CELLULAR KINETICS OF THE ANTI-MRBC RESPONSE IN CHICKENS

K. Dagg, S. P. Turner and F. Seto

Department of Zoology, University of Oklahoma, Norman, Oklahoma

The serum hemagglutinin (HA) titers and the frequencies of splenic plaque-forming cells (PFC) and rosette-forming cells (RFC) were compared for chickens immunized with either mouse or sheep erythrocytes (MRBC or SRBC). The antibody titer was greater for MRBC than SRBC, but the PFC and RFC values were similar. The anti-MRBC response profiles of 3- and 8-week-old birds showed that the antibody titers increased rapidly from the second day after immunization to maximum values in a week. The RFC and PFC levels were maximum at 4 to 5 days after immunization.

Comparison of the levels of spontaneous and antigen-induced RFC for MRBC in several lymphoid tissues showed that RFC were abundant in the spleen, less frequent in the blood, and infrequent in the thymus and bursa of immunized birds. The RFC numbers were negligible in all four tissues of unimmunized birds. The cellular changes elicited by MRBC immunization are similar to those reported for SRBC.

The HA, PFC and RFC assays for groups of birds exposed to various presumed immunosuppressive treatments as embryos or at hatching are compared and briefly discussed.

INTRODUCTION

Heterologous erythrocytes are effective immunogens in avian systems as well as in mammals. Early studies have shown that hemagglutinins are readily elicited by various mammalian erythrocytes in chickens and in isolated spleen cell preparations (1 – 4). Recent reports indicate that the avian cellular immune response to sheep erythrocytes (SRBC) is similar in many respects to that of mammals. Following the administration of SRBC, specific hemagglutinins appear in the blood within three days and reach peak concentration within a week (5). The antibody production is correlated with specific histological changes in the activated lymphoid organs. Lymphoid proliferation is accelerated, plasma cells appear in greater numbers (6), and new germinal centers appear in the spleen (5). Hemolytic plaque–forming cells (PFC) are abundant in the spleen but are less frequent in other lymphoid organs of immunized birds (6, 7). Cells with immunocytoadherent property appear in large numbers in the peripheral blood (8) as well as in the spleen (7, 9). These observations have been confirmed by many others.

We have now measured the levels of natural and immune hemagglutinins in the serum of embryos, baby chicks and juvenile chickens for several common heterologous erythrocytes. The natural agglutinin level was found to be lowest for sheep erythrocytes, modest for hamster and mouse red cells, and high for rabbit and rat erythrocytes. When the immune hemagglutinin–forming capacities of these erythrocytes were compared, the response was lowest for rabbit erythrocytes, moderate for hamster, sheep and rat cells, and high for mouse red blood cells. Mouse erythrocytes (MRBC) were chosen as the antigen for our experiments since the natural hemagglutinin background is generally low during the first two weeks after hatching and high hemagglutinin output can be elicited in young birds. Moreover the onset of responsiveness to this immunogen was earlier than with the other erythrocytes. MRBC has been found to be as effective as or better than SRBC in adoptive immunity experiments (10, 11), in kinetic analyses of chicken antigen-reactive units (12, 13), and in the antibody-mediated immune suppression model (14). This paper describes cellular kinetic studies with MRBC as antigen and compares the results with those obtained by others with the SRBC system.

MATERIALS AND METHODS

Inbred lines of White Leghorn chickens maintained in the Zoology Department were used for all experiments. All birds were allowed free access to Purina chicken feed and water.

Immunization with Heterologous Erythrocytes.

Appropriate doses of washed MRBC from a Swiss Albino mouse strain and SRBC (Brown Laboratories, Topeka, Kan.) were injected into the birds intracardially.

Hemagglutinin Titration.

Blood samples were obtained from the chickens by cardiac puncture. After centrifugation of the clotted blood, the serum was collected and frozen until antibody assay. Serial twofold dilutions of the serum in saline were prepared in Microtiter U–plates (Cooke Engineering Co., Alexandria, Va.). A 1% saline suspension of the appropriate RBC was added to each dilution. After incubation at room temperature for two hours, the hemagglutinin titer (HA) was read as the reciprocal of the highest $\log_2$ dilution of the serum with detectable macroscopic agglutination.

The PFC and RFC Assays.

A modification of the Cunningham monolayer assay for hemolytic PFC was used (15). Spleen cell suspensions were prepared and washed twice in Hank's solution (HBSS). The number of viable cells was determined by trypan blue dye exclusion in a hemacytometer. Cell viability generally exceeded 80%. All suspensions were diluted to a concentration of 5×10^6 viable cells/ml. Fresh serum from suitable chicken donors, diluted 1:4 in HBSS, was the complement source. The following mixture was prepared: 0.3 ml of spleen cell suspension, 0.3 ml of complement, 0.1 ml of 15% RBC in saline, and 0.5 ml HBSS. Aliquots (25μl) of the mixture were pipetted onto specially prepared microscope slides. Each slide was divided into three equal areas with adhesive tape strips which functioned as cover slip supports. A cover slip was carefully placed over each drop and a cell monolayer was allowed to form. Six or more replicates were prepared from each spleen. After incubation for 15 to 20 min in a moist 39 C chamber, the PFC were enumerated at 100× magnification.

The rosette-forming cell (RFC) immunocytoadherence assay was similar to the Haskill modification (16) of the original Zaalberg technique (17). Cell suspensions of spleen, thymus, and bursa were prepared as for the PFC assay. To obtain buffy coat preparations, whole blood was drawn from the donor by cardiac puncture, diluted in an equal volume of Alsever's anticoagulant, and centrifuged at low speed to sediment the erythrocytes. The overlying plasma and suspended leukocytes were transferred to a second tube and the cells were spun down. The supernatant was returned to the original tube and used to resuspend the pelleted blood cells. The separation process was repeated to recover more leukocytes. The leukocytes were washed four times in Alsever's solution and counted.

For the RFC assay a mixture consisting of 0.3 ml cell suspension (5×10^5 or 5×10^6 cells/ml), 0.05 ml of 15% RBC suspension, and 0.85 ml of HBSS was prepared. The mixture was centrifuged at moderate speed for 5 min and the pellet was stored in the refrigerator for 30 minutes. It was gently resuspended with a Pasteur pipette and 25 -μl aliquots of the mixture were transferred to slides described earlier for the PFC assay. Slides were prepared in duplicates for each RFC mixture. Rosettes, defined as clusters of four or more mammalian erythrocytes adhering to a chicken lymphoid cell, were counted under 100× magnification.

RESULTS

The immune responsiveness, as measured by HA titer and PFC and RFC counts, was

TABLE 1. *Comparison of the immune responses of chickens to sheep and mouse erythrocytes.*

Erythrocytes	Age of Birds (weeks)	Number	Antibody titer[a]	PFC Count[b]	RFC Count[c]
Mouse	3	4	10.2	5.6×10^2	
Sheep	2.5-3	8	6.6	8.4×10^2	5.9×10^1[d]
Sheep	8-10	2	8.5	48.0×10^2	

[a] Mean $\log_2$ hemagglutinin titers
[b] Geometric mean per 10^6 spleen cells
[c] Geometric mean per 10^6 spleen cells
[d] Sample of two birds

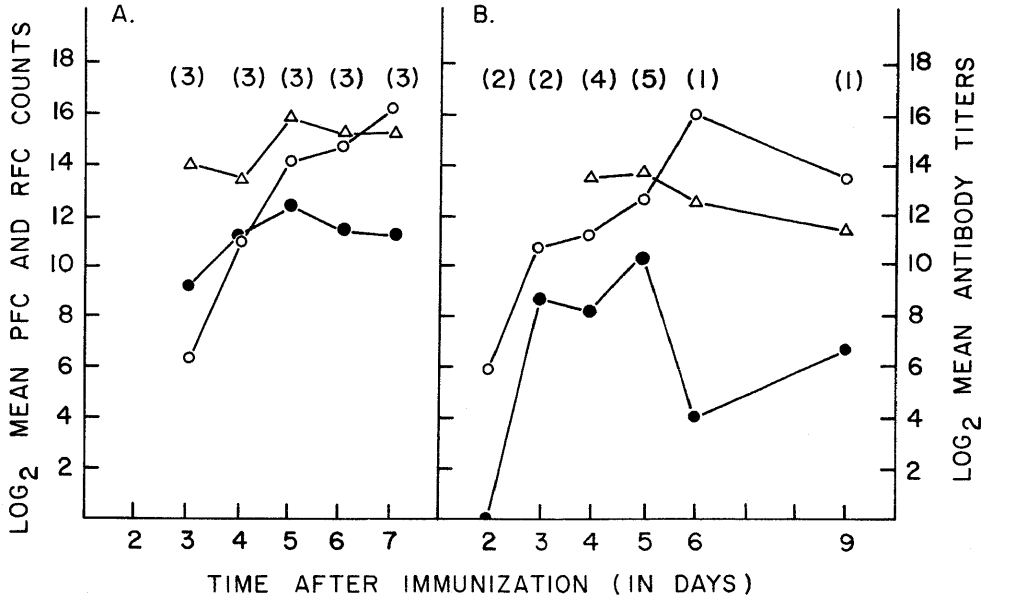

FIGURE 1. Anti-MRBC immune response profiles of 3-week-old (A) and 8-week-old (B) chickens. Number of birds in each sample indicated in parentheses; hemagglutinin titer in $\log_2$ units (○), splenic PFC (●), and RFC (△) are shown.

compared for groups of birds immunized with either MRBC or SRBC. The results are summarized in Table 1. The anti-MRBC response of this particular group of 3-week-old chicks is lower than that usually obtained in this laboratory. A more typical response is shown in the control group of Table 3. Even then it is apparent that the hemagglutinin response is higher with MRBC than SRBC (5). The PFC production, however, is of comparable magnitude, especially when the PFC value is compared with that of the control group shown in Table 3. The RFC values for the two birds immunized with SRBC (Table 1) are similar in magnitude to those obtained with MRBC immunization (Table 3). Included in Table 1 are the HA and PFC values for two older birds immunized with SRBC, obtained in a previous investigation (18).

MRBC was used as the immunogen in subsequent experiments reported here. The immune response of a group of 3-week chicks, immunized with 1 ml of a 1% MRBC suspension, and a group of 8-week birds, immunized with 1 ml of 2% MRBC, was measured. On various days after immunization, blood samples were drawn, the birds were sacrificed, and their spleens assayed for PFC and RFC. The results of the immune assays for the two groups are shown in Figure 1. In each group a typical hemagglutinin immune profile was obtained, with a low HA level at 2 to 3 days after immunization and a rapid rise to peak levels by the end of the week. The PFC and RFC profiles showed an earlier peak, with maximum values at about 4 to 5 days. In general, the RFC values were about 8 to 10 times that of the PFC. The profiles obtained for the two age groups are similar in magnitude.

The frequencies of spontaneous RFC in unimmunized birds and antigen-induced RFC in MRBC-primed birds were compared in several lymphoid organs. The RFC counts for the spleen, thymus, bursa, and buffy coat of unimmunized birds and those immunized 3, 4, 5 and 7 days earlier are summarized in Table 2. In the unimmunized birds the number of spontaneous RFC was very low in all organs assayed. Although the number of immunized birds assayed is small, it is clear that of the organs tested the frequency of RFC is greatest in the spleen, less in the blood, and least in the thymus and bursa.

Groups of birds that were exposed to various presumed immunosuppressive treatments as embryos or baby chicks were assayed for immune responsiveness three to four weeks later. One group received bone marrow cells from an incompatible donor

TABLE 2. *Spontaneous and immune anti-MRBC RFC counts in various organs of two-week-old chickens.*

Days[a]	No. of Birds	Mean RFC counts (in 10^3)[b]			
		Spleen	Thymus	Bursa	Buffy Coat
0	4[c]	0.017	0.0004	0.0007	0.0003
3	1	4.2	0.35	0.66	1.8
4	1	9.9	1.2	0.32	0.032
5	1	11.0	0.72	0.08	1.2
7	1	0.016	0.048	0.0	0.0

[a] Time after immunization in days
[b] Geometric mean of RFC counts of several slides
[c] Unimmunized birds; one 4 weeks and three 3 weeks of age.

at 16 days of incubation. Another group was administered the immunosuppressant drug cyclophosphamide (Cy) at 16 days of incubation. A third group included chicks that had received as 14-day embryos injections of a high-titer anti-MRBC serum. Some of these were later grafted with histocompatible thymus or bursa cells. When the HA production was tested at one week of age, all five groups of treated birds showed some evidence of depressed immunity as a result of the treatments. Birds that received MRBC at 14 or 17 days of incubation and again at hatching served as the antigen control group. In addition, a group of normal birds were used as untreated controls. All seven groups of birds were immunized with 1 ml 2% MRBC at 3 to 4 weeks of age, sacrificed 4 or 5 days later, and assayed for serum hemagglutinin and splenic PFC and RFC production. The results are summarized in Table 3.

The values obtained for the antigen control group are similar to those of the untreated control group, indicating that embryonic and neonatal exposure to MRBC does not significantly alter the immune response at 3 weeks of age. The hemagglutinin titer and PFC production of the group treated with Cy were significantly lower than values or both control groups ($P < 0.01$). The hemagglutinin responses of the remaining groups are not significantly different from those of the control groups. PFC production of the birds treated as embryos with anti-MRBC antibody and later grafted with thymocytes or bursacytes (groups 6 and 7) was significantly less than that of the untreated controls ($P < 0.05$ and < 0.01, respectively), but was not significantly different from the antigen control group value. This control may be more approriate for comparison with the treated groups since all were immunized at hatching with MRBC. The RFC values of groups 6 and 7 appear higher than normal; however, when compared with the untreated control group only group 7 gave a significantly greater value ($P < 0.01$), whereas compared to the antigen control group neither group gave a significantly higher RFC value. The hemagglutinin, PFC, and

TABLE 3. *PFC, RFC and hemagglutinin responses of immature chickens immunized with MRBC at 3-4 weeks of age.*

Pretreatment[a]	Age of Birds (weeks)	Time[b]	No. of Birds	Antibody titer[c]	PFC (in 10^3)[d]	RFC (in 10^3)[e]
1. None	3	4-5	6	12.7	3.6	2.9
2. MRBC	3	4	5	13.7	2.3	4.0
3. Bone marrow	4-5	4-5	8	14.6	1.3	1.2
4. Cytotoxan	3	4	8	8.4[f]	0.078[f]	1.5
5. Antibody	3-4	4	3	14.0	1.3	2.9
6. Ab, thymocytes	3-4	4	6	13.3	0.81[g]	8.9
7. Ab, bursacytes	3-4	4	3	14.3	0.22[g]	8.4[g]

[a] Experimental treatments as embryos or baby chicks (see text).
[b] Time after immunization in days
[c] Mean hemagglutinin titers in $\log_2$ units
[d] Geometric mean of PFC counts per 10^6 spleen cells
[e] Geometric mean of RFC counts per 10^6 spleen cells
[f] $P = 0.05$ or less compared to groups 1 and 2.
[g] $P = 0.05$ or less compared to group 1

RFC values for the antibody only and bone marrow groups (groups 3 and 5) are not significantly different from those of the controls. The immune response profile at 4 weeks of the bone marrow group (not shown) was similar in magnitude and pattern to that of the normal birds shown in Figure 1. Only the group exposed to Cy treatment exhibited a lasting immunosuppressive effect; all the others appeared to be unaffected by or to have recovered from the treatments.

DISCUSSION

Our experimental findings indicate that the cellular changes elicited by MRBC immunization are similar to those reported for the SRBC response in birds (7, 9, 19, 20). The numbers of PFC and RFC in the spleen increased rapidly from the third day after immunization with MRBC and reached peak concentration at about 5 days. In general the RFC frequencies are approximately 8 to 10 times the PFC numbers (21). The anti-MRBC hemagglutinin immune profile is similar to that for SRBC but of greater magnitude. The antibody appears in the blood 2 to 3 days after immunization, reaches a peak level by the 5-7th day, and then declines (5). The serum hemagglutinin level is generally lower in younger birds (5), but of the age groups tested in this study no consistent age dependency was evident. The immune competence to MRBC, as measured by the three parameters, appears to be well-developed even in 2- to 3- week-old chickens.

It has been reported that PFC are numerous in the spleen and less frequent in the thymus and bursa of birds immunized with SRBC (6, 22-24). Our value of approximately 7×10^3 PFC/10^6 spleen cells during the peak response to MRBC in 3-week-old birds is comparable to that reported by Sato and Glick (19) for the SRBC system, where similar assay techniques, route of antigen injection, and age of birds were used. No PFC were observed in the spleens of several young unimmunized birds used in this study. Background levels of splenic PFC for SRBC are generally low for older birds as well (20).

RFC for SRBC are reported to be abundant in the spleen and peripheral blood and infrequent in the thymus of immunized birds (8, 9, 17). The levels of spontaneous RFC for SRBC and guinea pig RBC are generally low in the spleen and other lymphoid tissues (25-28), although their numbers increase with age (29). Our counts indicated that the levels of anti-MRBC RFC in the various lymphoid tissues of unimmunized donors were negligible. The RFC were generally scarce in the thymus and bursa of immunized birds as well. They were abundant in the spleens of immunized birds and occurred in moderate amounts in the blood at 3 and 5 days after immunization.

Other groups of birds treated as embryos or as newly hatched chicks in various ways to modify their immune responsiveness were assayed at 3 to 4 weeks for PFC, RFC, and HA production. Except the birds treated with Cy, all groups appeared to have recovered from the immunosuppressive treatments by that time. Antibody-mediated suppression is known to be a temporary phenomenon in chickens (13), and recovery was expected to be nearly complete by 3 to 4 weeks after treatment. Cyclophosphamide treatment is known to produce a more extended immunosuppression (11), so the poor immune responsiveness observed in the Cy-treated birds was not unexpected.

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