FETAL RESPONSE TO ANTIGENIC STIMULUS

II. Antibody Production by the Fetal Lamb*, ‡

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Observations on the absence of an immune response in the immature young of a number of species and on the ability to induce in them a state of immunological tolerance of certain antigens have supported the hypothesis on the existence of an immunological "null" period during embryonic development of mammals. This state of immunological immaturity is widely assumed to persist throughout the intra-uterine existence of the fetus, and to terminate only at or shortly after birth (1–3); indeed, a varying degree of immunological incompetence has been observed in certain species for some time after birth (4, 5).

It has become increasingly evident, however, that the timing of immunogenesis may vary appreciably from one species to another. In the human the premature newborn may be stimulated to form antibody (6) and to develop delayed hypersensitivity (7), while the human fetus responds to congenital infection with plasma cell formation (8). The fetal calf *in utero* (9) and the immature opossum in the pouch (10) are able to produce antibody, the fetal guinea pig develops delayed hypersensitivity (11), and the fetal lamb seems able to reject homografts specifically (12).

There are two important purposes to be served by a study of the immune response of the fetus *in utero*. An appreciation of the factors involved in the ontogenesis of the immunological apparatus in the developing animal will contribute to an understanding of the fundamental nature of the immune response and its relationship to other biological phenomena. Secondly, the mammalian fetus offers an almost unique opportunity for the study of the

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immune response in an immunologic virgin, uncomplicated by the host of unrelated activities in which most "normal" animals are engaged.

The fetal lamb has proved to be an admirable experimental subject for this type of study. The placenta of the pregnant sheep does not permit passage of γ-globulin from mother to fetus; thus, any γ-globulin or antibody found in the fetal circulation is produced by the fetus. Further, the incidence of twinning in the pregnant sheep and the relative ease of the surgical procedure for intrafetal injection allow for the stimulation of only one sibling, reserving the twin fetus as a control.

In this paper we shall report on some of the characteristics of the antibody response of the fetal lamb following intra-uterine antigenic stimulation. Data will be presented on the variations in this response as a function of the gestational age of the fetus, the duration of the stimulus, the type of antigen employed, and the role of adjuvant. In Paper III of this series will be described the "immune" globulin response of these same fetuses, and its relationship to the antibody produced.

Materials and Methods

Pregnant Sheep.—The ewes employed in this study were of random mixed breed, from 3 to 7 years old. Through the courtesy of the Sheep and Fur Animal Research Branch, Animal Husbandry Research Division, Beltsville, Maryland, the servicing by the ram was done under controlled conditions such that the date of conception of the ewes could be established within 1 to 2 days. The gestation period of the ovine is 150 days.

Antigens Employed.—Twice-recrystallized ovalbumin was obtained from the Worthington Corp., Freehold, New Jersey. Ferritin was lot F74 from the Pentex Corp., Kankakee, Illinois, prepared from horse spleen. Purified diphtheria toxoid, lot PT78 (1325 Lf/mg) was obtained from the Massachusetts Department of Public Health. Bacteriophage φX 174 was prepared and purified as previously described (13). Heat-killed *Salmonella typhosa* O901 was obtained through the courtesy of Dr. Maurice Landy, National Cancer Institute, the National Institutes of Health, Bethesda. Viable BCG was kindly supplied by the Research Foundation, Chicago, as the lyophilized vaccine, Lot 10 BLP.

All injections were made intramuscularly in the fetus, usually in the rear limb. When two separate injections were made into the same fetus, both rear limbs were used. Bacteriophage was invariably injected in saline solution, using 10^{10} plaque-forming particles in 0.1 ml. Diphtheria toxoid was used in 100 μg doses, either in 0.1 ml of saline or in 0.1 ml of complete Freund's adjuvant prepared with 2 mg of viable BCG and with bayol F and arlacel A in customary proportions. All other intrafetal injections were of 0.25 ml of complete adjuvant containing 1.25×10^9 heat-killed *Salmonella*, 2 mg of viable BCG, 1 mg of ovalbumin, and in some instances 1 mg of ferritin.

Surgical Procedure for Intrafetal Injection.—The ewe at the desired stage of gestation was prepared by intravenous administration of ¼ grain of atropine HCl, 2 ml of koagamin coagulant, and 18 to 25 grains of pentobarbital sodium to provide light anesthesia. An endotracheal tube was introduced following topical tetracaine HCl spray in the throat, and ether-oxygen anesthesia maintained thenceforth. A midline incision was made along the linea alba posterior to the umbilicus for 6 to 8 inches. The gravid horn of the uterus could then be manipulated into position so that the portion of the uterus containing the posterior end of the fetus could be lifted through the incision. Holding the rear limb of the fetus against the uterine wall, an

uncontaminated 25-gauge needle could be directed through the uterus and fetal membranes and into the muscle of the fetal thigh, taking care to avoid the cotyledons and the blood vessels of the intervening tissues. In this manner, either or both rear limbs could be injected, and on occasion both twin fetuses could be injected in turn. In each instance, the site of the needle puncture in the uterus was carefully cleansed, and the uterine horn replaced in its normal position in the abdominal cavity. Closure was effected by employing heavy vetafil suture in cruciate stitches to close the peritoneum and fascia. Medium chromic gut in a loose continuous suture was used to close the subcutaneous fat and connective tissue. The skin was closed with a continuous mattress stitch of light vetafil. In most instances, 650,000 units of penicillin and 1.67 gm of dihydrostreptomycin sulfate were given intraperitoneally to the ewe, and 0.2 per cent nitrofurazone ointment applied liberally over the surgical field.

In all, 40 laparotomies were performed upon pregnant ewes in the 3 year period covered by this report. Of these animals, five were lost; one as an anesthetic death, two following the development of peritonitis, one as the result of herniation through the opened peritoneum, and one of a febrile illness of unknown etiology. In addition, one pair of twin fetuses was delivered stillborn to a toxemic ewe. In three other cases, hysterotomy after the desired period of stimulus revealed that one of the injected fetuses had suffered an intra-uterine death. In two of these three instances, the second twin had also been injected, but survived to furnish useful data.

Delivery of Fetal Animals and Collection of Specimens.—At the desired interval after intrafetal injection the fetus (and when present, its control twin) were delivered by hysterotomy, following essentially the same procedure as was outlined above for the initial laparotomy. Following exposure of the fetus through the incised fetal membranes and uterus, the maximum possible amount of fetal blood was withdrawn from the umbilical cord. Previously, blood had been taken for control purposes from the mother both at the time of the initial laparotomy and at the time of delivery of the fetus. In every instance, the serum was separated as rapidly as possible and stored in aliquots in the deep freeze.

Where one fetus was present, it and the corresponding blood were labeled with the mother's number -1; any twin was labeled with the mother's number -2. The newly delivered fetuses were immediately removed for autopsy, where the injection site could almost invariably be identified and so recorded.

Serologic Analyses.—The technic of Landy and coworkers (14) was employed for the examination of all maternal and fetal sera for the presence of *Salmonella* agglutinins, using a polyvalent *Salmonella* antigen. Antibodies against the mycobacteria were tested for by a modification of the tanned cell hemagglutination technic of Middlebrook and Dubos (15). The tanned erythrocyte hemagglutination technic of Boyden (16) was employed in the tests for antibody to ovalbumin and to ferritin. Diphtheria antitoxin was determined by the method of Fraser (17). Antibody to φX was determined by the standard bacteriophage neutralization assay (18). The results are expressed in terms of K, the first order inactivation constant which describes the neutralization of the phage by the antiserum in question. To determine whether the anti-phage antibodies were of the 19S β_{2M} macroglobulin variety, or 7S γ-globulins, advantage was taken of the susceptibility of the former to treatment with 2-mercaptoethanol (19). Sera were incubated in 0.1 M 2-mercaptoethanol at 37°C for 30 minutes, and then assayed for anti-phage activity as described above.

The Sensitivities of the Assay Procedures.—Since the fetal lamb responds differently to different antigens, it should be noted that the several serologic assay systems employed in this study are able to detect roughly the same order of magnitude of antibody concentrations. The assay for diphtheria antitoxin is able to detect about 0.001 unit of antitoxin/ml, equivalent to roughly 0.003 μg antibody protein/ml. The tanned cell hemagglutination technic can detect about 0.005 to 0.01 μg antibody/ml. In the bacteriophage neutralization test, a K value of 1 represents a serum yielding a precipitin arc in agar double diffusion, and is equivalent to at

least 1 μg antibody/ml. Since the phage neutralization technic can measure a K value of about 0.001, this test is capable of detecting anti-phage antibody at a concentration of more than 0.001 μg/ml. In the absence of reliable figures, it would appear reasonable to assume a sensitivity of the *Salmonella* agglutination procedure and the Middlebrook-Dubos technic of the order of 0.01 μg antibody/ml.

RESULTS

The two disadvantages encountered in the use of the pregnant sheep and the fetal lamb in experimental work relate to the size of the ewes and the physical difficulty in handling large numbers of them at one time, and to the fact that due to their breeding habits pregnant sheep are available for only a relatively short period once each year. It will therefore be appreciated that extensive studies employing many replicate animals for each experimental circumstance are impracticable. The present results therefore suffer from the disadvantage of small numbers; nevertheless, based in the present report on results obtained over a 3 year period, a generally consistent picture emerges.

Antibody Response as a Function of Fetal Age and Duration of Stimulus.— Laparotomies were performed on pregnant ewes at about the 70th day of gestation. One fetus in each ewe was injected intramuscularly in the hind leg with an antigen mixture containing 2 mg of viable BCG, 1.25×10^9 heat-killed *Salmonella typhosa*, 1 mg of crystalline ovalbumin, and 1 mg of ferritin, all emulsified in Freund adjuvant. At intervals from 10 to 60 days thereafter the fetuses were delivered by hysterotomy, bled from the cord, and the sera examined for antibody response on the part of the fetus. In addition, the sera of the control, uninjected twins delivered at the same time were examined serologically, as were control sera from the mothers taken at the time of initial injection of the fetus and at the time of surgical delivery. These data are recorded in Table I.

In no instance did any of the maternal sera show even a trace of anti-ovalbumin or anti-ferritin. As proved to be true of the general population of adult sheep, low, variable titers of *Salmonella* agglutinins, not exceeding 1:40, were found in many animals. These were not altered by intrafetal stimulus, indicating that the mothers had not responded to the antigen given the fetus. In a similar fashion, about half of all the adult ewes tested showed positive titers in the Middlebrook-Dubos test, not exceeding 1:64; here too, no change in titer followed intrafetal injection with BCG.

Four of the six fetuses injected at this time had twins, which were left uninjected for control purposes. In no instance was antibody found in any of these control animals with the assay procedures described above. In the stimulated animals, no detectable antibody could be found which corresponded to the *Salmonella* or the BCG components of the injection mixture. None of the fetuses made anti-ovalbumin antibody within the first 40 days following injection, and only 60 days after injection did a fetus show evidence of small amounts of antibody to this antigen. Ferritin, on the other hand, appeared to

provide a more effective stimulus to the fetal lambs, since all animals responded with anti-ferritin production, even as early as 10 days after injection of the antigen. There appeared to be no consistent relationship between anti-ferritin titer and the duration of stimulus in this experiment.

In order to examine the response of older fetuses to antigen, the same procedure as described above was employed in the sensitization of 89- to 101-day

TABLE I

Antibody Production by the 65- to 70-Day Fetal Lamb

Injected fetus received Freund adjuvant containing viable BCG, killed *S. typhosa*, ovalbumin, and ferritin.

Fetus No.	Gestation age at injection	Interval between injection and bleeding	Antibody titers*			
			Anti-ovalubmin	Anti-ferritin	Anti-*Salmonella*	Anti-mycobacteria
	days	*days*				
7013-1	70	10	0	128	0	0
7024-1	72	20	0	1024	0	0
7028-1	72	20	0	128	0	0
7023-1	71	29	0	16	0	0
7017-2	70	40	0	64	0	0
7032-1	65	60	64	2048	0	0
Control twins (uninjected)	Gestation age at delivery					
	days					
7024-2	92		0	0	0	0
7028-2	92		0	0	0	0
7023-2	100		0	0	0	0
7017-1	110		0	0	0	0

* Antibody titer is expressed as the reciprocal of the highest dilution of serum yielding a positive agglutination. All maternal sera were negative for anti-ovalbumin and anti-ferritin.

fetal lambs. The antigen mixture employed was the same, except that in a number of instances ferritin was omitted. The results of the antibody assays on these animals are recorded in Table II. Again, none of the sera taken from the respective mothers of the fetuses in question showed any evidence of an antibody response associated with the intrafetal stimulus; in every instance there was a complete absence of anti-ferritin and anti-ovalbumin. Nine of the thirteen fetuses in this study has twins, which are not immunized and were reserved for control purposes. In no instance did the control fetus show a trace of circulating antibody against any of the antigens employed to stimulate its sibling. This was even true of fetus 7021-1, whose injected twin showed one of the highest circulating antibody titers, both to ovalbumin and to ferritin, and confirms

the observation that interfetal exchange in the pregnant sheep is not a common occurrence.

In no instance was antibody found among the stimulated fetuses which reacted in the *Salmonella* agglutination or Middlebrook-Dubos tests. The results show that no fetal animal made antibody in response to ovalbumin earlier than the 30th day following injection. After this time, there seems to be a

TABLE II

Antibody Response in the 89- to 101-Day Fetal Lamb

All fetuses received Freund adjuvant containing viable BCG, killed *S. typhosa*, ovalbumin, and, in some instances, ferritin.

Fetus No.	Gestation age at injection	Interval between injection and bleeding	Antibody titers*			
			Anti-ovalbumin	Anti-ferritin	Anti-*Salmonella*	Anti-mycobacteria
	days	*days*				
7001-1	91	6	0	1,024	0	0
7039-2	91	6	0	1,024	0	0
495-1‡	89	10	0	—	0	0
877-1‡	89	10	0	—	0	0
2027-2	101	10	0	512	0	0
2495-1‡	90	20	0	—	0	0
2471-1‡	90	20	0	—	0	0
2252-1‡	90	33	512	—	0	0
1675-1‡	90	33	256	—	0	0
7009-1	101	30	64	256	0	0
7007-2	92	50	512	8,000	0	0
7021-2	93	52	4,000	16,000	0	0
7036-1	96	49	4,000	32,000	0	0

* Antibody titer is expressed as the reciprocal of the highest dilution of serum yielding positive agglutination.

‡ These animals received no ferritin in the injection mixture.

crude correlation between degree of antibody response and duration of stimulus, although this trend appears to be superimposed upon an appreciable individual variation in response.

Ferritin proved to be by far the most efficacious antigen in this experiment. Every fetus that received this antigen formed circulating antibodies to it, even as early as the 6th day after injection. Again, there seems to be a positive though crude correlation between the duration of stimulus and the level of anti-ferritin response by the fetus.

Antibody Response as a Function of the Nature of the Antigen.—It is apparent from the experiments described above that while the fetal lamb seems unable to respond to *Salmonella* and BCG with the production of circulating antibody, it is able to produce antibody against the two protein antigens employed,

ferritin and ovalbumin. To examine this situation further, a new class of antigenic substance, bacteriophage φX 174, was employed for fetal stimulation along with another protein antigen, diphtheria toxoid.

Fetal lambs whose gestation ages ranged from 60 to 118 days were injected *in utero* as outlined above. In the present experiment, the animals received 10^{10}

TABLE III

Antibody Response of the Fetal Lamb to Bacteriophage φX 174 in Saline in One Limb and Diphtheria Toxoid in Adjuvant in the Opposite Limb. The Effect of 2-Mercaptoethanol Treatment on the Antibody

Fetus No.	Gestation age at injection	Interval between injection and bleeding	Antibody titers*		
			Antitoxin	Untreated anti-φX	2 Mercapto-ethanol-treated anti-φX
	days	*days*	*units/ml*	*K*	*K*
2718-1	60	6	0	0.02	Not done
2097-2‡	60	10	—	2.3	0
7031-2	72	11	0	8.9	0
7005-2	91	10	0	7.3	0
7035-1	90	29	0	11.0	16.2
7027-1	101	10	0	228	14
7009-1	101	30	0	5.1	4.8
7026-1	118	10	0	23.0	0
Control twins (uninjected)	Gestation age at delivery				
	days				
7027-2	111		0	6.1	0
7009-2	131		0	0	0

* Anti-bacteriophage titers are expressed in terms of *K*, the first-order inactivation constant of neutralization.

‡ No toxoid given.

plaque-forming φX particles in 0.1 ml of saline intramuscularly in one rear limb, and 100 μg of diphtheria toxoid in complete Freund adjuvant intramuscularly in the opposite rear limb. They were allowed to respond to the stimuli for varying periods from 6 to 60 days thereafter and were then sacrificed by hysterotomy. The data for these animals, and for the uninjected control twins of two of these fetuses, are presented in Table III.

In no instance was circulating diphtheria antitoxin found in any of the immunized fetal lambs, regardless of the duration of the stimulus. Although it is not indicated in Table III, examination of these sera with the Middlebrook-Dubos technic (the fetuses having received complete adjuvant) also showed an absence of detectable antimycobacterial antibodies.

Every fetal lamb that received bacteriophage responded with the production

of circulating antibody which inactivated φX. Even the 60 day fetus allowed to respond for only 6 days produced small amounts of antibody to the phage. This antibody neutralized φX but not T_2, an immunologically unrelated phage. There seemed to be no clear-cut correlation between the antibody titer and the age of the fetus at injection. In no instance did any of the mothers possess anti-phage antibody which could be attributed to contamination of the mother during the process of injection of the fetuses, although several of the ewes possessed slight amounts of a phage-inhibiting activity in their sera both before and after fetal injection. One normal, uninjected control fetus (7027-2) possessed antibody to one of the antigens which its twin fetus had received. This control animal had an anti-phage titer of 6.1. Its twin had produced the remarkably high anti-phage titer of 228. Whether this response was due to accidental contamination of the control with virus during injection of its twin, or alternatively, to the interfetal transfer of antibody, is unclear. It may be mentioned that crossed circulations between non-identical lamb twins is reportedly an exceptionally rare phenomenon (20).

The Nature of the Anti-Phage Antibody.—It is an increasingly more common observation that the earliest response to antigenic stimulus, especially among young animals, consists of a β_{2M} macroglobulin antibody (6, 21). This macroglobulin antibody has been shown to be sensitive to treatment with 2-mercaptoethanol, whereas the 7S γ-globulin antibody is not inactivated by this treatment (19). We have therefore examined the antibody produced in the fetal lamb against bacteriophage φX, both with and without prior treatment with 2-mercaptoethanol. The effects of this treatment are recorded in the final column of data in Table III.

It may be seen that almost all of the anti-phage activity which the fetal lamb produced within the first 10 to 11 days after phage injection is sensitive to the destructive action of 2-mercaptoethanol, and is therefore presumably a 19S macroglobulin. Within this period only one fetus produced any 2-mercaptoethanol-resistant antibody, and this to the extent of only about 6 per cent of the total antibody titer in the animal which had produced by far the greatest anti-phage response of all the fetuses. In the fetuses allowed to respond to the phage stimulus for longer periods of time, the relative amount of 2-mercaptoethanol–resistant, 7S γ-globulin antibody increased.

A Preliminary Observation on the Effect of Adjuvant on the Antibody Response of the Fetal Lamb.—It was originally hoped to study the effect of adjuvant as an enhancer of antibody production in the immunologically virgin fetal lamb. It has been amply demonstrated that the adult animal, regardless of species tested, gives a heightened response to antigen in adjuvant. For this purpose, three sets of fetal lamb twins were employed, one sibling in each set receiving 100 μg of diphtheria toxoid in adjuvant and the other receiving the same dose of toxoid in saline solution. Each of the fetuses was given 10^{10} φX particles in saline in the opposite limb. All injections were intramuscular.

It proved to be impossible to study the effect of adjuvant on the response to diphtheria toxoid (Table IV), since the fetal lamb in no instance produced detectable circulating antibody to this antigen (see also Table III). The response to the bacteriophage injections which accompanied those of toxoid, however, proved of great interest (Table IV). The two surviving fetal lambs that had received phage accompanied by a simultaneous injection of adjuvant in the opposite limb gave anti-phage titers of 7.3 and 8.9. On the other hand, the three fetuses that received phage without accompanying adjuvant injection yielded anti-phage titers of 15.9, 20.9, and 38.4. While the data are incomplete,

TABLE IV

The Effect of Complete Adjuvant on the Anti-Bacteriophage Response of the Fetal Lamb

Fetus No.	Adjuvant injected*	Gestation age at injection	Interval between injection and bleeding	Antibody titers‡		
				Untreated anti-φX	2 Mercaptoethanol-treated anti-φX	Antitoxin
		days	*days*	*K*	*K*	*units/ml*
7031-1	No	72	11	20.9	0	0
7031-2	Yes	72	11	8.9	0	0
7005-1	No	91	10	38.4	0.79	0
7005-2	Yes	91	10	7.3	0	0
7010-1	No	91	10	15.9	0.31	0

* Diphtheria toxoid injected in one leg either in adjuvant or saline; all animals injected with phage in saline in other leg.

‡ Anti-bacteriophage titers are expressed in terms of K, the first order inactivation constant of neutralization.

the values suggest the possibility that the presence of adjuvant at a different site may have inhibited, rather than enhanced, the antibody response of the fetus to bacteriophage. As before, with the exception of a small amount of 2-mercaptoethanol-resistant 7S antibody in one animal (the best antibody producer), all of the anti-phage activity proved to be 19S protein susceptible to the action of 2-mercaptoethanol.

An Anamnestic Antibody Response at Birth.—A single fetal lamb (7036-1) was not sacrificed upon delivery at 145 days' gestation. On the day of birth it was given an intramuscular booster injection of the same adjuvant mixture (BCG, *Salmonella*, ovalbumin, and ferritin) that it had received *in utero* 49 days previously. At the time of birth, its anti-ovalbumin titer was 1:4,000 and its anti-ferritin titer 1:32,000; 7 days after the booster injection, it showed an anti-ovalbumin titer of 1:128,000 and an anti-ferritin titer of 1:128,000. These titers continued to increase, so that at 25 days of age, the lamb had an anti-ovalbumin titer of 1:500,000 and an anti-ferritin titer of 1:2,000,000.

During this period, however, the newborn lamb produced no detectable antibody against either *Salmonella* or BCG.

Antibody Response in the Adult Sheep.—Pairs of adult sheep were injected with the same amounts of each of the antigen mixtures employed in the lambs, in order to establish the fact that the different antigens employed for the stimulation of the fetal lambs are indeed antigenic in the ovine. Only against diphtheria toxoid was the antibody response poor; one of four ewes formed the minimal detectable antitoxin (0.001 unit/ml) in the 3 weeks following immunization. The antibody titers to ovalbumin, ferritin, bacteriophage, *Salmonella,* and BCG in the 3 weeks after immunization were all readily detectable. Had the same dosage per kilogram of body weight been used in the adults as was employed for the fetal lamb injections, the antibody response of the ewes would probably have been greater.

DISCUSSION

In striking contrast with the apparent development of immunologic competence only after birth in some species (4, 5), the present work has demonstrated that the fetal lamb *in utero* is able to respond to the intrafetal injection of antigen with the production of circulating antibody, often in rather substantial amounts. These data support previous observations that immunogenesis may occur in some mammalian species well before the end of gestation. Manifestations of one or another form of immunologic response have also been reported in the fetal guinea pig (11), the fetal calf (9), the fetal lamb (12), and the fetal human (8).

In none of the preceding examples is it known precisely at what stage of gestation this competence develops. In the present study, however, it is shown that as early as the 66th day of a 150 day gestation period, the fetal lamb is able to produce detectable circulating antibody in response to antigenic stimulus. It may be that even younger fetuses than this would be responsive; this will be tested in future studies. In this connection, we have speculated elsewhere (22) that immunogenesis in the human fetus may occur at about the 5th to 6th month of gestation, and that this event may be only one of several factors involved in the more general biological phenomenon of lymphoid maturation and of the ability of the fetus to mount a chronic inflammatory response to appropriate stimuli. Signs of immunologic competence have been sought in the human fetus (1, 23) in terms of the presence of such morphologic indicators as the plasma cell. Histologic examination of the pre- and postinjection fetal lamb (24), as of the fetal human (8), however, make it clear that the *readiness* to respond immunologically (competence) may offer no presently recognizable morphologic indicators. These may only appear *after* the immune response has been initiated.

The postulate of Burnet and Fenner (3) on the existence of an immunologic

"null" period during the early stages of development of the organism would seem to be incontrovertible. The youngest embryo certainly lacks a functioning immunologic apparatus, just as it lacks all of the other highly differentiated systems which it will subsequently develop. It is unclear, however, whether or not the acquisition by the fetus of immunological "competence" represents a discrete event of purely immunological significance to the organism, as is suggested by some current theories on the ontogenesis of the immunological apparatus (25, 26).

We have observed that the fetal lamb, for reasons unknown, recognizes a hierarchy among the various antigens employed in the present study. At no time during fetal existence or during the first weeks of life can the lamb be stimulated to the production of detectable circulating antibodies against diphtheria toxoid, *Salmonella typhosa*, or BCG. In this respect, the fetal lamb appears to be immunologically incompetent to respond to these antigens in the doses employed. On the other hand, the fetal lamb will produce antibody *in utero* against bacteriophage φX, ferritin, and ovalbumin. In the case of these three antigens, their hierarchical rank (φX better than ferritin better than ovalbumin) is manifested in several ways. In animals injected at a given gestation time with these antigens, the earliest substantial antibody response is found to be against the bacteriophage. With longer duration of stimulus anti-ferritin appears, and finally, in the case of the 90 day fetus, anti-ovalbumin some 3 weeks later. In general, the amounts of antibody produced against these three antigens follows the same order: anti-φX > anti-ferritin > anti-ovalbumin. While the 90- to 100-day-old fetal lamb requires about 30 days to produce the first signs of circulating anti-ovalbumin, it takes the 65- to 70-day-old fetus some 60 days to accomplish the same result. The first appearance in both instances comes at about 120 days' gestation. But during the entire period, the fetus is able to produce appreciable amounts of antibody to phage.

The point to be made here is that while in the process of producing anti-bacteriophage and anti-ferritin antibodies in significant amounts, the fetal lamb before the 120th day of gestation appears to be in an immunologic "null" state with respect to the antigen ovalbumin. Further, when the fetus commences production of anti-ovalbumin towards the end of gestation, it still appears unable to respond to immunization with diphtheria toxoid, *Salmonella typhosa*, or BCG. Competence with respect to these antigens is known to develop as the animals grow older (25). It should be recalled that only a single level of each of the antigens was used for intrafetal injection. The possibility exists that this dose might have been insufficient for certain antigens to induce an antibody response, or alternatively that it might conceivably have been too great, leading to tolerance of the antigen. In view of the results obtained in other species (5), the latter suggestion appears to be unlikely.

We must therefore consider the possibility that immunological maturation

may be a relatively slow process, perhaps occurring in stages (6). In terms of the clonal selection theory of immunogenesis (26, 27), the possibility to be entertained is that the clonal precursors appear and/or mature at different rates, rather than all in concert.

Alternatively, one might consider that "immunogenesis" is not a peculiarly immunologic process, but rather the by-product of a more general biological maturation or differentiation of multipotential reticuloendothelial cells, with the acquisition of more than merely a restricted immunologic prowess. In this view, the apparently increasing competence of the fetal lamb to the several antigens described above may only be an expression of the differences in "antigenicity" of the agents employed, all acting with greater or lesser success on the same slowly developing cellular elements. From either point of view, these data suggest (*a*) that it should be possible to induce tolerance to one antigen in an immature animal even while the animal is producing antibody to a second antigen, and (*b*) that the inability to induce an antibody response to a certain antigen, or the ability to induce tolerance of that antigen, does not of itself establish the absence of an over-all immunologic competence.

Some evidence has been obtained indicating that in contrast to its usual enhancing action in adult animals, complete adjuvant may act to depress the antibody response to bacteriophage φX in the fetal lamb. The injection of antigen in complete adjuvant induces in the fetal lamb (24), as in other animals, a very marked lymphoid response, involving in all probability a number of non-immunologic components (8). If it is true that the fetal animal is only slowly developing cells competent to respond to a variety of stimuli, then it may be supposed that the non-specific effect of adjuvant may be to preempt a part of this small number of cells for other functions, to the detriment of the specific immune response.

A final point in the data presented here deserves comment. The earliest antibody produced by the fetal lamb is sensitive to the action of 2-mercaptoethanol. By analogy with other studies on mammalian antibodies (6, 19), this presumably represents a rapidly sedimenting 19S β_{2M}-globulin with antibody activity. As we shall show in Paper III of this series, the first response to stimulus among the immune globulins of the fetal lamb is an increase in the amount of circulating β_{2M} protein. After more prolonged stimulation, and in older fetal lambs, antibody appears in the circulation which is resistant to the action of 2-mercaptoethanol. This later antibody is presumably of the 7S γ-globulin type. Thus the younger fetuses in their earliest response do not produce 7S antibodies as readily as the older fetuses and adult sheep. These observations are in accord with those made in other species (6, 21), in which it was shown that the earliest response to antigen, especially in the young animal, is predominantly a β_{2M} antibody.

SUMMARY

The fetal lamb *in utero* is able to form large amounts of specific antibody in response to antigenic stimulus as early as the 66th to 70th day of the 150 day gestation period. Among the several antigens employed, the fetal lamb responded earliest, and with the highest titers, to bacteriophage φX. Slightly less effective as an antigen was horse ferritin, while ovalbumin proved to be a weak antigen, especially in younger fetuses. Ineffective in stimulating an antibody response at any time during fetal or early neonatal life were diphtheria toxoid, *Salmonella typhosa*, and BCG. Thus, it may not be feasible to fix precisely the time of onset of immunologic responsiveness in a species, inasmuch as it appears to differ so greatly from one antigen to another.

The quantity of antibody found 10 days after φX immunization was not significantly different in fetuses injected at 60 to 120 days of gestation. The earliest anti-phage antibody produced by the lamb fetus is a macroglobulin sensitive to the action of 2-mercaptoethanol. Only in older fetuses with longer lasting stimuli were appreciable amounts of 7S γ-globulin antibodies formed.

The conformity of these observations to theories on the ontogenesis of the immune response is discussed.

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