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ANTIGENICITY OF THE CHORIO-ALLANTOIC MEMBRANE OF THE CHICKEN EMBRYO[1]

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In general, though not always, the substances which are foreign to or not normally present in the tissue of a respondent animal may be antigenic for the animal. Furthermore, recent studies indicate that not only organs like brain and the ocular lens, which are normally isolated from the lymph or blood circulation, possess organ-specific antigens, but also an organ so rich in blood supply as the heart may become antigenic to the animal itself and cause autoimmune responses under appropriate circumstances (1-4).

Extra-embryonic membranes such as chorions and amnions appear to be suitable targets for studying problems of organ specificity. They exist only for a certain period of time during gestation or at embryonic stage. It is possible that their constituent cells might possess some substances, among their proteins and polysaccharides, with unique antigenicity.

In the present study, the chorio-allantoic membrane (CAM) of the chicken embryo was chosen because of its easiness in handling, its similarity in embryonic origin with the amnion (5) and its wide use in virological research.

MATERIALS AND METHODS

Injection antigens. CAMs were removed from embryonating eggs of a flock of white Leghorns which had been maintained as a closed flock for years. Membranes were collected from different stages of development as required. 20 to 30 membranes were pooled, minced with scissors, and rinsed with cold saline to remove as much blood as possible.

CAM minces were used immediately or stored at -15 C for periods up to 2 months. Injection antigens were made by homogenization of the CAM mince in a Waring blendor for 5 minutes with twice volume of ice cold phosphate buffered saline at pH 7.2. After filtering through two layers of cheesecloth, the homogenates were used as such or emulsified with equal volumes of Freund's adjuvant (1.5 ml Arlacel A and 8.5 ml Klearol added with 50 mg of heat-killed and dried *Mycobacterium phlei* grown in Sauton's medium).

Test antigens. Antigens used for testing chicken sera were homogenates of the CAM, the whole embryo, or organs obtained from individual 11-day-old embryonating eggs. On the other hand, pooled homogenates of the organs were used for testing rabbit sera. All antigens were prepared by pestle-grinding with an equal amount of buffered saline at pH 7.2.

Immunizing procedures. Eight mongrel rabbits with a weight range of 1.5 to 3.0 kg were immunized according to the procedure recommended by Crowle (6). Rabbits A, B and C received respectively a primary subcutaneous injection of 3 ml of 11-day-CAM-adjuvant emulsion, and a secondary intramuscular injection of 3 ml of 11-day-CAM homogenate after 39 days. Rabbits D and E were immunized with 14-day-CAM antigens, and rabbits F, G and H with 20-day-CAM antigens.

Thirteen white Leghorn chickens weighing 1.0 to 2.5 kg each were used for immunization. They received 3 monthly intramuscular injections of 2 ml of 11-day-CAM-adjuvant emulsion.

All the animals were bled from the heart 9 days after the last injection. Pre-immune sera were also collected for control study. Immune sera were tested individually and never pooled. Chicken sera were aged at 4 C for 3 weeks or longer before use.

Serological method. The double diffusion method of Ouchterlony (7) was used with 1% Bacto-Noble agar (Difco) dissolved in phosphate buffered saline at pH 7.2 and merthiolate added at a concentration of 1/10,000. For chicken sera, salt concentration was raised to 8% (8). Patterns

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of 6 and 9 wells were used. The distance between wells was 5 mm for 6 place tests for chicken antisera, and 6 mm for 9 place tests for rabbit antisera. From 0.1 to 0.2 ml of the reactants was placed in the wells and the plates were read periodically for 14 days, incubated at 4-10 C in a moist chamber. Precipitin bands were recorded as a photogram, using a sheet of contrasty bromide paper exposed under the Ouchterlony plate by the light beam from a condenser enlarger (9).

RESULTS

Table I shows that rabbit anti-CAM sera contained antibodies producing 5 to 6 bands in the agar gel against the CAM of 11-day-old embryonating eggs. A similar number of bands was obtained regardless of the developing stage at which CAM was collected for immunization, and the antibodies cross-reacted in the similar pattern with the whole embryo and liver. Absorption test of Rose *et al.* (10) and inhibition test of Björklund (11) showed that they are antigens in common. It is apparent therefore, organ-specific antibodies and antigens specific and unique to the CAM were not demonstrated. *Figure 1* illustrates the agar-diffusion reaction produced by rabbit antiserum H.

Table II shows the reactions obtained by testing individual chicken serum against the CAM, the whole embryo, or various organs from individual 11-day-old embryonating eggs. The antisera of chickens immunized with the CAM of 11-day-old chicken embryos contained antibodies that reacted with certain, but not all, individual CAM of 11-day-old embryonating eggs. They reacted in the similar way or even with the production of more bands, with the whole embryo or liver of the same embryonating eggs, probably due to the different concentration among antigens. Their activity was inhibited by absorption with embryo homogenates. Furthermore, an antiserum producing a positive reaction with the CAM and embryo of one egg would frequently fail to elicit it with another one, which in turn gave a positive reaction to another serum. In other words, the reactivity of chicken antisera exhibited a high degree of selectivity. An example of Ouchterlony's reaction of chicken antisera which shows a single precipitation band between CAM and antiserum 7 is illustrated in *Figure 2.*

On the basis of these findings, it seems plausible that the precipitating antibodies obtained in chickens are isoantibodies. A possible interpretation of the present data is that only those antigens which were not shared by the respondent animal could elicit the formation of isoantibodies, and that the resulting antibodies would only react, by chance, with tissue samples derived from those animals which happened to possess the isoantigens in question. Along this line of reasoning, the existence of probably at least three isoantigens could be shown by agar gel diffusion precipitation test within the heterogenous flock of chickens under study.

All pre-immune sera showed negative precipitation.

It is interesting to note that certain chicken antisera reacted among each other to produce 1

TABLE I

Agar gel precipitation tests of rabbit anti-CAM sera against pooled antigens from 11-day-old embryonating eggs

Rabbit no.	Antiserum	Test antigens									
		CAM*	Embryo*	Liver*	Amnion†	Egg white†	Kidney†	Blood	Yolk sac†	Heart†	Gastro-intest. tract†
A	Anti-11-day CAM	5	5	5	4	3	2	2	4	4	4
B	Ibid	6	5	6	4	3	N	N	4	N	3
C	Ibid	4	4	4	4	4	3	N	3	N	4
D	Anti-14-day CAM	5	3	3	3	3	1	2	2	2	3
E	Ibid	5	N	2	3	2	N	N	2	N	3
F	Anti-20-day CAM	5	6	4	6	4	2	3	3	3	4
G	Ibid	5	5	5	4	3	3	2	2	4	4
H	Ibid	6	6	5	4	N	4	N	2	N	4

* The highest number of lines found in 3 to 5 tests done.

† The higher number of lines found in 1 to 2 tests done.

TABLE II

Agar gel precipitation tests of chicken anti-CAM sera against various organs from individual 11-day-old embryonating eggs

Antisera*	CAM	Test antigens																			
		Embryo		Liver			Amnion	Egg white		Kidney		Blood		Yolk sac		Heart	Gastro-intest. tract	
no.	a†b c d e	c	d	a	b	e	a	a	b	a	b	a	b	a	b	a	a	b
1	0 0 0 0 1	2	1	2	1	2	0	0	0	1	0	0	0	1	±	N	0	0
3	0 0 0 0 0	2	2	2	1	3	N	N	1	N	1	N	0	N	N	N	N	0
4	1**0 0 0 0	2	3	2	N	1	0	0	N	0	0	0	N	1	N	0	0	0
14	0 0N 0 0	N	1	1	0	1	0	N	N	0	0	0	0	0	0	±	1	0
5	0 0N 0 1	N	1	2	0	1	1	N	N	0	0	N	0	0	0	0	1	0
7	2 1 0 0 1	±	1	3	2	1	2	1	N	1	1	N	0	0	0	1	0	2
15	0 0N 0 0	N	1	2	0	1	N	N	0	N	0	0	0	N	N	N	N	0
8	N NN 0 0	N	N	NN		0	N	N	N	N	N	N	N	N	N	N	N	N
9	1 3N 0 0	N	1	1	3	0	0	N	N	0	0	0	0	1	±	0	N	N
23	2 1 1 1N	2	2	3	2	N	1	N	N	1	N	0	0	1	N	2	2	N
26	1 0 1 0N	2	2	2	1	N	1	0	0	3	1	0	0	1	N	0	1	0
27	1 0NNN	N	N	3	±	N	1	N	0	1	0	0	±	1	N	N	1	0
28	N 0NNN	N	N	N	0	N	N	N	0	N	N	N	0	N	N	N	N	N

*: Chicken anti-11-day CAM sera.
†: Individual embryonating eggs from which the antigens were obtained.
**: Number of precipitation lines.
±: Doubtful reaction.
N: Not done.

to 2 bands of precipitation in agar gel, whenever they were placed in the neighboring wells.

Burnet's technic of Simonsen phenomenon was tried to compare the number of immunologically competent leucocytes before and after immunization in chickens (12, 13, 14); unfortunately the data was not conclusive because inbred chickens were not available. Indirect complement fixation test was done using rabbit immune sera as an indicator serum (15), however no inhibiting antibodies were demonstrated in chicken antisera tested. Preliminary tests on 8 blood samples using tanned cell hemagglutination technic of Boyden (16) did not give evidence of the presence of CAM specific antigens.

DISCUSSION

Both affirmative and negative logical deductions are equally persuasive in the question of whether CAM possesses organ-specific antigens. This organ exists only in the embryonic stage as extra-embryonic membrane, therefore without getting into too much argument it is possible that it may prove foreign, accordingly antigenic, to grown chickens. On the other hand, a moderately differentiated tissue with rather simple physiologic functions as CAM is perhaps unlikely to develop organ-specific antigens. Final appraisal depends on concrete experimental findings, but only suggestive instead of conclusive inference could be made from the present study.

The present experiment failed to yield evidences to support the hypothesis that CAM possesses any unique organ-specific antigens. There are reasons to argue that under the conditions of our experiment, antibodies directed against organ-specific antigens of CAM, if the latter ever present, might have escaped detection to account for the negative result. CAM homogenates might contain such antigens attached to supra-molecular cellular elements which are capable of inducing antibody formation and yet too large to diffuse in the agar gel to be recognized. The antibodies produced could also be non-precipitating ones, which are known not to form visible precipitate with antigen even in 8% NaCl (17). Finally, heterologous interference of antibody formation (18) may also play a part, since the homogenate used for immunization is expected to contain many antigens. It is conceivable that such interference would occur provided the organ-specific antigens in CAM, if they exist at all, were

antigenically weak or quantitatively small.

Failure of the chickens to produce CAM-specific antibodies could hardly be interpreted as the result of immunological tolerance, effected by probable absorption of expendable CAM fractions *via* the umbilical veins. Rabbits did not produce CAM specific antibodies either.

It is interesting that the negative result of Ouchterlony's gel precipitation test is supplemented further by the negative finding obtained with the few experiments on tanned cell hemagglutination test and indirect complement fixation test. It is felt that our failure to demonstrate organ-specific antigens in CAM should be confirmed by fluorescent antibody technic of Coons. Using this method, several investigators were able to demonstrate human placenta and trophoblast localizing antibodies (19, 20).

The 11-day CAM was chosen for study because of its frequent use in virus research, and because it is almost fully developed at this stage. No attempts were made to study the possible ontogenic change in the antigenicity of CAM during its development as found in other organs (21, 22, 23).

SUMMARY

1. Experiments were conducted to see whether the extra-embryonic membrane, chorio-allantoic membrane (CAM), was organ-specific and antigenic to chickens.

2. Mongrel rabbits immunized with the CAM of 11-, 14-, and 20-day-old embryonating eggs developed antibodies producing 5-6 precipitation bands in Ouchterlony plates with the CAM of 11-day-old eggs. However, they cross reacted in a similar way with the whole embryo and liver. Absorption and inhibition tests further revealed that these were antigens in common. Organ-specific antibodies and antigens specific and unique to the CAM were not demonstrated.

3. Chickens immunized with the CAM of 11-day-old embryonating eggs produced antibodies that reacted occasionally with certain CAM of 11-day eggs. The reaction of chicken anti-CAM serum exhibited a definite selectivity. From zero to a maximum of 3 antigen-antibody systems were demonstrated in Ouchterlony plates depending on the combinations of individual sara with individual CAMs. It seems plausible that the precipitating antibodies observed were isoantibodies. They cross reacted with other organs too.

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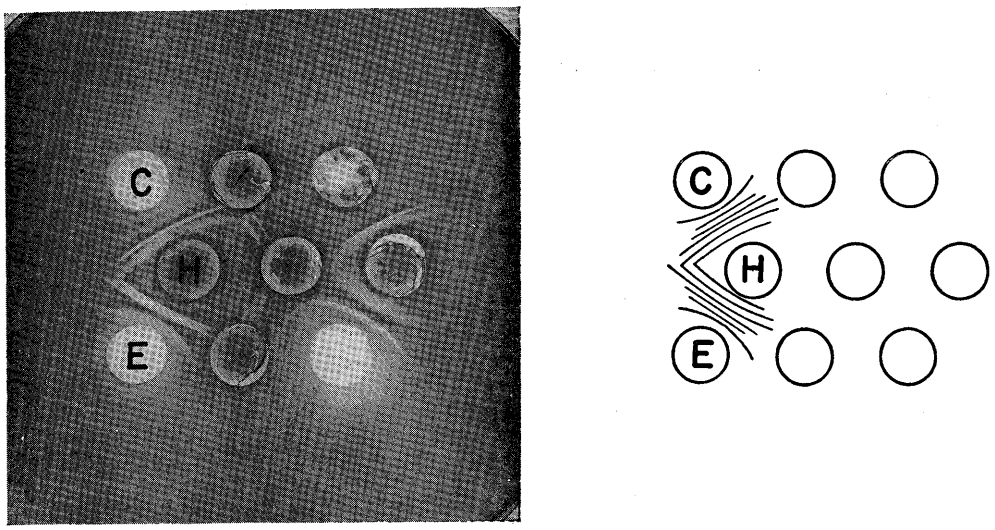

Fig. 1. Agar gel precipitation of rabbit anti-CAM serum H with the pooled CAM and embryo of 11-day-old embryonating eggs. Six similar precipitation bands occurred between antiserum H and CAM (C), and between antiserum H and embryo (E), although a line of partial identity, the 2nd one to the antiserum well, with a spur formation from the side of the CAM antigen was demonstrable.

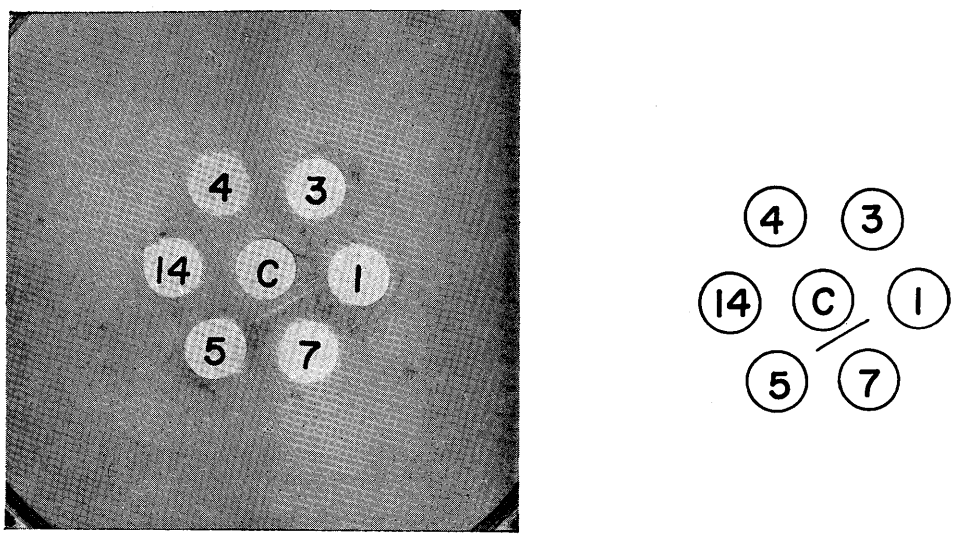

Fig. 2. Agar gel precipitation of chicken anti-CAM sera 1, 3, 4, 14, 5 and 7 with the CAM of an 11-day-old embryonating egg. A single precipitation band occurred between antiserum 7 and CAM.