B-ANTIGEN DISPARITY AS A FACTOR IN SUPPRESSION OF ANTIBODY FORMATION IN THE ALLOGENEIC *IN VIVO* SYSTEM OF CHICKENS

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Combinations of donors and hosts differing in B^1 and B^2 antigens were tested by Simonsen's graft-*versus*-host spleen assay. Splenomegaly was observed in 13-day embryo hosts which differed from donors in a B antigen. With 17-day embryo and day-old chick hosts, only the B^2B^2 to B^1B^1 combination consistently exhibited splenomegaly. Similar combinations were compared by the *in vivo* antibody assay, *i.e.*, by measuring antibody production of donor immunocytes cultured in embryo and baby chick hosts. The hemagglutinin response of 13-day embryo hosts was high, with titers significantly elevated in some B-antigen mismatches. With 17-day embryo hosts titers were reduced only in the B^1B^2 to B^1B^1 combination. Titers of baby chick hosts were decreased considerably in the mismatched combinations. Suppression of antibody production by grafted cells was correlated with B-antigen disparity between donor and host.

Early studies of antibody formation by transferred chicken spleen cells in presumed immunologically immature hosts have met with variable success (1 - 4). The poor immune response by transferred cells in older embryo and baby chick hosts was believed to be due to host rejection (5 - 7). Allograft responsiveness exists in the late chick embryo (8 - 10). Kinetic studies with the splenomegaly assay (7) and *in vivo* antibody assay of marker allogeneic immunocytes strongly support the emergence of allograft reactivity during the last third of embryo development. Antibody production by antigen-activated allogenic blood immunocytes was high when the immunocytes were cultured in 14- and 16-day chick embryo hosts, but significantly reduced in 18-day embryo and day-old chick hosts. The antibody production remained high, however, in cyclophosphamide-treated hosts of comparable ages. The suppression of the immune potential of the grafted cells was attributed to an incipient allograft reactivity of the host (11).

Earlier studies with inbred chickens clearly implicated the importance of genetic factors in skin graft rejection and graft-versus-host reactions (12 - 14) and, more recently, the B blood group was established as a major histocompatibility locus in chickens (15 - 17). Experiments with White Leghorn chickens homozygous for the B^1 and B^2 antigens were initiated to test the effects of these histocompatibility factors in the *in vivo* system. Various combinations of donors and hosts differing in the B^1 and B^2 antigens were first analyzed with the Simonsen's spleen assay to ascertain the histocompatibility interactions of the antigens in a graft-versus-host (GVH) model. The different combinations were further tested with the *in vivo* antibody assay to determine the effect of B-antigen disparity of donor and host on antibody production by allogeneic cells in embryo and baby chick hosts.

MATERIALS AND METHODS

The White Leghorn flock which was the source of eggs and chicks for the experiments was started with birds obtained through the generosity of Dr. T. Makinodan and Dr. J. F. Albright of the Oak Ridge National Laboratory, Oak Ridge, Tenn. The birds were blood-typed for B^1 and B^2 antigens and maintained as separate lines.

Splenomegaly assay

The magnitude of splenomegaly elicited in an immunologically unreactive host by immunocompetent donor cells is an indirect index of the graft-versus-host (GVH) reaction (8). Donors used in the splenomegaly assay were at least six weeks of age. Whole blood, obtained by cardiac puncture in an equal volume of sterile Alsever's solution, was centrifuged and enough supernatant fluid discarded to restore the original blood volume. One-tenth ml, 0.15 to 0.2 ml, and 0.3 ml of blood was intravenously injected into 13-day embryo, 17-day embryo,

and day-old chick hosts, respectively, with a 30-gauge needle attached to a ¼ to ½ ml tuberculin syringe. Spleen and body weights were determined six days after inoculation. Nine possible combinations of B^1B^1, B^2B^2 and B^1B^2 donors and hosts were tested for histocompatibility interactions.

In vivo antibody assay

Antigen-reactive cells are abundant in the blood of chickens immunized with mouse erythrocytes (MRBC). Whole blood was obtained by cardiac puncture in equal volume of Alsever's solution from donors primed with MRBC four days earlier. The blood was centrifuged; most of the supernatant fluid was discarded and replaced with a 4% MRBC suspension in sterile Alsever's solution. The donor blood, with the immunocytes, was mixed thoroughly with MRBC suspension and intravenously injected into embryo hosts or intraperitoneally into baby chick hosts. A 0.1 ml-volume of inoculum was administered to 13-day embryo hosts, 0.15 ml to 17-day embryos, and 0.3 ml to day-old chick hosts. The recipients were bled six days later for serum samples. Serum hemagglutinin titers were determined with the microtiter direct hemagglutination method with 1% MRBC saline suspension as antigen.

TABLE 1. *Spleen weight assay of host embryos inoculated with donor blood at 13 days of incubation.*

Donor antigens	Host antigens	No. of hosts	Mean spleen weight (in mg ± s.d.)
B^1B^1	B^1B^1	13	8.8 ± 1.1
B^1B^1	B^2B^2	10	9.1 ± 0.8
B^1B^2	B^1B^1	12	12.5 ± 4.5
B^1B^1	B^1B^1	43	12.5 ± 3.1
B^2B^2	B^2B^2	23	13.4 ± 3.6
B^1B^1	B^2B^2	25	23.4 ± 7.6[a]
B^1B^1	B^1B^2	12	21.0 ± 6.0[a]
B^2B^2	B^1B^1	25	35.6 ± 10.1[a]
B^2B^2	B^1B^2	10	27.7 ± 13.3[a]

[a] P of .001

TABLE 2. *Spleen weight assay of host embryos inoculated with donor blood at 17 days of incubation (A) and at hatching (B).*

Donor antigens	Host antigens	No. of hosts	Mean spleen weight (in mg ± s.d.)
(A)			
B^1B^1	B^1B^1	10	18.9 ± 5.2
B^1B^1	B^2B^2	3	17.5 ± 2.1
B^1B^2	B^1B^1	8	19.8 ± 5.7
B^1B^1	B^1B^1	13	16.7 ± 5.5
B^1B^2	B^2B^2	9	18.4 ± 3.6
B^1B^1	B^2B^2	5	20.4 ± 7.5
B^1B^1	B^1B^2	5	20.7 ± 3.7
B^2B^2	B^1B^1	11	27.1 ± 7.1[b]
B^2B^2	B^1B^2	12	18.1 ± 4.2
(B)			
B^2B^2	B^2B^2	15	33.4 ± 4.9
B^2B^2	B^1B^1	15	41.0 ± 13.0[c]
B^2B^2	B^1B^2	9	40.1 ± 7.5[c]
B^1B^1	B^1B^1	5	30.8 ± 6.9
B^1B^1	B^2B^2	7	40.9 ± 13.9

[b] P of 0.01
[c] P of 0.05

Nine possible combinations of B^1B^1, B^2B^2, and B^1B^2 donors and hosts were tested for antibody production in the *in vivo* model. The antibody production in matched and mismatched combinations with respect to the B-antigens were compared in particular for manifestations of allogeneic inhibition.

RESULTS

Splenomegaly assay

The results of Simonsen's spleen assay with 13-day embryo, 17-day embryo, and day-old chick hosts are summarized in Tables 1 and 2. In the 13-day embryo host series (Table 1) the mean spleen weights in those combinations where the GVH reaction is not expected are small and of normal sizes. Spleen sizes were smallest in groups in which tolerant B^1B^2 donor cells were inoculated into B^1B^2 and B^2B^2 hosts. Slightly larger spleen weights were obtained in the two combinations where donor and hosts were homozygous and alike in B antigen, and in the B^1B^2 donor to B^1B^1 host combination. Obvious splenomegaly was observed only in the four groups in which the hosts differed from the donor in B antigen, and which predisposes them to a GVH reaction. Thus, the B^1 and B^2 antigens in our system exhibited the typical histocompatibility interactions.

With 17-day embryo hosts, of the four donor-host combinations which exhibited splenomegaly in 13-day hosts, only the B^2B^2 donor to B^1B^1 host combination showed significant splenic enlargement. The three other groups were indistinguishable from the B-antigen matched control and B^1B^2 donor cells injected groups.

Of the combinations tested with day-old chick hosts, significant splenomegaly was

observed in B^1B^1 and B^1B^2 hosts inoculated with B^2B^2 donor cells. The apparently greater mean spleen weight in the B^1B^1 donor to B^2B^2 host combination did not differ significantly from that of the corresponding B^1B^1 control. The samples were small and highly variable in the two groups. The GVH-associated splenogmegaly is less apparent with older hosts.

In vivo antibody assay

The *in vivo* antibody assay of donor immunocytes cultured in 13-day embryo, 17-day embryo, and day-old chick hosts are summarized in Tables 3 and 4. Antibody production was generally high, with no apparent suppression, in all nine combinations of donor and host when 13-day embryo hosts were used (Table 3). The antibody

TABLE 3. *In vivo hemagglutinin titers in different donor-host combinations with respect to B antigen, in 13-day embryo hosts.*

Donor antigens	Host antigens	No. of hosts	Mean antibody titers (in $\log_2$ units ± s.d.)
B^1B^1	B^1B^1	7	10.4 ± 0.99
B^1B^2	B^2B^2	8	9.9 ± 0.68
B^1B^2	B^1B^1	13	9.7 ± 0.52
B^1B^1	B^1B^1	22	9.9 ± 0.90
B^1B^1	B^2B^2	23	11.3 ± 1.00[c]
B^2B^1	B^1B^2	6	11.2 ± 0.61[c]
B^2B^2	B^2B^2	17	8.8 ± 0.53
B^2B^2	B^1B^1	14	9.9 ± 1.23[b]
B^2B^2	B^1B^2	6	10.2 ± 0.93[c]

[b] P of 0.01
[c] P of 0.001

output was enhanced, moreover, in some B-antigen mismatched donor-host combinations compared with the corresponding matched groups.

With the exception of a reduction in antibody production in the B^1B^2 to B^1B^1 donor-host combination, no differences were apparent among the other combinations when 17-day hosts were used (Table 4). No allogeneic enhancement, as observed with 13-day hosts, was apparent in the older hosts. The results of experiments with two B^2B^2 donors and one B^1B^1 donor tested in B-antigen matched and mismatched combinations with day-old chick hosts are also shown in Table 4. With one B^2B^2 donor the mean antibody titers in B^2B^2 and B^1B^1 hosts were 11.6 and 10.1 $\log_2$ units, respectively. The difference in mean titers is significant. In another experiment diluted blood from B^2B^2 donor was inoculated into

TABLE 4. *In vivo hemagglutinin titers in different donor-host combinations with respect to B antigens of 17-day embryo hosts* (A) *and day-old chick hosts* (B).

Donor antigens	Host antigens	No. of hosts	Mean antibody titers (in $\log_2$ units ± s.d.)
(A)			
B^1B^2	B^1B^2	6	11.3 ± 0.69
B^1B^2	B^2B^2	6	11.8 ± 0.68
B^1B^2	B^1B^1	9	7.1 ± 1.29[c]
B^1B^1	B^1B^1	15	9.7 ± 0.79
B^1B^1	B^2B^2	14	10.2 ± 1.22
B^1B^1	B^1B^2	10	10.4 ± 1.29
B^2B^2	B^2B^2	9	10.1 ± 1.36
B^2B^2	B^1B^1	11	9.7 ± 1.23
B^2B^2	B^1B^2	8	10.5 ± 0.93
(B)			
B^1B^1	B^1B^1	11	8.0 ± 0.77
B^1B^1	B^2B^2	13	3.6 ± 1.22[c]
B^2B^2	B^2B^2	9	11.6 ± 0.55
B^2B^2	B^1B^1	11	10.1 ± 1.82[a]
B^2B^2	B^1B^2	7	6.5 ± 1.55
B^2B^1	B^1B^2	6	7.0 ± 1.32
B^1B^2	B^1B^1	5	3.0 ± 0.71[c]

[a] P of 0.05
[c] P of 0.001

B^2B^2, B^1B^2 and B^1B^1 hosts. Again reduction in antibody titer was obtained in B^1B^1 hosts. Thus among the donor-host combinations tested in day-old chick hosts, antibody production was significantly less in the B-antigen mismatched combinations than in the corresponding matched groups, the reverse of results observed with 13-day hosts.

DISCUSSION

The splenomegaly produced in immunologically unreactive hosts following the grafting of immunocompetent cells is one of the more constant features of the GVH reaction (18). When different combinations of donors and hosts, with respect to the B^1 and B^2 antigens, were tested by spleen assay of 13-day hosts, only in combinations where the hosts differed from the donor in a B antigen was there significant splenomegaly, all other combinations showed little or no increase in spleen weights. The magnitudes of the spleen weights observed are similar to those reported by others in experiments with inbred lines (13, 14), and the overall pattern of reactions compares well with that described in other histocompatibility systems involving B-antigen differences in chickens (15, 19).

The lesser GVH susceptibility of older embryo and baby chick hosts has been

reported before (6, 8, 20). Of the four combinations where the GVH reaction was expected, only the B^2B^2 to B^1B^1 donor-host combination among 17-day embryo hosts and the B^2B^2 to B^1B^1 or B^1B^2 combinations among baby chick hosts clearly manifested splenomegaly responses. The apparent lack of GVH reaction in the B^1B^1 donor to B^2B^2 host combination may be due to rejection of donor cells by an emergent host allograft potential. The absence of the GVH reaction with B^2B^2 or B^1B^1 donor cells in B^1B^2 hosts cannot be similarly interpreted and represents another form of allogeneic inhibition. Since isogeneic lines were not employed, the effects of other minor histocompatibility loci cannot be ruled out. Nonspecific defense mechanisms are better developed in older embryos and baby chicks (21, 22), and may cope effectively with the injurious aspects of the GVH reaction.

Antibody production by donor immunocytes in 13-day hosts was generally high in all nine donor-host combinations despite B-antigen disparity between donor and host in some cases. Inhibition indicative of host allograft reactivity was not apparent. High antibody titers were also obtained with 14- to 16-day embryo hosts in earlier studies with outbred material (11). Moreover significant enhancement of antibody formation was observed in those combinations in which the hosts differed from the donor in B antigen. Such allogeneic enhancement in immune responsiveness in the *in vivo* system is not unexpected; it has been observed by others (23, 24). Although the cause of allogeneic enhancement is unexplained, the increased response may be associated with release of mitogenic factors during the accompanying allogeneic tissue interactions (25).

With older hosts, instead of allogeneic enhancement, inhibition of antibody production was observed in some B-antigen mismatched combinations. Considering possible explanations of allogeneic inhibition (26), it is presumed that suppression of antibody formation in the older hosts is a consequence of their allograft responsiveness. Respectable antibody titers were obtained in the B-antigen matched control groups and in the cyclophosphamide-treated hosts, in our earlier studies, with outbred materials (11). The finding that disparity in B antigen between donor and host is a major factor in the suppression of antibody formation in the allogeneic *in vivo* system in chickens could well account for the similar decrease in donor cell immune responsiveness reported earlier with outbred chickens (11) and provide further support for the conclusions of earlier studies of a cellular immune potential in chick embryos (7, 8, 11).

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