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Humoral immune responses in foetal sheep

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Summary. A total of fifty-two foetal sheep between 49 and 126 days gestation were injected with polymeric and monomeric flagellin, dinitrophenylated monomeric flagellin, chicken red blood cells, ovalbumin, ferritin, chicken γ-globulin and the somatic antigens of *Salmonella typhimurium* in a variety of combinations. Immune responses were followed in these animals by taking serial blood samples from them through indwelling vascular cannulae and measuring the circulating titres of antibody. Of the antigens tested, ferritin induced immune responses in the youngest foetuses. A short time later in gestation, the majority of foetuses responded to chicken red blood cells, polymeric flagellin, monomeric flagellin and dinitrophenylated monomeric flagellin. Only older foetuses responded regularly to chicken γ-globulin and ovalbumin. However, antibodies to all these antigens were first detected over the relatively short period of development between 64 and 82 days gestation and this made it difficult to define any precise order in the development of immune responsiveness. Of the antigens tested only the somatic antigens of *S. typhimurium* failed to induce a primary antibody response during foetal life.

The character and magnitude of the antibody responses in foetuses changed throughout *in utero* development. Both the total amount of antibody produced and the duration of the response increased with foetal age. Foetuses younger than 87 days gestation did not synthesize 2-mercaptoethanol resistant antibodies or IgG_1 immunoglobulin to any of the antigens tested, whereas most foetuses older than this regularly did so.

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INTRODUCTION

The capacity to recognize and respond to foreign antigenic material is now known to develop *in utero* in many animals. As there is no transplacental exchange of maternal immunoglobulins in sheep, pigs and cattle (Brambell, 1970; Solomon, 1971), foetuses of these species can be considered to give authentic primary immune responses when they first react to an antigenic stimulus *in utero*. They provide a circumstance in which various aspects of antibody synthesis can be studied in the absence of extraneous immunoglobulin.

Many studies on developmental aspects of the immune response have been done in foetal sheep. The observation by Schinckel & Ferguson (1953) that foetal sheep could reject skin allografts, established that immune reactivity developed in this species before birth. Silverstein, Prendergast & Kraner (1964) extended this observation and found that while foetal sheep could reject skin allografts after 77 days gestation they could not do so before 67 days gestation. Silverstein, Uhr, Kraner &

Lukes (1963) also reported that foetal sheep produced humoral antibodies to a range of antigens at different stages of their development. They found antibodies to the bacteriophage ⌀X174 in foetuses of 66 days gestation, to ferritin at 80 days gestation and to ovalbumin at 123 days gestation. Foetal sheep, however, failed to produce detectable antibodies against diphtheria toxoid, *Salmonella typhosa* or BCG. From these results they concluded that the capacity of the developing foetus to respond to different antigens was established at different stages of development in a strict hierarchical sequence.

The earlier reports by Silverstein *et al.* (1963, 1964) have been reviewed in several subsequent publications (Silverstein & Kramer, 1965; Silverstein & Prendergast, 1970) and a very precise chronology of the time at which foetal sheep react to a range of antigens has been given. Silverstein & Prendergast (1970) stressed the 'remarkable precision with which the foetus develops competence to a given antigen at a given stage of gestation' and further observed that 'the first immunological activity of the foetus is in no way immature and that it has at its command all the essential components of the adult response'.

If immune responses to a variety of antigens emerge in an ordered sequence during development, and if these responses are fully mature from the time they are first present, then these findings have important implications for immunological theory and practice. The results obtained from studies on sheep have been interpreted as evidence that immunological diversity does not arise by somatic mutation (Silverstein & Prendergast, 1970). Additionally, if immune responses in the foetus are fully mature, then all the component systems involved must develop synchronously or some final developmental step must determine, in a quantal fashion, the expression of each specific immune response.

The experiments described in the present paper were designed to study the development of humoral immune responsiveness to a variety of antigens and to provide independent data on some kinetic aspects of the synthesis of specific antibody and immunoglobulins in foetal sheep.

MATERIALS AND METHODS

Animals

Virgin Merino and Merino-Border Leicester cross ewes, 2–4 year old, were mated with Merino, Border Leicester or Dorset Horn rams. Service dates were noted by fitting each ram with a harness and crayon and recording daily the ewes that were newly raddled.

Antigens

Sterile chicken red blood cells (CRBC) were collected into Alsever's solution, washed three times with 0·9% NaCl (w/v) solution and resuspended to a concentration of 5×10^9 cells/ml. Polymeric flagellin (POL) was isolated from *Salmonella typhimurium* (Strain SL870) according to the method of Ada, Nossal, Pye & Abbot (1964), diluted to 1 mg/ml in 0·9% NaCl solution and sterilized by UV-irradiation. Monomeric flagellin (MON) was prepared by acid hydrolysis of POL, chloramine-T being used to prevent repolymerization (Parish & Stanley, 1972). MON was purified by sucrose density gradient centrifugation, dialysed and diluted to a concentration of 1 mg/ml. MON was dinitrophenylated (DNP) according to the method of Eisen (1964) at a conjugation ratio of 2·5 DNP/MON. Cadmium-free ferritin (FER) was prepared from horse spleens by $(NH_4)_2SO_4$ precipitation. Crystallized, lyophilized, salt-free ovalbumin (OA) was obtained from Sigma Chem. Co. (Grade V). Both FER and OA were dissolved in 0·9% NaCl solution at a concentration of 10 mg/ml. Chicken gammaglobulin (CγG) was precipitated from normal chicken serum with 33% saturated $(NH_4)_2SO_4$. The precipitate was dissolved and reprecipitated with 33% $(NH_4)_2SO_4$, washed twice with 50% saturated $(NH_4)_2SO_4$, dialysed and adjusted to a concentration of 10 mg/ml. Estimates of protein concentration were made from optical density measurements at 280 nm. MON, DNP, FER, OA and CγG were sterilized by filtration through millipore membranes. Whole boiled *S. typhimurium* organisms were prepared as described by Campbell, Garvey, Cremer & Sussdorf (1970) and diluted to 1×10^{10} organisms/ml. The lipopolysaccharide (LPS) was isolated from *S. typhimurium* as described by Halliday & Webb (1965), adjusted to a concentration of 1 mg/ml, autoclaved and stored at $-20°$.

Haemagglutinating antibody assays

All assays were done in Microtiter V bottom trays (Cooke Eng. Co., Virginia) except the direct anti-CRBC antibody assays which were done in U bottom trays. Serial two-fold dilutions were made

with 25 μl Microtiter diluters and droppers. All antibody titres have been expressed as $\log_2$ of the reciprocal of the final dilution that showed partial agglutination.

Anti-CRBC antibodies were detected by adding a 0·5% (v/v) CRBC suspension to serial dilutions of serum in 0·25% (w/v) gelatin-0·9% NaCl solution. After mixing, the plates were incubated at 37° for 1 h. Antibodies to MON and POL were detected with sheep red blood cells (SRBC) coated with POL by the $CrCl_3$ method of Langman (1972), while antibodies to OA and FER were assayed by the $CrCl_3$ method of Poston (1974). SRBC coated with trinitrophenyl by the method of Rittenberg & Pratt (1969) were used in the anti-DNP antibody assays. Serum samples being assayed for antibodies to MON, POL, OA, FER and DNP were serially diluted in gelatin-0·9% NaCl solution, an equal volume of 1% sensitized SRBC was added and the plates stored at 4° for up to 4 h. Antibodies to CγG were detected by coating SRBC with a sub-agglutinating concentration of CγG anti-SRBC antibody by the method of Miller & Warner (1971), while SRBC passively coated with LPS were used to assay antibodies to *S. typhimurium* whole organisms or LPS. Both anti-CγG and anti-LPS assays were performed by diluting serum in 0·9% NaCl containing 1% heat-inactivated foetal calf serum, adding an equal volume of 1% sensitized SRBC and incubating the plates at 37° for up to 2 h.

The 2-mercaptoethanol (ME) sensitivity of antibodies was determined by incubating each sample of serum with an equal volume of 0·2 M 2-ME (Eastman Organic Chemicals) at 37° for 1 h. Uncoupled SRBC suspensions were used to detect non-specific agglutinins in the assays for anti-LPS, anti-CγG and anti-DNP antibodies, while SRBC coupled with gelatin by the two $CrCl_3$ techniques were used as controls in the POL and MON antibody assays and the OA and FER antibody assays respectively. All antigen coated SRBC supensions were titrated against known standard antisera before use and diluent controls were included.

Immunoglobulin determinations

Samples of sera from foetuses were examined by single-radial immunodiffusion to measure the concentration and class of immunoglobulins present. Mono-specific rabbit anti-sheep IgG_1, anti-IgG_2, anti-IgM and anti-IgA sera were prepared in a manner similar to that described by Brandon, Watson & Lascelles (1971) for cattle.

Surgical procedures

For the proposed experiments it was necessary to be able to procure samples of blood from foetuses sequentially over periods of 2–3 weeks following an antigenic challenge. Foetal sheep were removed from the amniotic cavity and exposed by the technique described by Smeaton, Cole, Simpson-Morgan & Morris (1969) and by Cole & Morris (1971). The external jugular vein, the facial vein or the lateral saphenous vein was cannulated with a clear vinyl tubing (Dural Plastics, Dural, New South Wales); the two most commonly used tubings had outside diameters of 0·96 mm or 1·20 mm. The cannula was secured in the vein with 5–0 silk ligatures, the skin incision closed and the tubing anchored to the skin of the foetus with a further 3–4 ligatures. The foetus was returned to the amnion and a short coil of tubing was left inside the amniotic cavity to allow for movement of the foetus. The amniotic fluid was returned and the foetal membranes reconstituted with a purse-string suture tied around the tubing. The uterus was closed with a continuous inverting silk suture. The remainder of the tubing was led out through a stab incision high in the abdominal wall of the ewe and the abdomen closed with interrupted silk mattress sutures. The tubing was filled with heparinized 0·9% NaCl (250 i.u./ml), sealed by tying a knot in the cannula and secured to the wool on the back of the ewe.

To collect blood samples the end of the cannula was washed with 70% ethanol-hibitane (ICI, Australia) solution and cut. The heparinized 0·9% NaCl in the cannula was withdrawn through a needle fitted tightly into the end of the cannula and a sample of blood (usually 2 ml) was withdrawn into a fresh sterile syringe. The cannula was refilled with sterile heparinized 0·9% NaCl and tied off.

RESULTS

Fifty-two foetal sheep between 49 and 126 days gestation were injected with up to four different antigens simultaneously; one into each limb of the foetus. Samples of blood were obtained (usually every 48 h) through the indwelling vascular cannulae and the serum samples assayed for the presence of antibody. It proved difficult to obtain serial

blood samples regularly from foetuses less than 70 days gestation and in some experiments on younger foetuses, blood samples were only obtained at the time of challenge and then at hysterotomy 14 days later.

Haemagglutinating antibody responses

(i) *To ferritin (FER).* Immune responses to FER were examined in twenty-nine foetuses injected between 49–126 days gestation. The earliest specific antibody detected following the subcutaneous (s.c.) injection of 1 mg of FER emulsified in Freund's complete adjuvant (FCA) was in serum obtained by hysterotomy 14 days after a 50 day old foetus had been injected (i.e. at 64 days gestation). Another foetus injected at 50 days gestation did not have antibody to FER when tested at 64 days. Two of six foetuses injected with FER between 58 and 60 days gestation and two of three foetuses injected at 69 days gestation did not produce any antibody during the 14 day period following injection but all foetuses injected after 70 days gestation responded. The anti-FER antibody synthesized by most foetuses was sensitive to 2-ME, although one 91 day old foetus, one 100 day old foetus and four of six foetuses injected after 106 days gestation synthesized 2-ME resistant anti-FER antibodies within 14 days of injection.

The magnitude of the anti-FER response was greater in the older foetuses, although the time after injection when antibody was first detected did not alter after 70 days gestation. The mean antibody titres of four foetuses injected at 70 days, of three foetuses injected at 100 days and of three foetuses injected at 120 days gestation are shown in Fig. 1. Antibody was detected in all groups by day 4 and antibody titres reached maximum levels between day 8 and day 10 in foetuses injected at 100 and 120 days gestation.

(ii) *To chicken red blood cells (CRBC).* Samples of serum were obtained by hysterotomy from four foetuses injected s.c. with 5×10^8 CRBC between 49–55 days gestation. No sample contained detectable antibody. Serum samples from three of twelve foetuses injected between 56 and 60 days gestation had low titres of antibody when collected 14 days after injection. All but one of seventeen foetuses of 69 days gestation or older responded to CRBC within 14 days of injection. The foetus which failed to respond when given a primary injection at 71 days gestation was re-injected with 5×10^8 CRBC at 85 days gestation via the indwelling intravenous cannula; antibodies were detected within 4 days of this second injection. Primary antibody responses to CRBC were usually detected by day 4 and invariably detected by day 8. The maximum titre of agglutinating antibody in the circulation usually reached levels between 4·0 and 7·0, while in one 120 days old foetus the titre reached 11·0. The maximum titres of antibody were generally higher in the older foetuses but this was not so in all cases. All foetal sheep which responded to CRBC synthesized 2-ME sensitive antibody and most of those injected after 87 days gestation also synthesized 2-ME resistant antibodies within 14 days.

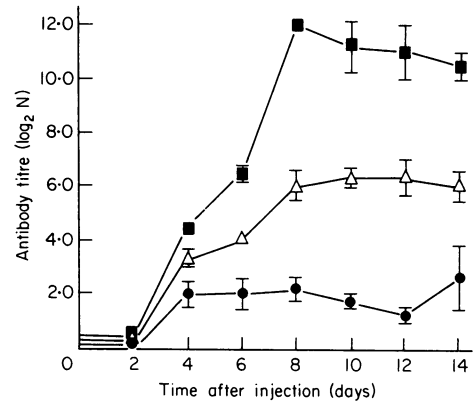

Figure 1. Mean total haemagglutinating antibody responses of foetal sheep to 1 mg FER in FCA. 70 days gestation (●); 100 days gestation (△); 120 days gestation (■).

(iii) *To polymeric flagellin (POL).* Only one of eight foetuses injected intramuscularly (i.m.) with 50 μg POL between 49 and 60 days gestation produced antibody within the ensuing 14 day period. The one foetus which responded was injected when 60 days old. Two foetuses, 69 and 71 days of age, were injected with 50 μg of POL and twelve other foetuses aged between 76 and 126 days were injected with 100 μg POL. Thirteen of these animals produced antibody by day 4 and one by day 6; their responses were maximum between days 4 and 8. The maximum haemagglutinating antibody titres in these foetuses ranged from 4·0 to 11·0 with the majority of animals injected after 110 days gestation reaching titres between 9·0 and 11·0. All the foetal sheep which responded to POL synthesized 2-ME sensitive antibody;

most foetuses older than 100 days also produced 2-ME resistant antibody during the first 14 days of the response. Although there was no relationship between the time that the first antibody appeared after injection and the age of the foetus, antibody persisted in the circulation over a longer period in older animals.

(iv) *To monomeric flagellin (MON).* Fifteen foetal sheep between 60 and 123 days gestation were injected i.m. with 100 μg MON. The onset of the antibody responses in two animals injected at 71 and 72 days gestation was delayed and the titres of antibody were lower than those in foetuses injected after 80 days gestation. Antibody was detected by day 4 in all foetuses injected after 80 days gestation and maximum antibody titres ranging from 5·5 to 9·5, occurred on day 4 or 6. There did not appear to be any correlation between the ages of these foetuses and the maximum titres of antibody. All the anti-MON antibody synthesized by these foetuses, with one exception, was sensitive to 2-ME.

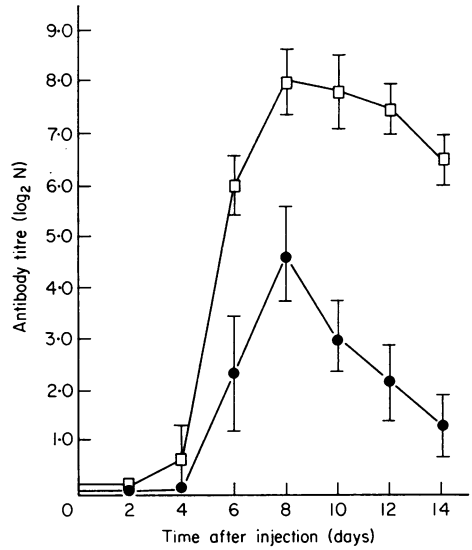

Figure 2. Mean total haemagglutinating antibody responses of foetal sheep to 1 mg CγG in FCA. 100 days gestation (●); 120 days gestation (□).

(v) *To ovalbumin (OA).* Thirty foetal sheep aged between 69 and 126 days gestation were injected s.c. with 1 mg of OA emulsified in 0·1 ml of FCA. The immune responses which followed the injection of OA varied considerably between foetuses in respect of the time at which antibody first appeared and the magnitude of the response. Of the six foetuses injected between 69 and 72 days gestation, two had detectable antibody by day 8, one by day 61, one by day 20 and one by day 30. One foetus failed to produce any detectable antibody. Anti-OA antibodies were present 8 and 16 days after challenge in two of three animals injected between 78 and 80 days gestation. After 90 days gestation most foetuses produced detectable antibody within 6–10 days. Maximum antibody titres, ranging from 3·0 to 8·0, occurred at various times after injection and did not seem to be related to the age at which the foetus was injected.

Almost all the specific antibody synthesized within the first 14 days of the response to OA was sensitive to 2-ME. After day 14 most foetuses synthesized 2-ME resistant antibodies and titres often reached levels between 8·0 and 12·0. Because of the variability in the onset of the responses, it was not possible to compare mean antibody titres in foetuses of different ages.

(vi) *To chicken gamma-globulin (CγG).* Twenty foetuses were injected s.c. between 59 and 123 days gestation with 1 mg of CγG emulsified in 0·1 ml of FCA. Antibody was not detected in three foetuses injected between 59 and 60 days gestation. In two animals injected at 71 and 72 days gestation low levels of antibody were detected by day 10. Antibody was detected in one of three animals injected between 78 and 81 days gestation and in two of three animals injected between 91 and 93 days gestation. Antibody was detected in these foetuses 5–6 days after the injection of CγG.

Only in foetuses older than 100 days gestation did good responses to CγG occur regularly. The mean antibody responses of three foetuses around 100 days gestation and of three foetuses around 120 days gestation are shown in Fig. 2. Except for one foetus, antibody was first detected in all of these animals on day 6 and maximum titres were usually reached by day 8. During the first 14 days of the response, the antibody formed by all but one of these foetuses, irrespective of their age, was sensitive to 2-ME. Many foetuses injected with CγG after 100 days gestation produced 2-ME resistant antibody, although in some cases this occurred quite late in the response.

(vii) *To dinitrophenylated MON (DNP).* Six

foetal sheep aged 59, 69, 70, 78, 100 and 119 days gestation were injected i.m. with 50–100 μg of MON-DNP. The foetuses injected at 59 and 78 days gestation did not respond to the hapten DNP whereas all the others did. Each animal that responded produced low titres of antibody which never exceeded 3·0. Although antibody was produced 4–6 days after the injection of antigen, the response was ephemeral and the antibody had disappeared within 6 days after it was first detected. None of the six foetuses produced antibodies to the MON carrier.

(viii) *To the somatic 'O' antigens of* S. typhimurium. Nine foetal sheep aged between 81 and 123 days gestation were injected i.m. with 1×10^9 boiled whole *S. typhimurium* organisms; a further nine foetuses aged between 80 and 121 days gestation were injected i.m. with 100 μg LPS from *S. typhimurium*. No foetus produced detectable haemagglutinating antibody to the LPS antigens even though serum samples were examined from some animals over periods of 20–30 days. Five of the animals which failed to respond to whole *S. typhimurium* organisms *in utero* were injected again between 80 and 140 days after birth. All of these lambs gave good antibody responses to *S. typhimurium* which were primary in character.

Immunoglobulin synthesis by foetal sheep

In Table 1 the results obtained in different animals, injected with several different antigens, have been collated to show the age at which foetal sheep first produced 2-ME resistant antibodies. The age at which these antibodies first appeared was quite consistent irrespective of the antigen injected; on average it was around 95 days gestation.

The class and concentration of immunoglobulin in serum samples obtained from forty-five foetuses during the first 14 days of their primary responses were analysed by single-radial immunodiffusion. Thirteen foetuses injected before 70 days gestation had a mean concentration (±se) of 35 ± 7 μg/ml of IgM in their sera 14 days after injection, although no IgG_1, IgG_2 or IgA was detectable during this time. Foetuses injected after 78 days gestation synthesized both IgM and IgG_1. The maximum mean concentrations of IgM detected within 14 days of injection were 151 ± 47, 110 ± 16 and 172 ± 44 μg/ml in groups of foetuses injected around 86, 108 and 121 days gestation, while the mean concentrations of IgG_1 were 70 ± 9, 134 ± 31 and 128 ± 14 μg/ml respectively. Although IgG_1 was not detected in some animals injected between 79 and 113 days gestation until day 14, it was detected by day 8 in all five foetuses injected around 121 days gestation. IgG_2 and IgA were not detected by radial immunodiffusion in any foetus during the first 14 days of a primary response.

Table 1. The appearance of 2-ME resistant antibody in the plasma of foetal sheep injected with various antigens

Age of gestation when injected (days)	Antigen	Age when 2-ME resistant antibody was first detected (days)
70	OA	92
79	POL	95
80	OA	96
80	CRBC	98
87	CRBC	93

When data on the time at which IgG_1 appeared in the serum of individual foetuses were collated, several foetuses were found which had been injected with antigens between 69 and 81 days gestation. IgG_1 was not detected in these foetuses until 87–90 days gestation—in one case 20 days after the antigen was injected (Table 2).

Variation in the immune response between foetuses

As in all physiological experiments done with healthy outbred animals there was a significant variation between the responses of foetal sheep, even when they were studied at the same stage of development.

In contrast to the relatively fixed stage of develop-

Table 2. First appearance of IgG_1 in the plasma of antigenically stimulated foetal sheep

Age of gestation when injected (days)	Antigens injected	Age when IgG_1 was first detected (days)
69	POL, CRBC, FER, OA	89
79	POL, CRBC, FER, OA	87
79	POL, CRBC, FER	87
80	CRBC, FER, OA, LPS	90
81	MON, FER, CγG, *S. tym.*	89

S. tym. = *S. typhimurium.*

ment at which foetuses first responded to POL and MON, the onset of competence to respond to FER, CRBC, CγG or OA was quite variable. Individual foetuses also varied in their responses to different antigens. There were three foetal sheep which did not respond to any of the antigens injected into them. One foetus injected at 49 days gestation did not produce detectable antibodies to CRBC, POL or FER whilst one 60 day old foetus failed to produce antibodies to CRBC or FER and another to CRBC, MON and CγG. In addition, two 69 day old foetuses failed to respond to FER, although one did respond to DNP and the other to CRBC and POL. Of ten foetuses injected simultaneously with both CγG and OA, eight produced antibodies to CγG before OA, while two produced antibodies to OA before CγG. This variability was highlighted in two foetuses injected with MON, CγG and OA at 72 days gestation; although responses to MON and CγG were detected in both animals within 14 days, antibodies to OA were first detected on day 8 in one foetus and on day 30 in the other. Irrespective of the age of the foetuses, essentially all antibody detected to MON and CγG during the first 14 days of the response was sensitive to 2-ME; however, again there were foetuses that were exceptions to this general finding.

Antibody responses in lambs

FER, CRBC, POL, CγG and OA were injected into 3–6 month old lambs in the same doses used in the experiments on foetuses. All these antigens induced good antibody responses in young lambs. In addition, these lambs responded to both LPS and boiled *S. typhimurium* organisms.

The responses to FER, POL and CγG in lambs showed similar kinetics to the responses observed in late term foetuses, except that most of the antibody synthesized to FER and POL was resistant to 2-ME. The responses to CRBC were lower than the responses detected in late term foetuses, while uniformly strong responses were detected to OA. Anti-OA antibody first appeared 7 days after the injection of antigen and most of the antibody formed was resistant to 2-ME. Four lambs injected with whole *S. typhimurium* organisms and 2 lambs injected with LPS all produced specific antibody by day 4. The maximum titres occurred at day 7 and reached values between 5·0–7·0 and 3·5–5·0 for *S.typhimurium* organisms and LPS respectively.

DISCUSSION

The results of the experiments reported here have bearing on two important propositions; first, that immunological competence develops in a strictly determined sequential fashion to various antigens during foetal life and second, that once reactivity is established against an antigen, the specific immune response is fully developed and adult-like in character (Silverstein *et al.*, 1963; 1964; Silverstein & Kraner, 1965; Silverstein, Parshall & Uhr, 1966; Silverstein & Prendergast, 1970). In regard to the second proposition, the data are unequivocal; the immune responses to FER, POL and CγG were significantly greater in older foetuses. The titres of antibody were significantly higher and the duration of the response much longer in older animals. Additionally, antibody to CγG and MON appeared earlier in animals injected after 80 days gestation. The enhanced antibody responses in older foetuses occurred concomitantly with the extensive lymphopoiesis which occurs during the last half of gestation (Fahey & Morris, 1974). It seems that a similar maturation of immune responsiveness occurs in the foetal pig and the dog in which the plaque-forming cell response to sheep red cells has been shown to be greater in older animals (Schultz, Wang & Dunne, 1971; Jacoby, Dennis & Griesemer, 1969). Thus the experiments demonstrate that a considerable development occurs both qualitatively and quantitatively in the immune response beyond the time when the animal is first capable of responding to an antigen.

Older foetuses, as well as synthesizing greater quantities of antibodies, also showed significant qualitative differences in their immune response. Foetuses less than 90 days gestation did not synthesize 2-ME resistant antibodies irrespective of the time of challenge or the nature of the antigens injected; after 90 days an increasing proportion of foetuses produced 2-ME resistant antibodies to CRBC, POL, FER and OA. Similarly, foetuses younger than 87 days did not synthesize IgG_1 following antigenic stimulation.

The question of whether or not there is a hierarchy in the development of immunological competence (Silverstein & Kraner, 1965; Silverstein & Prendergast, 1970) can be examined by comparing the capacity of foetal sheep of different ages to produce detectable antibody to various antigens. The data have been arranged to give an idea of the probability

Table 3. The proportion of foetal sheep at different stages of gestation which produced antibody within 14 days of being injected with various antigens

Days gestation	Antigen								
	FER	CRBC	POL	MON	CγG	DNP	OA	*S. tym.* whole orgs.	*S. tym.* LPS
46 to 55	1/2*	0/4	0/3	NT	NT	NT	NT	NT	NT
56 to 65	4/6	3/12	1/5	0/2	0/3	0/1	NT	NT	NT
66 to 75	4/6	4/5	2/2	2/2	2/2†	2/2†	2/6	NT	NT
76 to 85	3/3	2/2	2/2	2/2	1/3	0/1	1/3	0/1	0/1
86 to 95	3/3	4/4	3/3	3/3	2/3	NT	5/5	0/2	0/1
96 to 105	3/3	NT	1/1	2/2	3/3	1/1†	3/4	0/2	0/2
106 to 115	3/3	2/2	2/2	2/2	2/2	NT	3/5	0/2	0/2
116 to 126	3/3	5/5	5/5	2/2	4/4	1/1†	7/7	0/2	0/3

* No. of animals producing antibody/No. of animals injected
† Weak responders.

NT = not tested.
S. tym. = *S. typhimurium.*

that a foetus of a given age will respond to a particular antigen, but it needs to be stressed that the results relate only to the responses observed to a single dose of antigen, injected by one route, in the one physical form, together with other antigens. Inferences drawn from these data may not be valid beyond these experimental constraints.

In Table 3 the period of gestation between 46 and 126 days has been divided into 10 day intervals, and the number of animals in each decile which synthesized antibodies within 14 days of being injected with a particular antigen, is given as a fraction of the number of animals tested. The 14 day interval was chosen arbitrarily to allow some judgement to be made on the competence of the foetus when it was first injected, or shortly thereafter. The order the antigens are listed in Table 3 represents an attempt to depict a sequence in the development of competence, however, the differences described between the onset of competence to CRBC, POL and MON and between CγG and DNP are again arbitrary. FER was placed first due to the fact that there was one 50 day old foetus which responded to this antigen and because of five animals injected with FER and CRBC at or before 60 days gestation, four produced antibodies to FER but not CRBC, while only one did the converse. If Table 3 is interpreted in terms of when the majority of foetal animals tested can respond to a particular antigen, a hierarchy can be drawn up in which responsiveness to FER appears around 60 days gestation, for CRBC, POL and MON around 70 days gestation, for CγG and OA around 90 days gestation and for the somatic antigens of *S. typhimurium*, not at all.

If, on the other hand, the results are used to decide the earliest age at which any foetus produced detectable antibody to a given antigen, the outcome of the analysis has a different emphasis. Table 4 lists the age at which antibody to six antigens and two different forms of one of these antigens first appeared in foetal sheep. All of these responses arose during a period between 64–82 days gestation. Leaving aside the failure of the foetuses to respond to *S. typhimurium* and acknowledging the limitations of the experimental protocol, it would not be unreasonable to interpret the data as showing that there is a relatively short period of development during which the foetus acquires the ability to respond to a range of antigens. Both lines of argument can be interpreted as indicating a sequential development of immunological competence, although the second interpretation condenses the time scale of this developmental sequence substantially.

The summary of results shown in Table 3 also highlights another aspect of the appearance of immunological competence in foetal sheep. Although antibody responses to POL and MON occurred in all foetuses injected after 69 days gestation, the responses to CγG, OA and to a lesser extent CRBC and FER, gave an imprecise indication of the onset

Table 4. The first appearance of haemagglutinating antibody to antigens injected into foetal sheep older than 50 days gestation

Antigen	FER	CRBC	POL	OA	MON	DNP	CγG	*S. tym.*
Age of gestation at which antibody first detected (days)	64	72	74	76	78	78	82	> 143

S. tym. = *S. typhimurium.*

of immune competence to these antigens. Silverstein & Prendergast (1970) injected 50–60 foetal sheep with OA and never observed a response prior to 118 days gestation. They stated that only one of 20–30 foetal sheep injected after 125 days gestation failed to respond. The 'remarkable precision' in the onset of competence to OA reported by Silverstein & Prendergast (1970) was not found in the breeds of sheep or with the ovalbumin preparation used in our experiments.

There are several obvious criticisms of the experiments described in this paper. It can be argued that there are more sensitive assays for antibody than the haemagglutination assay. No matter what assay is used, however, an empirical decision has to be taken as to whether or not an immune response to a particular antigen has taken place. The haemagglutinating antibody assays described can detect from 30–100 ng/ml of antibody (unpublished data), and we consider this concentration to be near the lower level of physiological significance. Because several antigens were injected simultaneously, antigenic competition may have played some role in the apparent sequential development of competence, although injecting antigens into different sites should have reduced this likelihood (Taussig, 1973). After 90 days gestation the vast majority of animals responded to all of the antigens injected; the exception being the somatic antigens of *S. typhimurium*. Since all animals received similar treatments it may be inferred that antigenic competition was not the reason why the younger foetuses failed to give immune responses to some antigens.

The lack of reactivity of foetuses to the somatic antigens of *S. typhimurium* was not genetically determined, for foetuses that failed to respond to these antigens *in utero* produced specific antibodies when injected 80–140 days after birth. The inability of foetuses injected with MON–DNP to respond to the carrier, MON, also remains unexplained, particularly as other foetuses of a similar age produced antibodies to uncoupled MON.

It is important to reiterate that as only the humoral haemagglutinating antibody responses of the foetuses were followed, other components of the immune response may have gone undetected. In this context Silverstein *et al.* (1963) found that foetal sheep failed to produce antibody to BCG; recent experiments, however, demonstrated that while 100 day old foetal sheep did not produce antibody until 4 to 5 weeks after being injected with BCG, the peripheral blood leucocytes obtained 12–14 days after vaccination responded specifically to PPD *in vitro* (Fahey, 1977).

These results report only one aspect of the phenotypic expression of immune responses of foetuses injected with various antigens. As such they have nothing to contribute to the question of how immunological diversity arises. The experiments only detected the presence or absence of a fully functional humoral immune mechanism and therefore the results cannot be interpreted in terms of how genetic information within the lymphocyte is expressed. If, as it seems, the foetus becomes competent to produce antibody to a wide range of antigens around mid-gestation, this could be due to the emergence, at this period of development, of lymphocytes with the appropriate specific receptors on their surfaces (Decker & Sercarz, 1974; Symons, Binns, Lay & Walters, 1977). An increase in the percentage of peripheral blood leucocytes with membrane bound immunoglobulin has been reported to occur around mid-gestation in foetal sheep and this could indicate that immunological competence is related to the appearance of immunoglobulin bearing lymphocytes (Symons & Binns, 1975). However, just as likely, the findings could be due to the functional maturation of any of the manifold components of the immune mechanism, without which antibody would not be produced. If

this were so, the cells that are responsible for recognizing different antigens could have developed at an earlier stage of gestation than when antibody can first be detected and they may have developed either sequentially or in unison. Recent studies on the ontogeny of antigen-binding cells in chickens (Lydyard, Grossi & Cooper, 1976) and some studies in mice (Yung, Wyn-Evans & Diener, 1973) appear to show that cells capable of binding different antigens appear at different stages of development. However, other studies in mice, which employed techniques of similar sensitivity for the different antigens, showed that there was no restriction in the variety of specificities expressed by antigen-binding cells in foetal mice, either with respect to the kinds of antigens bound or to the avidities of binding (Spear, Wang, Rutishauser & Edelman, 1973; D'Eustachio & Edelman, 1975).

The general conclusions that can be drawn from these studies are that foetal sheep develop the capacity to synthesize humoral antibodies to a wide variety of antigens over a short period of time around mid-gestation. Subsequently, the antibody response develops throughout the remainder of gestation, leading to the production of greater amounts and different classes of antibody in older foetuses.

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REFERENCES

Ada G.L., Nossal G.J.V., Pye J. & Abbot A. (1964) Antigens in immunity. I. Preparation and properties of flagellar antigens from *Salmonella adelaide*. *Aust. J. exp. Biol. med. Sci.* **42**, 267.

Brambell F.W.R. (1970) The transmission of passive immunity from mother to young. In: *Frontiers of Biology*, Vol. 18. North-Holland Publishing Co., Amsterdam.

Brandon M.R., Watson D.L. & Lascelles A.K. (1971) The mechanism of transfer of immunoglobulin into mammary secretion of cows. *Aust. J. exp. Biol. med. Sci.* **49**, 613.

Campbell D.H., Garvey J.S., Cremer N.E. & Sussdorf D.H. (1970) *Methods in immunology* (2nd edn), p. 128. Benjamin, New York.

Cole G.J. & Morris B. (1971) The growth and development of lambs thymectomized *in utero*. *Aust. J. exp. Biol. med. Sci.* **49**, 33.

Decker J.M. & Sercarz E.E. (1974) Early simultaneous appearance of antigen-binding cells in the foetal sheep. *Nature (Lond.)*, **252**, 416.

D'Eustachio P. & Edelman G.M. (1975) Frequency and avidity of specific antigen-binding cells in developing mice. *J. exp. Med.* **142**, 1078.

Eisen H.N. (1964) Preparation of purified anti-2, 4-dinitrophenyl antibodies. *Meth. med. Res.* **10**, 94.

Fahey K.J. (1977) Humoral and cell-mediated immune responses in foetal sheep following vaccination with BCG. *Aust. J. exp. Biol. med. Sci.* **55**, 419.

Fahey K.J. & Morris B. (1974) Lymphopoiesis and immune reactivity in the foetal lamb. *Ser. Haematol.* **7**, 548.

Halliday W.J. & Webb M. (1965) A plaque technique for counting cells which produce antibacterial antibody. *Aust. J. exp. Biol. med. Sci.* **43**, 163.

Jacoby R.O., Dennis R.A. & Griesemer R.A. (1969) Development of immunity in foetal dogs: Humoral responses. *Am. J. vet. Res.* **30**, 1503.

Langman R.E. (1972) The use of erythrocytes sensitized with flagellar antigens from Salmonella for the assay of antibody and antibody-forming cells. *J. immunol. Method.* **2**, 59.

Lydyard P.M., Grossi C.E. & Cooper M.D. (1976) Ontogeny of B cells in the chicken. I. Sequential development of clonal diversity in the bursa. *J. exp. Med.* **144**, 79.

Miller J.F.A.P. & Warner N.L. (1971) The immune response of normal, irradiated and thymectomized mice to fowl immunoglobulin G as detected by a haemolytic plaque technique. *Int. Arch. Allergy*, **40**, 59.

Parish C.R. & Stanley P. (1972) Chemical and biological properties of bacterial flagellin following iodination and oxidation by chloramine-T. *Immunochemistry*, **9**, 853.

Poston R.N. (1974) A buffered chromic chloride method of attaching antigens to red cells: Use in haemagglutination. *J. Immunol. Method.* **5**, 91.

Rittenberg M.B. & Pratt K.L. (1969) Antitrinitrophenyl (TNP) plaque assay. Primary response of Balb/C mice to soluble and particulate immunogen. *Proc. Soc. exp. Biol. (N.Y.)*, **132**, 575.

Schinckel P.G. & Ferguson K.A. (1953) Skin transplantation in the foetal lamb. *Aust. J. biol. Sci.* **6**, 533.

Schultz R.D., Wang J.T. & Dunne H.W. (1971) Development of the humoral immune response of the pig. *Am. J. vet. Res.* **32**, 1331.

Silverstein A.M. & Kraner K.L. (1965) Studies on the ontogenesis of the immune response. In: *Molecular and cellular basis of antibody formation* (ed. by J. Sterzl), p. 341. Czechoslovak Academy of Sciences, Prague.

Silverstein A.M., Parshall C.J. & Uhr J.W. (1966) Immunological maturation *in utero*: Kinetics of the primary antibody response in the foetal lamb. *Science*, **154**, 1675.

Silverstein A.M. & Prendergast R.A. (1970) Lymphogenesis, immunogenesis, and the generation of immunological diversity. In *Developmental aspects of antibody formation and structure* (ed. by J. Sterzl and I. Riha), p. 69. Academic Press, New York.

Silverstein A.M., Prendergast R.A. & Kraner K.L. (1964) Foetal response to antigenic stimulus. IV. Rejection of skin homografts by the foetal lamb. *J. exp. Med.* **119**, 955.

SILVERSTEIN A.M., UHR J.W., KRANER K.L. & LUKES R.J. (1963) Foetal response to antigenic stimulus. II. Antibody production by the foetal lamb. *J. exp. Med.* **117**, 799.

SMEATON T.C., COLE G.J., SIMPSON-MORGAN M.W. & MORRIS B. (1969) Techniques for the long-term collection of lymph from the unanaesthetized foetal lamb *in utero*. *Aust. J. exp. Biol. med. Sci.* **47**, 565.

SOLOMON J.B. (1971) Foetal and neonatal immunology. In: *Frontiers of Biology*, Vol. 20. North-Holland Publishing Co., Amsterdam.

SPEAR P.G., WANG A.-L., RUTISHAUSER U. & EDELMAN G.M. (1973) Characterization of splenic lymphoid cells in foetal and newborn mice. *J. exp. Med.* **138**, 557.

SYMONS D.B.A. & BINNS R.M. (1975) Immunoglobulin-bearing lymphocytes: Their demonstration in adult sheep and ontogeny in the sheep foetus. *Int. Arch. Allergy*, **49**, 658.

SYMONS D.B.A., BINNS R.M., LAY C.A. & WALTERS D.E. (1977) Ferritin and ovalbumin binding blood lymphoid cells in foetal sheep. *Int. Arch. Allergy*, **53**, 104.

TAUSSIG M.J. (1973) Antigenic competition. In: *Curr. Top. Microbiol. Immunol.* **60**, 125.

YUNG L.L.L., WYN-EVAN T.C. & DIENER E. (1973) Ontogeny of the murine immune system: Development of antigen recognition and immune responsiveness. *Europ. J. Immunol.* **3**, 224.