The Effect of B-Cell Immunosuppression on Age-related Resistance of Chickens to Marek’s Disease

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SUMMARY

Chickens were bursectomized by cyclophosphamide treatment at hatching. At 8 or 9 weeks of age, bursectomized and unbursectomized hatchmates, free from prior infection, were challenged with pathogenic Marek’s disease virus. Oncogenicity of the virus inoculum was confirmed by inoculating 1-day-old susceptible chickens. At the time of virus challenge, blood cells from the cyclophosphamide-treated chickens were able to mount a vigorous graft-versus-host reaction in allogeneic embryos. This ability indicated that the thymus function was intact. There were no significant differences in Marek’s disease response of bursectomized and unbursectomized chickens, in spite of a severe defect in the bursa-dependent functions in the bursectomized chickens. Some bursa-deficient chickens had non-proliferating, presumably regressing lesions in peripheral nerves. Because these lesions lacked plasma cells, it was concluded that the plasma cell may not play a functional role in recovery from Marek’s disease.

INTRODUCTION

Susceptible chickens develop extensive lymphoproliferation upon exposure to MDV. The lymphoproliferation most commonly occurs in peripheral nerves and often results in paralysis and death of affected chickens. However, chickens develop natural resistance to MD with increasing age (2, 3, 5, 24, 26, 30, 32). The exact age at which resistance becomes operative seems to vary with different genetic lines of chickens (5, 6) and is perhaps dependent upon several unknown factors.

As a result of the observation that chickens exposed to MDV at 8 weeks of age or older did not succumb to clinical MD, although many had microscopic lesions 10 weeks after inoculation, Witter et al. (32) postulated that age resistance to MD may be mediated through lesion regression. This conclusion was confirmed in a subsequent study (26) in which 2 age groups of chickens, 12 week old and 1 day old, were simultaneously inoculated with MDV, and a representative number of chickens in each age group were examined on a chronological basis for lesions of MD. The results clearly revealed that gross and microscopic lesions developed in both age groups. However, in the older chickens, the lesions disappeared and the chicken survived, whereas, in the younger chickens, a high rate of mortality was accompanied by a continued presence of gross and microscopic lesions. Although the phenomenon of lesion regression in MD had been noted earlier (Refs. 1 and 16; R. L. Witter, unpublished data), our observation provided the 1st experimental model that could be exploited to investigate the basic mechanisms underlying lesion regression.

In our initial study (26), there were no striking differences in the incidence and levels of anti-MD antibody between the groups of chickens that regressed MD lesions and those that did not. In contrast, however, a positive role of humoral immune competence was evident in Calnek’s study (5) in which acquisition of age resistance seemed to parallel the ability of chickens to produce antibody. Thus, the involvement of humoral immunity in age-related resistance to MD needed further clarification.

In this paper, we describe our attempts to obtain direct evidence for the role of humoral immunity in MD resistance by studying the response of older chickens rendered deficient in the bursa-dependent functions by cyclophosphamide treatment. The results indicated that such immunodeficient chickens remained fully resistant to MD.

MATERIALS AND METHODS

MDV. The 20th cell culture passage of a cell-associated preparation of clone 19 of the JM strain (20) of MDV was used. This virus, highly pathogenic for susceptible chickens, was propagated and assayed in duck embryo fibroblasts (33).

Chickens. White Leghorn chickens, highly susceptible to MD at hatching, were a cross between lines 15 and 7 (15 x 7) (29) being maintained at this laboratory. Parent stock survived a natural exposure to MDV; thus, the progeny possessed MD antibody at hatching.

Newly hatched chickens either were used at 1 day of age or were held until 8 or 9 weeks of age in vinyl canopy isolators maintained at a positive pressure with filtered air. Before deliberate virus challenge, older chickens were tested for freedom from inadvertent exposure to MDV. Fifteen to 17% of chickens in each isolator were tested for viremia and...
AGP antibody (26). Because cyclophosphamide-treated birds were not expected to respond by antibody production, they were tested for MDV infection by viremia alone in the 1st experiment. In the 2nd experiment, 5 untreated hatchmates were intermingled with the cyclophosphamide-treated birds, and at the time of virus challenge, the untreated birds were tested for freedom from virus infection by viremia and antibody. In both experiments, all chickens were free from MDV infection at the time of virus challenge.

**Cyclophosphamide Treatment.** Chickens were inoculated intraabdominally with a total of 12 mg of cyclophosphamide (Cytoxan; Mead Johnson Laboratories, Evansville, Ind.),2 1 injection of 3 mg at each of the 1st 4 days after hatching. The cyclophosphamide-treated and untreated birds were monitored for B- and T-cell functions. The T-cell function was tested by measuring the ability of 8- or 9-week-old cyclophosphamide-treated birds to mount a graft-versus-host reaction in chicken embryos of unrelated genetic source. Several parameters were used to assess the B-cell function: (a) the ability to produce antibodies against MDV (tested by the AGP and immunofluorescent tests), (b) the presence of germinal centers in cecal tonsils; and (c) morphological alterations of lymphoid cell elements in the bursa of Fabricius.

**Graft-versus-Host Reaction.** Blood was obtained by venipuncture and mixed 5:1 with a 3% solution of sodium citrate. Two-tenths ml of citrate whole blood from each chicken was deposited on the artificially dropped chorioallantoic membrane of each of eight 12-, or 13-day-old chicken embryos of commercial source (purchased as unincubated fertile eggs from the Rainbow Trail Farms, St. Johns, Mich.). Five days after inoculation, spleens from individual embryos were weighed and mean weights were determined for each group.

**Seroology.** Procedures for AGP test and indirect immunofluorescent tests have been described (7, 19).

**Experimental Plant.** Two similar experiments were conducted. The various experimental groups are given in Table 2. Briefly, in the 1st experiment, 2 groups of 63-day-old and 1 group of 1-day-old chickens of cross 15 × 7, free from prior MD exposure, were simultaneously inoculated intraabdominally with 5.2 × 10⁸ plaque-forming units of MDV. One of the 2 older groups had been treated with cyclophosphamide at hatching. One group each of 63-day-old and 1-day-old chickens were left as uninfected controls. An additional uninfected control in the older group included 12 cyclophosphamide-treated chickens. Each infected or uninfected group was housed in a separate isolator maintained at a positive pressure with filtered air. Chickens dying during the 1st week of life were excluded from the data. In those dying thereafter, if diagnosis could not be reached on gross examination, sections of the right vagus nerve, right brachial and sciatic plexuses, and gonads were examined for histological lesions of MD. The experiment was terminated 11 weeks after MDV challenge. At that time, serum samples obtained from surviving chickens in each age group (including controls) were tested for MD antibody by the AGP and immunofluorescent tests, and all chickens were examined for gross and microscopic lesions of MD. In certain groups, in addition to the sections of peripheral nerves and gonads, bursa and cecal tonsils were also sectioned and stained with hematoxylin and eosin or methyl green pyronine. The bursas were examined for lymphoid elements in the follicles and the number of germinal centers present in a cross-section through a cecal tonsil was determined.

The 2nd experiment was similar in design to the 1st, except that the older groups were 56 days of age at the time of MDV inoculation and the inoculum consisted of 7.9 × 10⁸ plaque-forming units per chicken. The responses in both experiments were analyzed by the χ² method.

**RESULTS**

**Effects of Cyclophosphamide Treatment.** Because the main objective of this study was to determine the effect of B-cell deficiency on resistance to MD, it seemed important to carefully assess the effectiveness of cyclophosphamide in accomplishing adequate bursectomy. The effectiveness was assessed by comparing the B-cell functions of chickens that received cyclophosphamide at hatching and MDV at 8 or 9 weeks of age with those of chickens that received MDV alone at 8 or 9 weeks.

**Bursa of Fabricius.** In general, bursal morphology in the cyclophosphamide-treated group was altered as described by Linna et al. (14). In contrast to the untreated group, bursa of Fabricius in the cyclophosphamide-treated group was consistently much smaller in size, and the plicae were thin and ill defined. In some treated birds, grossly identifiable bursal tissue was missing although, by taking a section of the area of the usual location of bursa, microscopic remnants of bursal tissue could usually be seen.

Histologically, the bursal wall and follicular reticulum were intact, but in most chickens the follicles were devoid of lymphoid cell elements (Figs. 1 and 2). Fibrosis was quite prominent. Of 51 cyclophosphamide-treated chickens whose bursal remnants were examined microscopically, in 48%, all follicles in the sections examined had undergone lymphoid cell degeneration (Fig. 2). In 40%, an occasional follicle either escaped degenerative changes and appeared normal or was partly degenerated (Fig. 3); in 12%, bursal tissue appeared morphologically intact. However, birds with partly intact bursal tissue did not necessarily produce detectable antibody to MDV. Among chickens that had occasional morphologically normal follicles in the cross-sections of the bursa, only 3% had antibody; whereas among cyclophosphamide-treated chickens with morphologically intact bursa, 83% had antibody.

Bursal degenerative changes described above could not be attributed to the effects of MDV (12, 13, 18) because a similar spectrum of changes was observed in 24 control chickens that were treated with cyclophosphamide but not

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Resistance to MD in Immunosuppressed Chickens

with MDV, and bursas of cyclophosphamide-free chickens exposed to MDV at 8 or 9 weeks of age were morphologically normal in appearance at the end of the experiment.

Germinal Centers and Antibody Production. Cecal tonsils represent nodular collections of lymphocytes in the gut wall near the entrance of the ceca. The bursa-dependent elements in the cecal tonsils are represented by plasma cells and germinal centers. We counted the number of germinal centers in a cross-section through 1 tonsil as a quantitative measure of peripheralized bursal cells. Although this quantification was at best subjective because of the variation in size of the tonsils among birds, there was an excellent correlation between the presence of germinal centers in the tonsils and the ability of chickens to produce antibody (Charts 1 to 3). Of a total of 51 cyclophosphamide-treated birds examined in both experiments, 45 lacked germinal centers in cecal tonsils. Of these 45 chickens, 44 were examined for antibody for MDV, and all were negative by the AGP as well as the immunofluorescent test. Of the 6 chickens that had germinal centers in their cecal tonsils, 5 had MD antibody. Germinal centers and MD antibody were consistently detected in all of the 52 MDV-inoculated chickens that did not receive cyclophosphamide.

The MD antibody responses of cyclophosphamide-treated and untreated groups are compared in Charts 2 and 3. Of 53 cyclophosphamide-treated chickens in both experiments, 47 lacked antibody detectable by the immunofluorescent test (Chart 2); 51 were negative by the AGP test (Chart 3). Variable titers of antibody were detected in all chickens in the groups that were not treated with cyclophosphamide.

Graft-versus-Host Reaction. When blood from immunologically competent chickens is injected into allogeneic embryos, a graft-versus-host reaction occurs, manifested by splenomegaly (8, 27). Because this reaction is based on an immunological attack mounted by thymus-dependent cells (9), the graft-versus-host reaction has been often used to
was not significant in individual experiments, the $x^2$ was 7.14 that cyclophosphamide treatment had no effect on the ability of chickens treated with cyclophosphamide at hatching to lack MD antibody and thus were included in the bursectomized groups. If the chickens that lacked antibody and germinal centers were assumed also to lack MD antibody and thus were included in the bursectomized groups.

Bursectomy in both experiments did not significantly alter the incidence of MD. In Experiment 1, the MD response of 21 bursectomized chickens was compared with that of 27 chickens with intact bursa. The bursectomized group had a total MD incidence of 33\%, compared with an incidence of 59\% in the unbursectomized group ($p > 0.05$). In Experiment 2, the MD incidence of 7 and 26\%, respectively ($p > 0.05$). Simultaneously inoculated 1-day-old chickens of both the treated and untreated groups were examined to determine whether the lesions were of the same genetic background as the older groups developed a 90\% or higher incidence of MD in both experiments. These results indicate that the MDV inoculum was highly pathogenic and confirm our earlier finding (32) that the reduced MD response especially evident at the level of mortality and gross lesions in older birds was age dependent. Although the fact that there was a lower incidence of total MD (particularly of nonproliferative lesions) in the groups treated with 12 mg of cyclophosphamide than in the untreated groups was not significant in individual experiments, the $x^2$ was significant if the data from the 2 experiments were combined ($x^2 = 5.86; p < 0.05$). This combined difference may be due to cyclophosphamide although, in a previous study (21), MD was ameliorated with high doses of cyclophosphamide (16 to 20 mg) and was apparent only at the level of gross lesions; the total incidence of MD remained unaffected. The mechanism of the effect of cyclophosphamide on lymphoma formation in MD needs clarification.

In Experiment 1, the bursectomized birds had a higher incidence of mortality and gross lesions than the unbursectomized hatchmates. However, these differences were not significant in this experiment ($p > 0.05$) and did not exist in the 2nd experiment.

Nerve sections of all chickens diagnosed as having MD were examined to determine whether the lesions were of the proliferative or nonproliferative (presumably regressing) type. The criteria used to distinguish between proliferative and nonproliferative lesions were the same as described before (26). As we have reported previously (26), a proliferative lesion was considered to be characterized by the presence of small and medium lymphocytes mixed with blast cells and MD cells (16), whereas lesions containing no blast cells or MD cells but some plasma cells were considered nonproliferative. In both experiments, the incidence of proliferative lesions in bursectomized groups tended to be slightly higher than in unbursectomized birds, but these differences were not significant ($p > 0.05$). Notably, nonproliferative lesions were present in the bursectomized groups in the absence of plasma cells.

**DISCUSSION**

Immune surveillance in tumors, i.e., the concept that the prevention of development of a neoplasm is determined by the immune competence of the host, has been noted in many tumor systems (4, 28). If resistance to MD has an immunological basis, our results strongly suggest that, in this disease, cell-associated immune mechanisms rather than the humoral factors may play the principal role. Chickens treated with cyclophosphamide were fully capable of overcoming infections with a virulent MDV inoculum in the absence of detectable antibodies. Thus, assuming that age resistance in this study was expressed through lesion regression (32, 26), it may be concluded that lesion regression in MD proceeds independent of antibody directed against the virus or virus-induced antigens.

Similar evidence for the lack of involvement of humoral immunity in innate resistance was also obtained in another study in which surgically bursectomized, agammaglobulinemic chickens of genetically resistant Line 6 remained refractory to virulent MDV (25). This observation was confirmed in Line 6 and in Line N (another genetically resistant line) by additional experiments in which chickens were bursectomized by cyclophosphamide treatment (J. M. Sharma, unpublished data). On the other hand, we recently noted (21) that herpesvirus of turkeys failed to confer immunity against MD in chickens pretreated with high doses of cyclophosphamide. Although a transitory defect in the T-cell function in the initial stages of cyclophosphamide

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Charts 2 and 3 show the titers of immunofluorescent (IF) and AGP antibody to MD, respectively.

<table>
<thead>
<tr>
<th>Chart 2</th>
<th>IF Antibody Titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expt. 1</td>
<td>~320, 160, 80, 40, 20, &lt;20</td>
</tr>
<tr>
<td>Expt. 2</td>
<td>~320, 160, 80, 40, 20, &lt;20</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chart 3</th>
<th>AGP Antibody Titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expt. 1</td>
<td>8, 4, 2, 1, 0</td>
</tr>
<tr>
<td>Expt. 2</td>
<td>8, 4, 2, 1, 0</td>
</tr>
</tbody>
</table>
### Table 1

**Effect of cyclophosphamide treatment on the graft-versus-host reaction**

Whole citrated blood (0.2 ml) was deposited on the chorioallantoic membrane of 13- (Experiment 1) or 12-day-old (Experiment 2) embryos. Spleens were weighed 5 days after inoculation.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>No. of chickens</th>
<th>Cyclophosphamide (12 mg)</th>
<th>No. of enlarged spleens/total no. of spleens examined</th>
<th>Spleen wt (mg) of embryos*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4</td>
<td>+</td>
<td>12/13</td>
<td>104.1 (79.7-136.0)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>-</td>
<td>15/18</td>
<td>97.1 (77.0-122.4)</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>+</td>
<td>27/30</td>
<td>40.1 (32.6-49.3)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>-</td>
<td>21/28</td>
<td>42.3 (33.5-53.5)</td>
</tr>
</tbody>
</table>

*Chickens were treated with cyclophosphamide during the 1st 4 days of hatching. The graft-versus-host test was done when chickens were 9 weeks (Experiment 1) or 8 weeks (Experiment 2) of age.

* Mean spleen weight of un inoculated embryos was 10.4 mg and 12.4 mg in Experiments 1 and 2, respectively.

### Table 2

**The effect of B-cell function deficiency on the response of 15 × 7 chickens exposed to MDV at 56 or 63 days of age**

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>B-cell function</th>
<th>% MD</th>
<th>Lesion morphology* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDV (12 mg) Age (days) MD antibody* Germinal centers in cecal tonsils*</td>
<td>Dead* Gross lesions at termination* Total*</td>
<td>Proliferative</td>
<td>Nonproliferative</td>
</tr>
<tr>
<td>1</td>
<td>21</td>
<td>+</td>
<td>63</td>
</tr>
<tr>
<td>27</td>
<td>+</td>
<td>63</td>
<td>26/26</td>
</tr>
<tr>
<td>27</td>
<td>+</td>
<td>1</td>
<td>6/6</td>
</tr>
<tr>
<td>10</td>
<td>+</td>
<td>63</td>
<td>0/8</td>
</tr>
<tr>
<td>14</td>
<td>+</td>
<td>1</td>
<td>0/6</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>28</td>
<td>56</td>
</tr>
<tr>
<td>30</td>
<td>4</td>
<td>56</td>
<td>29/30</td>
</tr>
<tr>
<td>59</td>
<td>4</td>
<td>1</td>
<td>4/5</td>
</tr>
<tr>
<td>10</td>
<td>+</td>
<td>56</td>
<td>0/6</td>
</tr>
<tr>
<td>15</td>
<td>+</td>
<td>1</td>
<td>0/6</td>
</tr>
</tbody>
</table>

* A proliferative lesion was characterized by the presence of blast cells and MD cells, whereas a nonproliferative lesion lacked these 2 types of cells.

* Antibody was tested by AGP and immunofluorescent tests.

* A cross-section through a cecal tonsil was examined in each chicken.

* Chickens that died during the observation period and had gross or microscopic lesions of MD.

* Chickens that died with gross lesions + those found to have gross lesions when killed at the end of the experiment.

* Total, chickens in the above 2 categories + those with microscopic lesions at the end of the experiment.

* Number positive/total tested.

* NT, not tested.

Treatment (14, 23) may have contributed to the lack of vaccine protection, cyclophosphamide-treated birds were severely deficient in the bursa-dependent functions at the end of the experiment.

Firm conclusions on the mechanism(s) of various resistance models in MD (i.e., age related, genetic, and vaccine-induced resistance) must await a better understanding of the pathogenesis of the disease. Although MD has been generally considered a neoplasm, this view has not attained uniform acceptance: 1st, because the nature of the target cell in MD, i.e., the cell that undergoes neoplastic transformation or provokes lymphoid cell proliferation, is not clearly established; and 2nd, because the lesion morphology in MD in some chickens has an inflammatory rather than a neoplastic appearance. However, it was recently shown (11, 22) that most lymphoid cells constituting MD lesions are of T origin, mixed with a minor proportion of B-cells. Our results are consistent with the above observation that T-cells are the principal participants in MDV-induced lesions because, in this study, proliferative lesions developed in
chickens deficient in B-cells. Similarly, hormonal or surgical bursectomy does not affect the incidence of MD (6, 10, 15, 17).

The plasma cell is a common accompaniment of MD lesions in chickens (16, 31). The studies of Payne and Biggs (16) on the chronological development of MD lesions indicated that, in most susceptible chickens, the lymphoid cell infiltration is progressive and results in clinical disease and death. However, in some chickens the initial proliferative lesion does not progress to gross tumor formation but assumes an inflammatory appearance and regresses. The inflammatory type, presumably regressing lesion is characterized by the marked presence of plasma cells. In birds infected at an older age also, we noted that plasma cells were often present in a nonproliferative lesion (26). Because plasma cells are principally involved in antibody production, the appearance of these cells in a nonproliferative lesion has implied that antibody may be actively involved in the pathogenesis of MD, particularly in lesion regression. Our results contradict the prevailing role attributed to plasma cells in MD pathogenesis. Because lesions presumably regressed in the absence of plasma cells in cyclophosphamide-treated chickens, the presence of plasma cells apparently does not serve a functional role in recovery from MD lesions. Nonproliferative lesions in cyclophosphamide-treated birds consisted of a collection of small and medium lymphocytes and lacked blast-type cells as well as plasma cells.

These studies also confirmed our earlier reports on age-related resistance to MD. The low incidence of mortality due to MD in the older chickens was in contrast to the high incidence of mortality in the simultaneously infected 1-day-old chickens. These results demonstrated the existence of age-dependent resistance in this disease. Many older birds had microscopic lesions at the end of the experiment, but most of these lesions were of a nonproliferative, regressive type as compared with a high incidence of progressive proliferative lesions in the younger age group. These observations support earlier findings that age resistance is expressed mainly at the level of mortality and gross tumor formation and is mediated through lesion regression (26, 32).

Cyclophosphamide was the method of choice for inducing B-cell deficiency because this drug is easier to administer than the laborious surgical procedures and eliminates not only the stem cells residing in the bursa of Fabricius but also the cells that peripheralize into other areas in the body (14). Indeed, the B-cell deficiency rendered by cyclophosphamide treatment was quite complete and long lasting. Most of the chickens treated with cyclophosphamide remained incapable of producing antibody and had no detectable evidence of B-cells at peripheral sites, such as in cecal tonsils. In fact, there was an excellent correlation between the lack of production of antibody and the absence of germinal centers in cecal tonsils (14). Germinal centers in the cecal tonsil were not found in any of the antibody-free chickens, whereas germinal centers could be consistently found in the presence of antibody. Although cyclophosphamide reportedly causes lymphoid depletion in the thymus (14), this defect occurs early in the treatment and is temporary, and full T-cell function is restored within a few weeks of treatment with the drug (14, 23). Circulating blood cells from cyclophosphamide-treated 8-week-old chickens used in this study had fully restored ability to mount graft-versus-host reaction in allogeneic embryos. Thus, the study reported here was not confounded by a deficiency in the cellular immune functions, but measured the effect of the B-cell deficiency alone.

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