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ACQUIRED IMMUNITY IN OPOSSUM (DIDELPHIS VIRGINIANA) EMBRYOS[1]

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Opossum pouch young were immunized with bacteriophage (f2, $\phi\chi$-174, and T4), haptens (2,4 dinitrophenyl (DNP), fluorescein conjugates (FTC), and 1-dimethylaminophthalene (DNS)), or proteins (ribonuclease (RNAase), sperm whole myoglobin, and lysozyme). The haptens were placed on three carriers (bovine serum albumin, hemocyanin, or bovine γ-globulin. The sera obtained from animals at sacrifice 2 weeks after immunization were studied by bacteriophage neutralization with either the intact organisms or T4 coupled to one of the haptens or to one of the proteins. When the levels of antibodies were compared with the age of the pouch young, a distinct hierarchy of responsiveness was observed. The order of appearance of responsiveness was: bacteriophage f2, DNP, bacteriophage $\phi\chi$-174, FTC, bacteriophage T4, and RNAase. This hierarchy could not be explained as a difference in sensitivity of the assay, since the assays were of similar sensitivity in each case.

It has been suggested that development of the immunologic capacities of the fetus is a well ordered, genetically determined process (1, 2). However, data supporting this hypothesis have been difficult to obtain since most animal models are complicated, requiring intervention *in utero* (1, 3). The North American opossum (*Didelphis virginiana*) provides a useful alternative model because of the early developmental stage at which these animals are born and enter the maternal pouch (4, 5). When in the pouch, opossum young can be manipulated directly for immunization (5, 6) and for modification of their immune systems (7).

The object of the paper is to provide information as to the nature of the hierarchy of responsiveness to antigens in intact embryos. We report the results of immunization of opossum pouch young with bacteriophage, haptens placed on three different protein carriers, and three small proteins. Bacteriophage neutralization techniques (8) were used to provide similar degrees of sensitivity for estimation of the antibody activities to each of the antigens.

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MATERIAL AND METHODS

Animals. Opossums were obtained from local trappers and in some cases the newly caught animals had young in their pouches. The ages of these animals were estimated by previously established criteria of body weight and of tissue differentiation (4, 5). In other cases, animals were bred in captivity in facilities generously provided by the School of Veterinary Medicine at New Bolton Center. The ages of pen-bred animals could be precisely determined by making daily inspections of the maternal pouches with the day of birth counted as day 0. Adult animals were maintained on canned dog food and water *ad libitum*. They were ordinarily kept in large open air pens but they were housed in stainless steel cages in our animal facilities when young were found in the pouches.

Antigens. The bacteriophage f2 and $\phi\chi$-174 were obtained commercially (Miles Laboratory, Kankakee, Ill.) and bacteriophage T4, obtained initially from Dr. L. Pizer, was prepared and purified in our laboratory by standard methods (9). The proteins bovine serum albumin (BSA),[3] bovine γ-globulin (BGG), hemocyanin

[3] Abbreviations used in this paper: BSA, bovine serum albumin; BGG, bovine γ-globulin; Hcyn, hemocyanin; Lys, lysozyme; RNAase, ribonuclease;

(Hcyn), hen egg white lysozyme (Lys), ribonuclease (RNAase), and sperm whale myoglobin (Myo) were obtained from commercial sources.

The hapten, 2,4 dinitrophenyl (DNP) was covalently linked to the proteins by reacting equal weights of each protein with equal weights of sodium dinitrobenzenesulfonate at pH 9.5, 37°C for 16 hr. The unreacted hapten was removed by extensive dialysis. The protein conjugates prepared in this way had molar ratios of DNP to protein of 33:1 (per 100,000 m.w.) for Hcyn, 30:1 for BSA, and 29:1 for BGG. Fluorescein conjugates (FTC) were made by incubating a 10-fold excess of the protein (by weight) with fluoresceinisothiocyanate (FITC, Isomer I, Sigma, St. Louis, Mo.) at 37°C (pH. 9.5) with slow stirring for 16 hr. These preparations were then dialyzed extensively. The extent of conjugation was 6:1 for BSA, 7:1 for Hcyn (per 100,000 m.w.), and 16:1 for BGG. Conjugation with dansyl was carried out for BSA and BGG by reacting 1-dimethylaminophthalene-s-sulfochloride (DNS chloride, Sigma) in dioxane at pH 7.5, 3°C, for 6 hr. The unreactive hapten was removed by dialysis. Conjugation with Hcyn was done by a method described by others (10). The DNS-chloride was dissolved in acetone and subsequently was reacted with Hcyn (3°C, pH 7.5) until clear. The precipitate was extensively dialyzed against 0.2 M KCl at 3°C.

The concentration of bacteriophage used for immunization varied (Table I). The concentrations of the hapten conjugated proteins were 2.5 mg/ml in Freund's complete adjuvant (CFA) or in Freund's incomplete adjuvant (IFA). The proteins RNAase, Myo, and Lys were each used in concentrations of 2.5 mg/ml in CFA or IFA for immunization. In many instances RNAase was included with the hapten conjugates for immunization. In each case, approximately 0.01 ml of the antigen preparations was injected subcutaneously into each embryo.

Antibody assays. Antibodies against the haptens and against the proteins Lys, RNAase, and Myo were assayed with the bacteriophage T4 conjugated to each of these antigens. DNP-T4 (11, 12), FTC-T4 (8), and DNS-T4 (8) were prepared by previously described methods. Myo-T4 (8), Lys-T4, and RNAase-T4 were prepared by a modification of the technique of Haimovich and Sela (13). The antibody assays were carried out with the agar overlay technique (9). Our earlier experiments (8) indicate that the sensitivity of these assays is nearly identical, measuring as little as 0.1 ng/ml. The reactions in most cases were carried out at 37°C for 60 min to maximize the sensitivity of the assay. Because of the high level of natural reactivity of control serum with DNP-T4 the reactions were carried out at varying dilutions of serum for 10 min so that neutralization constants (K) could be calculated.

In order to establish standards for designating a positive reaction, we studied control serum from unimmunized opossums of each of five age groups as well as maternal serum for anti-DNP activity. The mean and S.D. for each control group were then calculated and positive responses for anti-DNP were recorded when the K for serum from immunized young was greater than 2 S.D. from the mean of the controls. The natural antibody activity for the other antigens was much less and its level seemed not to be age dependent. Standards for these antibody responses were established by evaluating the serum from unimmunized opossums which were 3 to 6 months of age when bled. The means of the ratios of plaques at time 60 min to those at time 0 min were established for each antigen. Results of similar phage neutralizations with serum from immunized animals were gathered and those in which neutralization was more

TABLE I

Responses to varied amounts of bacteriophage in animals 25 days and older

Age	Low Dose Group			High Dose Group		
	f2[a]	ϕX-174[a]	T4[a]	f2[a]	ϕX-174[a]	T4[a]
days	10^7	1.2×10^7	2×10^6	10^{10}	10^{10}	10^{10}
25–29	0:1[b]	0:1	0:2	4:5	2:5	2:5
30–39	1:7	2:9	0:9	5:8	7:9	5:8
40–49	4:27	4:29	0:29	5:5	4:5	3:5
50+	3:8	5:8	0:8	8:8	8:8	8:8

[a] Antigens.

[b] The ratios indicate the number of positive responses:total animals or pool of animals tested.

Myo, sperm whale myoglobin; DNP, 2,4 dinitrophenyl; FTC, fluorescein conjugates; FITC, fluoresceinisothiocyanate; DNS chloride, 1-dimethylaminophthalene-s-sulfochloride; CFA, Freund's complete adjuvant; IFA, Freund's incomplete adjuvant.

than 2 S.D. from the mean of the unimmunized controls were considered to be positive.

RESULTS

Response to bacteriophage. Preliminary studies using bacteriophage f2 $\phi\chi$-174, and T4 emulsified in CFA indicated that adult opossums responded vigorously to each of these antigens and that the antibodies formed to these antigens do not cross-react with each other.

The effect of varying the amount of antigen on the immune response of pouch young was studied (Table I) in opossums older than 20 days. One group (low dose) received 10^7 f2, 1.2×10^7 $\phi\chi$-174, and 2×10^6 T4 bacteriophage particles in CFA. The second group of opossum young (high dose) were given 10^{10} particles of each virus in CFA. Each animal was sacrificed 14 days after immunization. Only 16% of the low dose animals responded to bacteriophage f2, 21% responded to bacteriophage $\phi\chi$-174, and none of the animals produced antibodies when immunized with bacteriophage T4. In contrast, 61% to 77% of the animals receiving the high dose responded immunologically.

In addition to these older opossums, 12 embryos between 8 and 22 days of age were given the higher dose of bacteriophage (Table II). The youngest animals responding to bacteriophage in this experiment were 15 days old when immunized with bacteriophage f2 or $\phi\chi$-174 and 20 days of age when given bacteriophage T4. Statistically significant differences ($p > 0.01$) were recognized when the incidence of positive responders 20 days or older was compared with the incidence of positive responders in the 8- to 19-day age group.

Response to haptens. Three haptens (DNP, FTC, and DNS) were used in this portion of our study. Each of the haptens was conjugated to three different protein carriers. Adult opossums had good immune responses to DNP and FTC but failed to respond to DNS.

The results obtained by immunizing opossum embryos of varying ages with each of the hapten-carrier combinations are recorded in Table III. As expected no embryos responded to DNS regardless of the carrier used but some animals of each age group produced anti-FTC when the carrier was Hcyn. The youngest responsive animal immunized with FTC-Hcyn was 19 days of age, and the 6 younger unresponsive animals ranged from 11 to 18 days of age. In contrast, no opossums which were younger than 30 days responded to FTC-BSA and no opossums less than 50 days of age responded to FTC-BGG. Positive responses were recorded in approximately 60% of animals older than 50 days of age.

The response of opossum embryos to DNP proved to be more difficult to interpret. As indicated in Table III, a high proportion of the animals tested responded to DNP on each of the

TABLE II

Responses to 10^{10} bacteriophage in animals less than 25 days of age

Age	f2	$\phi\chi$-174	T4
days			
8	0:2[a]	0:2	0:2
9	0:1	0:1	0:1
11	0:1	0:1	0:1
15	1:2	1:2	0:2
16	1:1	0:1	0:1
20	1:3	2:3	1:3
22	0:2	1:2	0:2

[a] The ratios indicate the number of positive responses:total animals or pool of animals tested.

TABLE III

Effect of carriers BSA, Hcyn, and BGG

Age	BSA			Hcyn			BGG		
	DNS	FTC	DNP	DNS	FTC	DNP	DNS	FTC	DNP
days									
8–19	0:1[a]	0:6	5:6	0:3	1:7	1:3	0:4	0:4	2:3
20–29	0:5	0:11	3:4	0:3	1:7	4:5	0:2	0:4	3:3
30–39	0:6	3:8	5:6		1:2	1:2		0:2	2:2
40–49	0:6	2:6	4:6	0:7	2:7	7:9	0:6	0:6	7:10
50+	0:7	2:7	4:6	0:3	3:3	3:3	0:4	3:4	3:4

[a] The ratios indicate the number of positive responses:total animals or pool of animals tested.

three protein carriers. The youngest responding animals were 11 days of age when immunized with DNP or BSA, 17 days with Hcyn, and 15 days with BGG. No animals younger than 15 days were tested with the BGG-hapten combination. As mentioned earlier, anti-DNP activity was present in many unimmunized control animals and increased with age. Thus, the activity of serum from older unimmunized opossum embryos was very nearly as great as that seen in serum of unimmunized adults. However, the antibody levels were substantially greater in immunized than in unimmunized animals and the level of antibody activity reached was independent of the protein carrier (Fig. 1) except that Hcyn was somewhat less effective than were the other two carriers.

Response to proteins. Three small proteins were employed in this portion of our study (Table IV). The youngest opossum responding to RNAase was 30 days of age and 21 younger animals failed to respond to RNAase. Although older opossum embryos were somewhat more frequently responsive, they still represented only a small fraction of the total number of animals studied in any one age group. Our experience with Lys and Myo was less extensive with none of the 13 embryos tested responding to Myo. Seventeen animals were immunized with Lys but only 4 produced detectable antibodies. The youngest of these responsive animals was 35 days of age.

Effect of adjuvant. Four litters of opossums were studied to identify the effect of adjuvant on the immune response in pouch young. One-half of each litter was given antigen emulsified in CFA and the remaining opossums were given antigens emulsified in IFA. The haptens were conjugated to BSA. The results of these studies are included in Table V. There was no difference in antibody responses to each of the four antigens used.

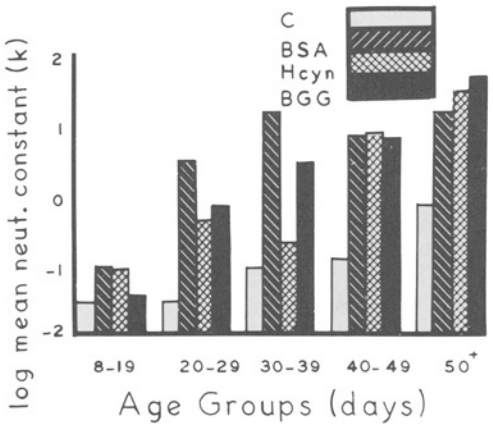

Figure 1. The average log mean neutralization constants (K) for anti-DNP are included for unimmunized controls and animals immunized with DNP on each of three protein carriers.

TABLE IV

Response to proteins

Age	RNAase	Myoglobin	Lysozyme
days			
8–19	0:13[a]		
20–29	0:8		0:1
30–39	2:18	0:9	2:9
40–49	3:27		
50+	2:16	0:7	2:7

[a] The ratios indicate the number of positive responses: total animals or pool of animals tested.

DISCUSSION

The principal objective of this work was to test the hypothesis that a hierarchy of responsiveness to antigen exists in intact animals. In order to do this, we selected three groups of antigens representing intact viruses, haptens, and small proteins as antigens. Bacteriophage neutralization was selected as the method of antibody assay to standardize the sensitivity of antibody detection (8). These studies indicate that a hierarchy of antigen responsiveness exists when the population of opossum embryos is considered as a whole, but that there is considerable variation among embryos with regard to their responsiveness to individual antigens. Figure 2 summarizes the results of our studies. It shows the earliest time during development at which the responses to individual antigens were

TABLE V

Effect of adjuvant on the immune response

Antigen	CFA	IFA
Bacteriophage	6:10[a]	5:6
FTC	0:4	1:5
DNS	0:4	0:5
RNAase	0:5	1:8

[a] The ratios indicate the number of positive responses: total animals or pool of animals tested.

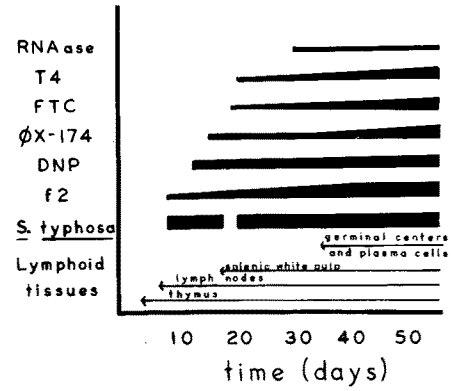

Figure 2. Development of lymphoid tissues and immune responses to *S. typhosa;* bacteriophage f2, ϕX-174, and T4; haptens DNP and FTC; and RNAase in opossums. The percentages of positive animals are indicated by the thickness of the bars.

found, the proportion of embryos responding to each antigen at any given time, and the maturation of the lymphoid tissues in opossum embryos. The protein Myo and the hapten DNS are excluded since none of the test animals responded to these antigens. Although four embryos responded to Lys, the numbers of available test animals younger than the youngest responder was so small that no conclusion as to the beginning of responsiveness could be reached.

The present studies suggest that animals first responded to bacteriophage f2 at 13 days. Previous observations (6) indicated that animals as young as 8 days of age can respond to this antigen. Similarly, earlier studies (5) had shown that opossums as young as 8 days of age responded to antigens of *S. typhosa*. By the 8th day in the maternal pouch, opossums have an immature thymus as well as primitive lymph nodes (4, 5). The initial response of developing opossums to bacteriophage ϕX-174 was first seen at 15 days with four younger animals not responding to this antigen. The thymus has, by 15 days, become more elaborate in having clearly defined cortical and medullary divisions and the embryonic lymph nodes have become more prominent. By the 20th day, when the youngest opossum responsive to T4 was found, the spleen had begun to acquire perivascular lymphoid elements. We have indicated a deletion in responsiveness to *Salmonella typhosa* between 17 and 20 days since our earlier experiments (5) showed a failure of responsiveness to this antigen in a small group of opossum embryos studied at this time. This observation should be tested with larger numbers of opossums. Thus, if the youngest responsive animal is used to establish the position of a particular antigen in a hierarchy, it seems that initiation of immune responses to three bacteriophages and to a bacterium is spread over a 12 day period.

Similar observations have been made with the other antigens used in this series. For example, the youngest animal responding to RNAase is 30 days with 21 younger animals failing to respond. However, in contrast to what was seen with bacteriophage only a small proportion of animals at any stage of maturation in the pouch responded to RNAase. Our preliminary studies suggest that peak antibody responses to RNAase may not be reached in adult opossums until the 3rd week post immunization. This could account for some of the failures to immunize pouch young with this antigen since these animals were sacrificed 2 weeks after immunization. The relative infrequency of immune responses to FTC, and Lys, and the absence of immune responses to DNS and Myo remain unexplained except for the observation that Lys, Myo, and DNS are poor antigens in adult opossums.

A study in sheep (1, 2) also showed a hierarchy with respect to certain antigens. However, because of differences in the antigens used in opossums and sheep only the responses to ϕX-174 and *S. typhosa* can be compared directly. Sheep responded to immunization with ϕX-174 at 40 days of gestation, at which time a discrete thymus was not present. An early response was also generated with this antigen in opossums but it appeared, as did response to each antigen used by us, only after lymphocytes were formed in the thymus. Sheep and opossums differ significantly with regard to their responses to *S. typhosa*. Sheep failed to respond to this antigen at any time during development but opossums responded to *S. typhosa* at a very early stage of development (5), coincident with the time of their responsiveness to bacteriophage f2.

Recent experimental work in ontogeny of the immune response has emphasized the differential rate of development of T and B cell populations (14, 15). Such studies indicate that B cell functions appear early in development and

rapidly express a wide repertoire of antigen-specific cell receptors (15) but T cell functions develop later (14). These observations suggest that the hierarchy of responsiveness in the systems examined most probably reflects T cell development and differentiation. Although it seems likely that both sheep and opossums have both T and B cell populations of lymphocytes, neither has been explicitly demonstrated in these animal species. However, the fact that opossums which are as young as 15 days of age respond to certain haptens makes it likely that a system analogous to T cells has developed by this stage of maturation. The variability and often low degree of hapten substitution on protein carriers used in these experiments probably makes further speculation as to the relationships between development of these animals and T-B cell maturation unwarranted.

We did not formally test any of the other T cell functions such as delayed hypersensitivity of graft rejection. Previous work indicated that thymectomy modified the development of lymphoid tissues in opossum embryos (7) and unpublished observations (16) suggest that lymphocytes from adult opossums do not respond in mixed lymphocyte cultures. Studies by other investigators (17) have indicated that opossum embryos can reject skin allografts applied as early as the 12th day in the maternal pouch.

Although our comparisons of immune responses with CFA and IFA are limited, they suggest that the form of presentation of the antigen to these embryos makes little difference in their ability to respond to given antigens. Similar results were obtained when antigens were presented to adult opossums in CFA or in saline (18). The study of dose response in embryos which we carried out with bacteriophage suggests that dose of antigen will be significant in the study of ontogeny of the immune response. We hope to pursue this further, especially with regard to haptens (e.g., FTC) and small proteins (e.g., RNAase) where the immune response is variable.

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