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The Ontogeny of Complement Activity. Complement Titers in the Developing Chick Embryo During Graft-*Versus*-Host Reactions.* (31586)

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This report summarizes our experience with titrations of total complement (C′) activity in the developing chicken embryo undergoing a graft-*versus*-host (GVH) reaction. Using a rapid convenient assay for total C′ activity in chicken serum, it was found that (a) C′ activity is present in the embryonic chick, and (b) C′ titers are elevated rather than decreased during the GVH reaction in the embryonic chick.

Material and methods. GVH reaction in the chick embryo. GVH reactions, based on the methods of Simonsen(1) and Cock and Simonsen(2), were produced as previously described(3). In each of these experiments 0.1 ml of heparinized whole blood from an adult White Leghorn chicken was injected into the vitelline circulation of each of a group of 2 to 6 13-day embryos of this same outbred strain. Five days later, on the 18th day of incubation, following exsanguination by direct puncture of the vitelline circulation, the eggs were opened, the spleens removed, and the weight of each individual spleen recorded in milligrams.

Collection of serum. Freshly drawn whole blood was allowed to stand at room temperature for ½ hour and at 0 to 4°C for 2-4 hours. Serum was collected by centrifugation and assayed immediately for total C′ activity. Although adult chicken serum can be frozen and stored for subsequent C′ assay, fetal chicken serum sometimes shows precipitation on freezing and is thus unsatisfactory for subsequent study.

Preparation of indicator cells. The washed rabbit erythrocyte was used as the indicator particle in this reaction. Rabbit blood was collected in sodium EDTA. The erythrocytes were washed twice in 0.01 *M* EDTA in veronal-buffered saline and twice in the veronal-buffered saline with supplemental Mg^{++} and Ca^{++}, (GVB^{++}), as described by Mayer(4). The suspension was standardized to 2×10^8/ml cells in GVB^{++}, the buffer in which the lytic reaction was to be performed. The rabbit erythrocyte is approximately twice the size of the sheep erythrocyte, and contains proportionately more hemoglobin.

Preparation of chicken-antiserum against rabbit erythrocytes. This antiserum was prepared by immunizing a group of adult White Leghorn chickens with washed rabbit erythrocytes according to the schedule of Nelson(5). The animals were given 1.0 ml of a 20% suspension of these cells intraperitoneally on day 1, another 1.0 ml of this suspension intravenously on days 3, 5, 7, and 9, and 1.0 ml of a 50% suspension intraperitoneally on day 11. Serum was harvested on day 17; it had an agglutinating titer of 81,920 when tested by adding 0.1 ml of this serum to 0.1 ml of a washed 2% suspension of rabbit erythrocytes. The serum was heated at 56°C for 30 minutes to destroy C′ activity.

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TABLE I

	Amount of serum (ml)	GVB (ml)	Antibody 1:100 (ml)	Rabbit erythrocytes, 2×10^8/ml	Serum dilution
Test serum dilutions a)	1.0	—	.1	0.1	1:10
b)	.5	.5	.1	0.1	1:20
c)	.3	.7	.1	0.1	1:33
d)	.2	.8	.1	0.1	1:50
#1. Test serum blank	.5	.7	—	—	1:20
#2. Cell + antibody blank	—	1.0	.1	0.1	—
#3. Complete hemolysis	—	Water–1.1	—	0.1	—

Assay procedure. Rabbit erythrocytes were selected as the indicator target for the action of chicken C′. These cells, whether unsensitized or hypersensitized with the appropriate heated chicken antiserum, lysed more readily than sheep erythrocytes when these were compared in the presence of equal amounts of chicken C′. The buffer system used was that described by Mayer(4); 0.15 *M* NaCl was found to be superior to 0.12, 0.09, 0.06, and 0.03 *M* NaCl in supporting hemolysis in isosmotic solutions. The concentrations of divalent cations (Ca^{++} and Mg^{++}) previously found optimal for guinea pig C′ activity (4) also were within the range optimal for expression of chicken C′ activity.

Reactions were carried out in low reaction volumes with small numbers of target cells, to increase the sensitivity of the assay. Cells in a total volume of 0.1 ml, antibody in 0.1 ml and the test chicken serum in 1.0 ml were allowed to react at 37°C for 60 minutes; 3.0 ml of saline was added, the tubes centrifuged, and hemoglobin determined in the supernate in a Beckman DU spectrophotometer at 412 mμ. The final assay, based on those described by Mayer(4), was performed as in Table I, beginning with a 1:10 (or an alternately convenient) starting dilution of the test serum. The optical density observed was corrected for spontaneous lysis (blank #1) and hemoglobin in the test serum (blank #2), and the per cent lysis was determined by dividing this corrected optical density by the optical density of the "complete hemolysis" standard. The 50% point was then determined. The small amount of heated immune serum was added to each reaction tube to insure optimal sensitization of the indicator rabbit erythrocytes. Addition of larger amounts of antibody to the assay tubes did not increase the C′ activity of either the grafted or the control embryo sera.

Presensitization was not carried out, since we observed excessive agglutination during centrifugation in the washing steps when the optimal amount of antibody was used. Maximal C′ activity was observed with antibody dilutions up to 1:200 (0.0005 ml of serum), and in all the assays 1:100 dilutions of antibody were used to assure full sensitization. Visible agglutination occurred in all the reaction tubes after 30-45 minutes, but this did not interfere with the 50% point determinations. This assay provided a satisfactory measure of total C′ activity within the range of our concern, titers from $<1:10$ to $>1:200$. Within these limits the slopes of the hemolysis curves in the vicinity of the 50% point are equal.

Results. Results of these experiments are presented in Table II. Complement activity could be identified and measured in each 18-day embryo studied. Sera from the untreated 18-day-old embryos had a mean titer of 9. The hemolytic activity measured was identified as C′ because: (a) no lysis occurred without the addition of heated immune chicken sera, and in the range of limited antibody, the amount of lysis was proportional to the amount of immune serum added; (b) sodium EDTA in concentrations of 0.01 *M* completely inhibited this lytic reaction; and (c) the lytic activity also was completely inhibited by heating the test embryo sera at 56°C for 30 minutes. The complement titer of the untreated embryo sera is markedly lower than that in the serum of the adult chicken, where titers in the range of 200-400 are seen.

TABLE II. Complement Titers in Chick Embryos Undergoing Graft-*Versus*-Host Reactions.

Donor chicken	Embryonic recipients: Spleen wt (mg)	C′ titer	Donor chicken	Embryonic recipients: Spleen wt (mg)	C′ titer
1	59	17	6	115	18
	37	14		82	19
	35	10		113	16
	25	10			
	39	15	Mean	63	21
	41	19			
2	54	14	Uninjected	12	10
	43	15	controls	13	8
				16	9
3	74	66		10	7
	45	26		14	5
	64	16		11	8
4	91	33		12	10
	90	14		12	16
5	70	10			
	98	45	Mean	12	9
	36	14			

It is clear from the accompanying table that C′ titers are increased rather than decreased during this reaction. Only 1 of 8 uninjected control embryos had complement titers of greater than 10, whereas all 19 embryos with graft-*versus*-host reactions had titers of at least 10, and 16 of 19 embryos had titers greater than 10. In many instances the C′ titer was increased several-fold over the highest titers observed in the control embryos. The mean titer in the injected embryos was 21 as compared to 9 in the control embryos.

The results presented here represent a summary of experiments utilizing the blood of 6 donor chickens, performed on the same day. They have been confirmed with two additional experimental series.

C′ activity was also tested in serum separated from 13-day-old embryos. No total C′ activity was detected in any of 5 such specimens.

Discussion. The presence of C′ in the newly born has been reported by numerous investigators in several species, including man (6-10), pig(11), and cattle(12,13). C′ activity has been detected in fetal sheep sera by Rice and Silverstein(14), but did not appear until toward the end of the gestational period. By contrast, we have detected C′ activity in fetal calf serum as early as the 12 cm stage, which would correspond to the late 1st or early 2nd trimester(13). Thus, our finding C′ activity in chicken embryo serum is not surprising, nor is the low ratio of embryonic to adult serum C′ titer, also the usual finding in mammalian embryos or newborns. However, in mammals it could not be ascertained whether C′ was being actively synthesized by the fetus or was being passively transferred from the mother. In the incubating chicken egg, in which no placental connection between the developing embryo and its parent is present, detectable C′ activity appeared sometime between the 13th and 18th days of incubation, indicating that the fetus is capable of forming hemolytic C′. Complement may be stored in the yolk contents, as gamma globulins are known to be stored(15), but this does not account for the increase in serum C′ during the graft-*versus*-host reaction. This is in concert with the observed failure of material-fetal exchange of complement in complement deficient mammals.

Complement activity in the chick was measured by techniques similar to those described by Rose and Orlans(16), in their studies of chicken complement(16-19). However, in our assay 50% lysis was determined using approximately ⅓ of the total number of indicator cells and approximately ½ the total volume of reactants used in that assay system. In further contrast, (a) rabbit rather than sheep erythrocytes were used as the indicator cells, (b) immune chicken rather than immune horse serum was used for sensitization, and (c) sensitization was performed in each reaction tube in lieu of presensitization of the entire indicator cell suspension. Titers in our assay seem to be 2-4 times higher than those in the assay of Rose and Orlans(16). Our main purpose was to find a reproducible measure of 50% hemolysis, and we did not establish linearity over the entire range of lysis.

C′ titers in the GVH reaction were previously reported to be depressed in young rats undergoing homologous disease(20). Our experience here stands in contrast since the fetal chick undergoing a GVH reaction showed increased C′ titer. However, differences in the

nature of the graft-*versus*-host reaction in the chick embryo and the runting syndrome in the rodent, and in timing of the study, might account for the apparent discrepancy. Our observations seem compatible with evidence we have previously presented that certain cellular immune reactions may occur without drop in the C′ titer, and perhaps without involvement of the humoral C′ system at all(21). Detailed studies based on frequent sampling throughout the entire course of graft-*versus*-host reactions in chick embryos and the several other forms of homologous disease of rodents are indicated.

The fact that foreign leukocytes were introduced into the embryo makes it impossible to conclude that the rise in C′ titers observed did not depend upon these adult donor cells. However, the normal or high serum complement levels observed in neutropenic(22), eosinopenic(23), lymphopenic(10), and thrombopenic(22) states in man, and the normal total C′ titers in all of the various forms of agammaglobulinemia(10) make it seem unlikely that these circulating elements and the activities of the donor lymphoid cells account directly for the rises in C′ titer observed. Indeed it would appear that the best explanation at present for the increased concentration of complement in response to graft-*versus*-host reaction in the chick embryo is the "acute phase" response of complement previously reported by Boltax and Fischel (24).

Summary. 1. A method for titration of total C′ activity in the chicken is briefly described. 2. Chick embryos on the 18th, but not 13th, day of incubation have demonstrable C′ activity by this assay. 3. The 18-day-old chicken embryo responds to the stimulus of the graft-*versus*-host reaction with a rise of C′ titer.

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