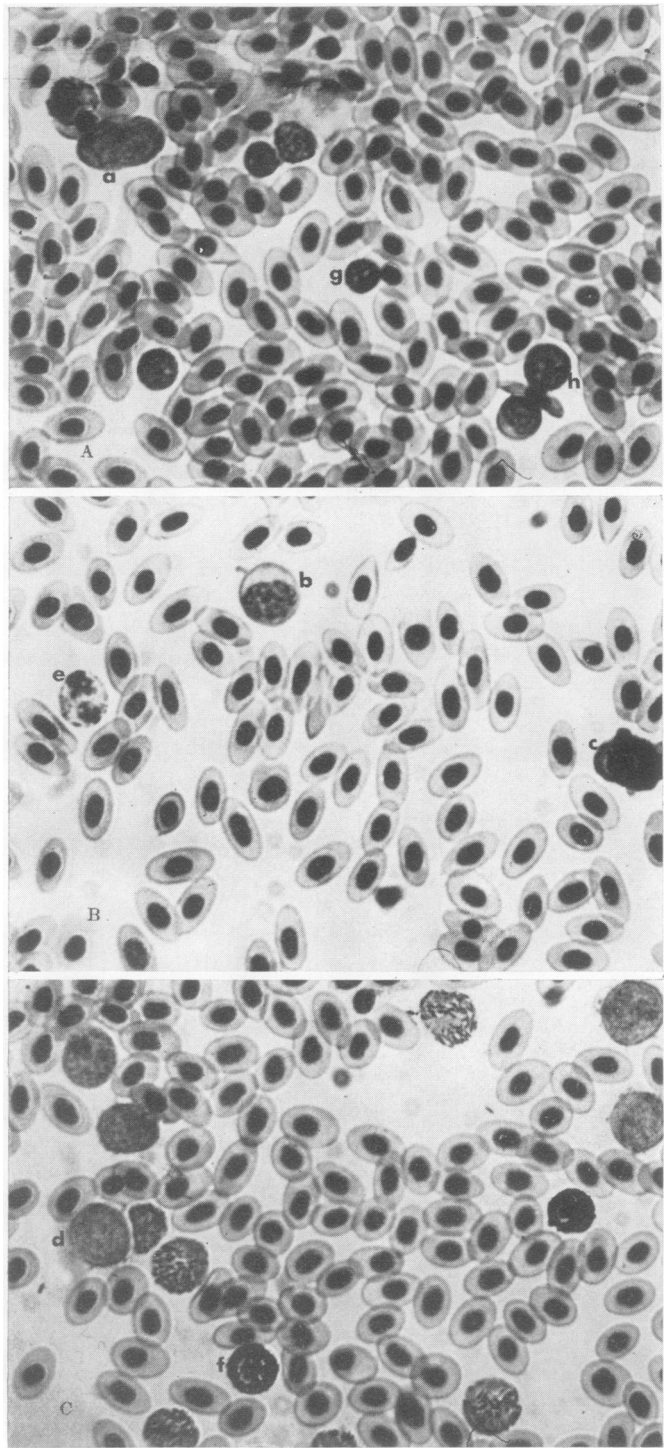

FIG. 3. *White blood cells from Marek's disease-infected chickens. (A) a, large lymphocyte; g, small lymphocyte; and h, medium-sized lymphocyte. (B) b, large lymphocyte; c, large blast cell; and e, heterophil. (C) d, blast cell; and f, basophil. Wright's stain, ×750.*

TABLE 3. *Blast cell counts of control and infected chickens*

Determination	Blast cells per mm³			
	Control	Control-antigen	Responsive	Unresponsive
Mean	336	626	1,730	2,975
Range	293–380	202–1,860	132–8,412	400–20,285

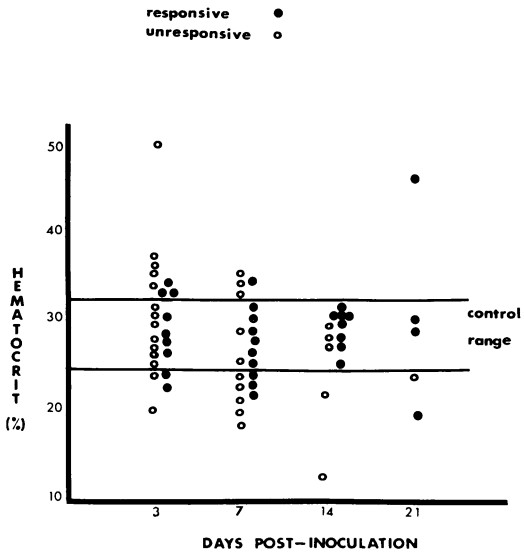

FIG. 4. *Relationship of response to packed cell volume in control and Marek's disease-infected chickens.*

animal. Previous work has shown (9) that MD causes a decreased primary antibody response to soluble antigens. In the only other report concerned specifically with determination of immunologically deficient conditions in MD (11), antibody responses to both soluble and particulate antigens in MD-infected chickens were lower than controls. Some (4 of 29) of the infected chickens were completely unresponsive to antigenic challenge. In this study, the phenomenon of unresponsiveness was found to be characteristic of at least one-half of all infected birds in four different experiments utilizing more than 200 chickens. Even in the responsive infected group there was a significant depression of immunological reactivity.

Other oncogenic and nononcogenic virus diseases have been found to cause a depressed immunological response (13); among these, Friend, Rowson-Parr, and Rauscher viruses affect the primary and secondary responses in mice, and Gross and Moloney murine leukemias have a depressive effect on primary responses. Junin,

TABLE 4. *Effect of antigen injection on acute leukosis*

Antigen injected	Immune response	Mortality (%)	Gross lesions (%)
Yes	Unresponsive	80	93
Yes	Responsive	63	81
No		28	36

lymphocytic choriomeningitis virus, murine cytomegalovirus, measles, influenza virus, and rubella have all been implicated in causing general depressions in immunological competence.

In the present study, alterations in the peripheral blood cell picture concomitant with the progression of MD-induced leukemia were correlated with immunological responsiveness. To measure both the MD-specific and antigen-specific induced cellular changes, two groups of control chickens were used. One consisted of the disease control and the other maintained as the antigen control.

Antigen injection in control chickens caused a slight leukocytosis, but white blood cell counts that were consistently above 50,000 cells per mm³ in infected chickens were considered leukemic. The normal range (although some counts were outside this range) for total white blood cell counts for control chickens was 20,000 to 30,000 cells/mm³ with a coefficient of variation within experimental animals of 10 to 20%. Other work (8) has shown that increases in total leukocyte counts (up to 300,000 cells per mm³) are associated with MD. In the present study, white blood cell counts of the infected chickens at 1, 2, and 3 weeks postinoculation with antigen were significantly higher than control values.

Descriptions of blood cellular changes in leukosis (2, 4, 5, 15, 17) have previously been described as consisting of total white blood cell count increases. These studies did not differentiate between MD and diseases of the avian leukosis complex, so specific induced changes have not previously been determined.

Correlation of white blood cell counts with responsiveness to antigenic challenge in MD-infected chickens revealed that twice as many unresponsive chickens (54%) were leukemic as similarly treated but responsive chickens (24%). This indicated a possible correlation between ability to commit functional antibody-producing cells and susceptibility to neoplastic alterations of precursor cells, since it has been shown that blast cells and immature plasma cells contain antibodies but do not release the antibody until they reach a fully differentiated stage (1). In the unresponsive group, those cells which synergistically function

in either antigen recognition or production of specific antibody are more deeply involved by invasion of the transforming infectious agent(s) than similar cells in the responsive chickens. Alternatively, unresponsive chickens could be more readily predisposed to secondary invasion by neoplastic cells and thus exhibit higher incidences of leukemia.

Examination of blood smears for characterization of cell changes in the different groups showed that in both control groups there were some cell type differences. As would be expected, the lymphocyte populations differed in that the control group which received antigen had increased numbers of medium-sized lymphocytes with cytoplasmic blebs. These extrusions serve to release various gamma globulins into the serum and have been previously termed stimulated lymphocytes (6). The lymphocyte population of non-antigen-injected chickens consisted primarily of smaller type lymphocyte. The occurrence of abnormal blood cellular constituents in MD-infected chickens was very apparent. The most characteristic of these cell dyscrasias were populations of very large mononuclear cells; specifically, the large lymphocyte and blast cell (hemocytoblast) types were found in abundance.

In terms of response to antigenic insult, unresponsive chickens had significantly higher numbers of circulating blast cells than responsive infected chickens. Both of these groups had significantly more of these cells than controls. Blast cells represent a circulating transitional cell-type which are in the process of differentiating into any one of many different types of cells. The possibility exists that in MD these cells are unable to undergo complete differentiation into antibody-forming cells. This may be reflected quantitatively in that the unresponsive group of chickens had significantly more blast cells than the responsive group, which only manifested a depressed antibody response when compared with controls.

One of the common sequelae associated with bone marrow transformations induced by acute leukemia is the occurrence of anemia. The leukemia of MD causes a transient anemia (4, 8, 16, 17). Chickens with the high hematocrit values could possibly reflect bone marrow erythropoetic alterations induced by the MD agent.

Finally, infected chickens which were injected with antigen were more susceptible to the pathological effects of Marek's disease. It has been suggested (12) that a depression of response mechanisms was a prerequisite for neoplastic growth conditions for some of the murine leukemias.

LITERATURE CITED

1. Abramoff, P., and N. B. Brien. 1968. Studies of the chicken immune response. I. Correlation of the cellular and humoral immune response. J. Immunol. **100**:1204–1209.
2. Blakemore, F. 1934. The leucocytes of fowl blood with special reference to fowl paralysis. Vet. Rec. **14**:417–422.
3. Cho, B. R., S. G. Kengy, and U. H. Kim. 1968. Atypical cells in the peripheral blood of chickens exposed to Marek's disease agent. Can. J. Comp. Med. **32**:562–567.
4. Furth, J. 1935. Lymphomatosis in relation to fowl paralysis. Arch. Pathol. **20**:379–428.
5. Johnson, E. P., and B. V. Conner. 1933. Blood studies of fowls with various forms of lymphomatosis (fowl paralysis). J. Amer. Vet. Med. Ass. **36**:324–343.
6. Lucas, A. M., and C. Jamroz. 1961. Atlas of avian hematology. Agr. Monogr. no. 25:50, U.S. Department of Agriculture, Washington, D.C.
7. Pappenheimer, A. M., L. C. Dunn, and V. Cone. 1929. Studies on fowl paralysis (neurolymphomatosis gallinarum). I. Clinical features and pathology. J. Exp. Med. **49**:63–86.
8. Patterson, L. T., S. K. Wade, D. L. Evans, and J. N. Beasley. 1969. Hematological changes in acute leucosis (Marek's disease). Poultry Sci. **48**:1857.
9. Payne, L. N. 1970. Immunosuppressive effects of avian oncogenic viruses. Proc. Roy. Soc. Med. **63**:16–19.
10. Peterson, R. A., R. Hendrickson, and R. A. Good. 1963. Reduced antibody forming capacity during the incubation period of passage A leukemia in C3H mice. Proc. Soc. Exp. Biol. Med. **114**:517–520.
11. Purchase, H. G., R. C. Chubb, and P. M. Biggs. 1968. Effect of lymphoid leukosis and Marek's disease on the immunological responsiveness of the chicken. J. Nat. Cancer Inst. **40**:583–592.
12. Salaman, M. H. 1967. The effect of some leukemogenic viruses on immune reactions. Proc. 3rd Symp. Comp. Leukemia Res. **31**:92–96.
13. Salaman, M. H. 1970. Immunodepression by mammalian viruses and plasmodia. Proc. Roy. Soc. Med. **63**:11–15.
14. Salaman, M. H., and N. Wedderburn. 1966. The immunodepressive effect of Friend virus. Immunology **10**:445–458.
15. Seagar, E. A. 1933. Cellular reactions in the blood in neurolymphomatosis gallinarum (fowl paralysis). Vet. J. **89**:557–561.
16. Vickers, J. H., C. F. Helmoboldt, and R. E. Luginbuhl. 1967. Pathogenesis of Marek's disease (Connecticut A. isolate). Avian Dis. **11**:531–545.
17. Warthin, A. S. 1907. Leukemia of the common fowl. J. Infec. Dis. **4**:369–381.