ANTIBODY PRODUCTION IN CHICK EMBRYO HOSTS BY ALLOGENEIC DONOR CELLS

Frank Seto

Department of Zoology, University of Oklahoma, Norman, Oklahoma

A modified *in vivo* model is described in which immunologically activated immunocytes from blood are cultured in chick embryos for kinetic studies. Whole blood from allogeneic chicken donors, immunized 3 or 4 days earlier with mouse erythrocytes (Mrbc) as antigen, when mixed with Mrbc and intravenously injected into 14-day chick embryo hosts produced high levels of antibody 6 to 8 days later. Control embryos inoculated with antigen only were negative. When the amount of donor blood or antigen in the inoculation mixture was varied, direct dose-dependent relationships were observed between donor cell dose and antibody output, as well as between antigen concentration and antibody output. The 6-day mean antibody titers were considerably less when older (18-day) embryos were used as hosts than when 14-day embryos were similarly used. Although cyclophosphamide pretreatment of 14-day hosts did not alter antibody production significantly, similar treatment of 18-day recipients did result in marked enhancement of the antibody response.

The immune state is normally activated by antigen stimulation but may be passively acquired by receiving antibodies produced in another animal. A third, temporary immune condition termed adoptive immunity, is affected by the grafting of immunologically activated cells. Although the phenomenon was known for over a half century, only recently, with the increasing relevance of immunology to transplantation biology, has its importance become appreciated. Although of interest in itself, adoptive immunity provides the basis for a useful experimental model, the *in vivo* culture system (1), in which immunologically unreactive hosts are used to grow antibody-forming cells from spleen and lymph nodes. Extensive cytokinetic analyses have been made of mouse spleen populations by injecting them intravenously, or by enclosing them in diffusion chambers and implanting them into X-irradiated, isogeneic adult mice (1, 2). Most early experiments have dealt with mammals (3, 4), but birds have been used occasionally (5-7). Attempts to study antibody formation by transferred spleen cells in allogeneic baby chick and chick embryo hosts have met with variable success (5, 8-10). Because of the great success of the experimental model in the mammalian system, the approach was utilized for similar antibody kinetic studies with the chicken.

This paper describes preliminary experiments with a modified *in vivo* technique in which immunologically passive 14-day chick embryos were used to grow immunocytes from blood of immunized allogeneic donors.

MATERIALS AND METHODS

Juvenile and adult White Leghorn chickens were donors. White Leghorn and hybrid California Gray x White Leghorn embryos served equally well as recipients. These were obtained from a local commercial source.

Mouse erythrocytes (Mrbc) from Swiss albino mice were obtained, in Alsever's solution, by cardiac puncture. The cells were washed three times in 0.15 M NaCl, packed by centrifugation, and antigen suspensions of known concentrations were prepared. The amount of antigen (Mrbc) intravenously injected varied according to the age and size of the donor chicken. Three or four days after immunization, whole blood was obtained in an equal volume of sterile Alsever's solution by cardiac puncture, and after centrifugation enough supernatant was discarded to restore the original blood volume. One-tenth ml of whole blood, with or without 4×10^7 Mrbc as antigen, was intravenously injected into the chorioallantoic vessel of each 14-day host embryo with a ¼ ml tuberculin syringe and 30 gauge needle. Six days later the embryos were bled from the chorioallantoic vessel with a 1 ml tuberculin syringe and 27 gauge needle. Antibody titers

of the separated serum samples were determined in duplicate by the Microtiter (Cooke Engineering, Alexandria, Va.) hemagglutination technique.

Experiments were conducted to determine antibody production as a function of (a) duration of *in vivo* culture, (b) amount of donor cells inoculated, (c) antigen concentration, and (d) host treatment with cyclophosphamide (CX). A different donor was used in each experiment. The numbers of assay embryos varied with each experiment and are included in the tables.

RESULTS

Time study of antibody production by donor cells in embryo hosts

Embryos were inoculated with a mixture of donor blood and antigen (Mrbc). At various times after injection, embryos were bled and the serum samples tested for anti-mouse hemagglutinin titers. The results of two tandem experiments are summarized in Table 1. When the mean antibody titers, measured in $\log_2$ units, were plotted as the function of days of *in vivo* culture, the antibody level increased from a negligible level at 3 days to a peak level at about 6 to 8 days and then declined. Similar results were obtained when sheep erythrocytes were used as the immunizing and test antigen.

Antibody production as function of donor cell dose and antigen concentration

In one experiment whole blood from an immunized donor was mixed *in vitro* with different amounts of antigen and 0.1 ml of the mixture was injected into embryos.

TABLE 1. *Effect of duration of culture on antibody production by donnor cells.*

	Experiment A		Experiment B	
Days in culture	Number of antibody producing embryos[a]	Antibody titer ($\log_2$)[b]	Number of antibody producing embryos	Antibody titer ($\log_2$)
3	0/7	0.0		
4	9/9	4.3 ± 2.3		
5	9/9	6.8 ± 1.6		
6	8/8	9.5 ± 0.7	17/17	10.6 ± 1.4
8	7/7	10.1 ± 1.1		
10			16/16	8.2 ± 2.0
14			11/11	6.5 ± 1.8

[a] Number of embryos producing antibody/total number of embryos examined.
[b] Mean antibody titer of all embryos in sample, with standard deviation.

TABLE 2. *Antibody levels in hosts inoculated with varying concentrations of antigen (Experiment A) or donor cells (Experiment B) in the inoculation mixture.*

Experiment	Amount of donor blood (ml)[a]	Number of mouse erythrocytes (10^6)	Number of antibody producing embryos[b]	Antibody titer ($\log_2$)[c]
Control	—	40.0	0/6	—
A	0.1	40.0	6/6	12.1 ± 0.20
	0.1	10.0	6/6	12.1 ± 0.49
	0.1	2.5	7/7	11.1 ± 0.79
	0.1	0.6	7/7	10.2 ± 1.07
	0.1	0.2	8/8	8.3 ± 2.10
B	0.05	40.0	6/6	12.0 ± 1.5
	0.025	40.0	7/7	8.0 ± 2.4
	0.0125	40.0	5/7	4.9 ± 3.5
	0.00625	40.0	3/7	2.2 ± 2.8

[a] Total volume of donor blood in a constant volume (0.1 ml) of inoculum.
[b] Number of embryos producing antibody/total number of embryos examined.
[c] Mean antibody titer of all embryos in sample, with standard deviation.

The mean 6-day serum antibody titers of the different experimental groups were compared (Table 2, Experiment A). Antibody production was independent of dose in the higher antigen concentrations, but significantly reduced at the lowest dose ($P<.01$). In another experiment, various dilutions of blood (½, ¼, ⅛, 1/16) mixed with a constant amount of antigen were injected into groups of host embryos. Results of this experiment are shown also in Table 2 as Experiment B. The data indicate a dose-dependent relationship between donor blood and antibody production. Calculation of F values resulted in $P<.001$ for all groups and $P<.01$ between groups.

Antibody production in CX-pretreated host embryos

Groups of 14-day and 18-day embryos were exposed a day before inoculation to 0.8 and 1.9 mg of CX, respectively. Controls were 14-day and 18-day untreated embryos. All were then inoculated with the standard mixture of donor blood and antigen. Mean 6-day antibody titers of the different host groups are summarized in Table 3. Antibody production in embryo hosts injected at age 14-days is uniformly high and, with the exception of Experiment B ($P<.05$), no significant difference was found in CX-treated and untreated hosts. Compared to 14-day hosts, the antibody production in 18-day hosts was significantly reduced ($P<.001$). CX-pretreatment of 18-day hosts resulted in significantly higher ($P<.001$) titers.

DISCUSSION

Despite extensive use of the *in vivo* approach for analysis of antibody formation in mammals (1-3), its application in other vertebrate groups has been sporadic. Moderately successful experiments have been reported with allogeneic donor-host combinations in chickens in which spleen cells were propagated in immunologically immature hosts (6, 7, 11, 12). This report confirms the feasibility of the *in vivo* culture model for analysis of immunocyte kinetics.

Blood from immunized donors provides a more convenient source of antigen-sensitive cells than does the spleen (13). In our experiments, whole blood was mixed *in vitro* with antigen and injected intravenously into host embryos. Immunocytes of the blood produced high levels of antibody in the host embryos and the time profile of antibody production in the *in vivo* system was similar to that reported in the intact bird following antigenic stimulation (14). Host embryos do not respond to antigen, as in-

TABLE 3. *Antibody production by donor cells in 14-day and 18-day cyclophosphamide-treated (CX) and nontreated embryo hosts.*

Experiment	Group	Host embryos[a]	Amount of donor blood (ml)	Number of antibody producing embryos[b]	Antibody titer (log_2)[c]
A	I	14	0.1	6/6	10.6 ± 1.9
	II	14 CX	0.1	8/8	10.4 ± 1.4
	III	18	0.1	4/7	2.0 ± 2.2
	IV	18 CX	0.1	8/8	4.9 ± 1.5
B	I	14	0.1	8/8	9.3 ± 0.8
	II	14 CX	0.1	9/9	11.1 ± 1.1
	III	18	0.2	6/6	4.0 ± 1.1
	IV	18 CX	0.2	9/9	9.7 ± 2.1
C	I	14	0.1	6/6	13.3 ± 1.4
	II	14 CX	0.1	7/7	12.7 ± 0.9
	III	18	0.2	4/4	6.1 ± 1.8
	IV	18 CX	0.2	4/4	11.5 ± 0.4

[a] Age of host embryo in days, and treatment with cyclophosphamide (CX).
[b] Number of embryos producing antibody/total number of embryos examined.
[c] Mean antibody titer of all embryos in sample, with standard deviation.

dicated by negative results with antigen injection only. Low antibody titers were observed when donor blood alone or blood from unprimed donor with antigen was used (13), a response thought to be due to naturally occurring background levels of immunocytes.

Dose-dependent relationships between antibody production and the amount of donor cells or antigen concentration are clearly evident in this study, although not as precisely demonstrated as with the isologous transfer system in mice (1). Earlier studies with chickens were not sufficiently quantitative to demonstrate these dose-dependent functions (10, 15). The reduction in antibody production by transferred cells in older embryo hosts is better explained by assuming host rejection of donor cells (8, 12, 16) rather than by the inability of the embryo system to support antibody formation (9). Our studies clearly show that the embryo environment can support antibody formation. Earlier studies have shown that X-irradiation and chemical immunosuppressant treatment of embryos decrease the graft-versus-host response to allogeneic cells and delay the subsequent development of hemagglutinin-forming and cellular immune capacities (14, 17, 18) and are consistent with the view of a host-versus-graft capacity in embryos (16). Thus, treatment of older host embryos with cyclophosphamide should enhance antibody production by the donor cells according to this interpretation, and this was actually observed. The rather variable results reported by others with allogeneic *in vivo* system in birds, especially when older embryos and baby chicks were used as hosts (7-10, 15), are explainable not only by differences in techniques, but also by complications of graft and host cell interactions (16). Consistent results are obtainable by using younger embryos as hosts.

The occurrence of antibody-producing cells in the circulating blood during the immune reaction has been demonstrated by a number of techniques in mammals (19, 20). Immunocytes, as detected with the *in vivo* technique, also occur in the peripheral blood of chickens following antigen stimulation (13). Antigen-sensitive cells reacting in the *in vivo* system probably include memory cells which were produced during immunization (21) and became antibody-formers upon subsequent antigen exposure.

ACKNOWLEDGMENTS

This project was aided by the Faculty Research Fund of the University of Oklahoma.

Cyclophosphamide (Cytoxan) was generously supplied by Mead Johnson and Co., Evansville, Ind.

REFERENCES

1. J. F. Albright, T. Makinodan, and E. E. Capalbo, Proc. 9th Congr. Intern. Soc. Blood Transf. Mexico, (1964), pp. 301-308.
2. P. Nettesheim and T. Makinodan, J. Immunol. 94: 868-876 (1964).
3. N. A. Mitchison, J. Cell. Comp. Physiol. 50 (suppl. 1): 247-264 (1957).
4. J. Sterzl, Folia Biol. 3: 1-9 (1957).
5. L. R. Sibal and V. H. Olson, Proc. Soc. Exp. Biol. Med. 97: 575-579 (1958).
6. Z. Trnka, Nature 181: 55 (1958).
7. J. Sterzl, Nature 183: 182 (1959).
8. Z. Trnka and I. Riha, Nature 183: 546 (1959).
9. B. W. Papermaster, S. G. Bradley, D. W. Watson, and R. A. Good, Proc. Soc. Exp. Biol. and Med. 102: 260-264 (1959).
10. E. P. Cohen, J. S. Ingraham, and D. W. Talmadge, Proc. Soc. Exp. Biol. Med. 113: 75-78 (1963).
11. V. Haskova and J. Svoboda, Folia Biol. 5: 8-17 (1959).
12. P. Isacson, Yale J. Biol. Med. 32: 209-228 (1959).
13. F. Seto, Amer. Zoologist 8: 814 (1968).
14. F. Seto and W. G. Henderson, J. Exp. Zool. 169: 501-512 (1968).
15. B. W. Papermaster, S. G. Bradley, D. W. Watson, and R. A. Good, J. Exp. Med. 116: 1191-1208 (1962).
16. F. Seto, Transplantation 5: 1280-1228 (1967).
17. F. Seto and J. F. Albright, Develop. Biol. 11: 1-24 (1965).
18. F. Seto, Proc. Okla. Acad. Sci. (in press).
19. L. Hulliger and E. Sorkin, Nature 198: 299 (1963).
20. R. Kearny and W. J. Halliday, J. Immunol 95: 109-112 (1965).
21. S. T. Ellis, J. L. Gowans, and J. C. Howard Cold Spring Harbor Symp. Quant. Biol. 32 395-406 (1967).