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Prenatal Immunization of the Lamb to Brucella; Secondary Antibody Response *in utero* and at Birth*

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Summary. Pre-natal immunization has been investigated as a means of overcoming the non-reactivity of newborns to bacterial somatic antigens. Normal newborn lambs evidenced little or no antibody response to killed *Brucella abortus* whereas the same dose of antigen elicited relatively high levels of antibody in foetuses late in the gestation period. When foetal lambs were immunized at mid-gestation they responded slowly and with low levels of antibody. The response was adequate, however, to prime for a secondary one *in utero* and at birth. The secondary response *in utero* occurred at the same time and with antibody levels as high as those attained in adult sheep on secondary stimulation. When secondarily stimulated at birth, a significant percentage of the neonates responded rapidly with high levels of antibody. As customarily found in older animals, IgG immunoglobulin appeared later and regressed more slowly than IgM.

INTRODUCTION

Humans and several other species can respond immunologically in foetal life (Šterzl and Silverstein, 1967). At birth, however, when there is need for active immunization against certain infectious diseases, the newborn appears relatively non-reactive. Many species do not respond markedly to Brucella or Salmonella somatic antigens until several weeks of age (Šterzl and Silverstein, 1967; Evans and Smith, 1963; Smith, 1960). Biberstein, Kennedy, Robinson and McGowan (1966) and Osburn and Kennedy (1966) reported that the foetal lamb infected with *Brucella ovis* synthesized specific antibody. Although active invasion of lymphoid cells by bacteria may have preceded antibody production, nevertheless this finding showed that under certain conditions the foetus could process somatic antigen and respond with antibody synthesis. Investigating the antibody response of the normal foetal lamb to Brucella antigens we found that the foetus synthesized high levels of anti-Brucella agglutinins when induction of the response was initiated late in gestation (Richardson, Beck and Clark, 1968). This contrasted markedly with the low levels attained when the response was initiated in newborn lambs.

The objective of the work reported here was to use the Brucella-foetal-lamb system to determine if non-reactivity of the newborn lamb to Brucella antigen could be circumvented

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by primary immunization *in utero* followed by secondary antigenic stimulation at birth. In the course of the investigation the secondary antibody response *in utero* also was examined.

MATERIALS AND METHODS

Antigen. Brucella abortus Type 1 antigen was prepared two to four subcultures after the organisms were isolated from infected cows. The culture was grown on Tryptose agar (Difco) in 5 per cent CO_2 for 72 hour at 37°, washed from the surface with 0·85 per cent NaCl in 0·5 per cent phenol, and stored at 4°. Prior to use, the killed antigen was washed twice and diluted in saline to contain 10^{10} brucellae per ml as determined by a Libby photronreflectometer.

Immunization of foetal and neonatal lambs. Crossbred lambs were injected intracardially *in utero* with 1 ml of Brucella antigen as follows: Laparotomies were performed on anesthetized ewes 70 or more days after breeding. The foetus was gently manipulated into position to permit intracardiac injection without exteriorizing or otherwise traumatizing the uterus. With thumb over the foetal heart, a 1-inch 22-gauge needle, with 2·5-ml syringe attached, was inserted through the uterine wall at a point just off the end of the surgeon's thumb. Upon entering the heart, a blood sample was obtained. Syringes were exchanged and the plunger was slightly retracted to assure proper location of the needle before the antigen was injected. The procedure for obtaining additional blood samples or for second injections of antigen was the same.

The lambs were born naturally and reared with the ewe except as noted. Prior to lambing the udder was taped so that the lamb could not nurse until the blood sample designated as '0 hour' had been obtained from the lamb. Neonates were then injected with 10^{10} killed brucellae by the intravenous route.

Determination of anti-Brucella agglutinin levels. Brucella antibody was determined by the standard test tube agglutination method using USDA strain 1119 antigen and incubation for 48 hour at 37°. Sera that were negative by the macromethod were retested by the microagglutination method of Claflin, Smithies and Meyer (1966).

Determination of immunoglobulin class. To estimate the percentage of IgM and IgG agglutinin, sera were diluted 1:5 in 0·125 M 2-mercaptoethanol in pH 7·4 phosphate-buffered saline and heated for 1 hour in a 37° water bath. Agglutination determinations were carried out without alkylation of the treated serum.

Density of the agglutinin was determined by discontinuous sucrose gradient centrifugation using a 39 L rotor at 114,000 ***g*** for 18 hour. Visible bands at the sucrose interfaces were removed with a 27-gauge needle and the tube drained. The pellet and each interface-fraction were diluted with saline to the original volume of the sample.

RESULTS

PRIMARY IMMUNIZATION OF THE NEWBORN LAMB

Since it had been found that foetal immunization could routinely be accomplished with 10^{10} brucellae (Richardson, Beck and Clark 1968), normal newborn lambs were injected with the same dose of antigen to establish the anti-Brucella response after birth. Fig. 1 shows the average agglutinin levels of sera from forty-one unimmunized lambs and from twenty-nine lambs receiving 10^{10} brucellae at birth. Anti-Brucella agglutinin is present in low titre in the serum of adult ewes with no history of disease and no experimental

exposure to *Brucella*. Although lambs do not passively acquire maternal antibody via the placenta, they obtain antibody from colostrum during the first 24–48 hours of life, before the intestinal barrier develops. Seven of the forty-one unimmunized lambs had nursed before the first serum sample was obtained. The average titre at 0 hour was 1/6; by day 2 the average was 1/42. Thereafter, it declined steadily.

Of the lambs receiving antigen immediately after birth, four had nursed before blood samples were obtained. The average titre was 1/7 at 0 hour and 1/37 on day 2. The level regressed through day 8, paralleling the slope of the unimmunized group. Thereafter, the agglutinin of fourteen lambs increased. Maxima were reached from days 11 to 18 and

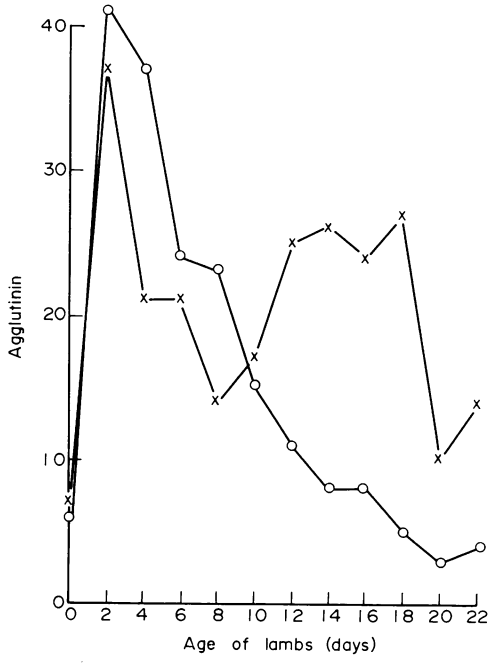

FIG. 1. Antibody levels of neonatal lambs. (×) Average agglutinin levels of twenty-nine lambs immunized at birth with 10^{10} killed *Brucella abortus*. (○) Average agglutinin levels of forty-one normal lambs.

ranged from 1/10 to 1/320 with 1/40 for seven of the responding lambs. Fifteen lambs did not evidence any increase in agglutinin. In lambs with high levels of maternal antibody, a low response could have occurred and been obscured, i.e. six lambs had agglutinin titres of 1/80 and one 1/160 on day 2; by day 12 the titres were still 1/10–1/40.

PRIMARY IMMUNIZATION OF THE FOETAL LAMB

A total of sixty-four foetal lambs have been immunized with 10^{10} brucellae. The agglutinin levels of some of the lambs were followed after birth for 2–6 weeks. Fig. 2 shows data from a representative group of six lambs immunized 25–35 days prenatally. As with all lambs immunized 20 days or more prenatally, anti-Brucella agglutinin was maximal at birth or within 24 hours. Although the agglutinin levels of lambs immunized late in the gestation period varied considerably between individual lambs, the extent of the response in general significantly exceeded that of lambs immunized at birth.

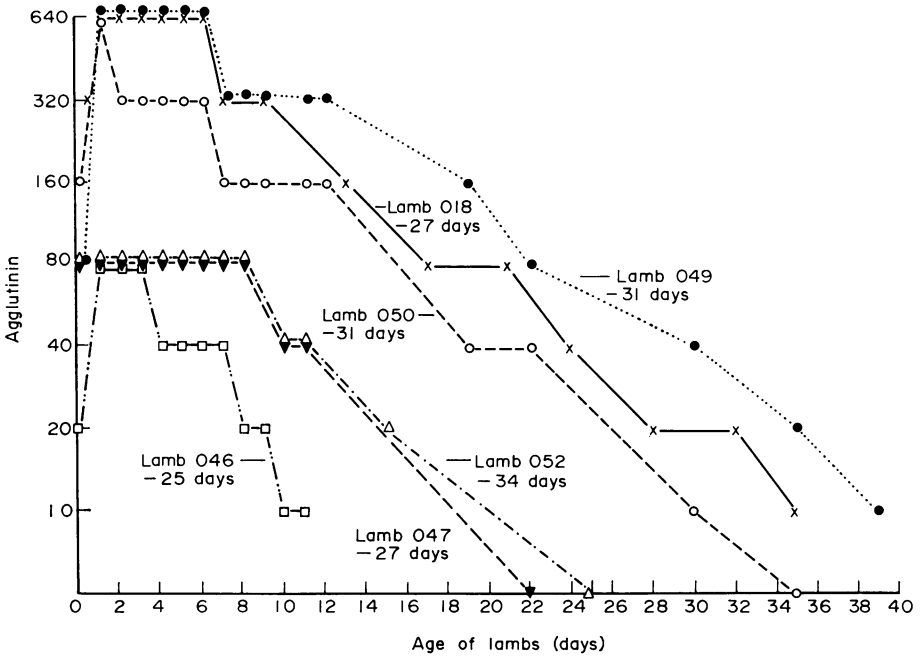

FIG. 2. Antibody response of neonatal lambs immunized 25–35 days prenatally with 10^{10} brucellae

TABLE 1

BRUCELLA ANTIBODY RESPONSE OF FOETAL LAMBS

Ewe No.	Foetus	Number of injections *in utero*	Day after primary*	Day after secondary of single or twin†	Agglutinin titres (reciprocal) Macro	Micro
99	Single	1	21	—	20	128
106	Single	1	21	—	20	256
107	Single	2	18	—	20	128
			49	0	40	256
			55	6	640	NT‡
108	Single	2	18	—	80	512
			39	0	20	256
			45	6	1280	NT
110	Single	1	21	—	< 10	128
112	Twin A	2	18	—	10	128
			39	0	40	256
			45	6	1280	NT
			Twin A Twin B			
	Twin B	1	18 —	—	< 10	< 2
			39 0	—	< 10	< 2
			45 6	—	< 10	< 2
114	Single	1	21	—	< 10	64
119	Single	2	18	—	10	128
			39	0	40	512
			45	6	5120	NT
120	Single	1	18	—	< 10	< 2

* 10^{10} brucellae 50–65 days prenatally.
† 10^{10} brucellae.
‡ NT, not tested.

To examine the response earlier in foetal life, blood was sampled *in utero*. The response of nine foetal lambs to Brucella antigen was relatively low and slow when it was initiated 50–60 days prenatally (Table 1). (The period of gestation is 145–150 days.) At 18–21 days, when agglutinin levels late in gestation were maximal, six foetal lambs had macroagglutinin levels of 1/10–1/80 and three were negative. Microagglutinin levels of antibody were present in two of the latter. Owing to surgical accidents and sacrifice for tissue samples, only four of the immunized foetuses remained for testing at 39 or 49 days. The agglutinin levels of three of the four were higher than at 3 weeks. Higher levels might have been attained and receded in the intervening weeks.

SECONDARY IMMUNIZATION OF THE FOETAL LAMB

To determine if the foetal lamb was capable of mounting a secondary response to *Brucella*, the four immunized foetuses and the unimmunized twin B of ewe 112 were injected with antigen after the blood was sampled at 39 or 49 days. Maximum antibody levels of newborn lambs and adult sheep receiving a second injection of 10^{10} brucellae were regularly elicited by day 5 or 6. Therefore, foetal blood samples were obtained 6 days after the second stimulus *in utero*. As Table 1 shows, the agglutinin titre in one foetal lamb was 1/640, in two 1/1260 and one 1/5120. The blood of twin B of ewe 112 was negative. Thus, the secondary antibody levels of the foetal lambs were as high at 6 days as those attained in adult sheep on secondary stimulation.

SECONDARY IMMUNIZATION OF THE NEWBORN LAMB

The rapid, marked secondary response of foetal lambs to Brucella antigen demonstrated that the newborn must be equally immunocompetent. To determine the reactivity of newborn lambs to a second stimulus of antigen, fourteen foetal lambs were primarily immunized 33–52 days before birth. At birth the sera of twelve of the fourteen lambs contained macroagglutinin levels of 1/10 and 1/20, one had a microagglutinin level of 1/64 and one was negative (Table 2). The newborns received a second injection of 10^{10} brucellae, and blood samples were obtained 3–4 times weekly for 3 or more weeks. As Table 2 shows, ten of the thirteen neonates with Brucella agglutinins present at birth manifested secondary responses with maxima by days 5–7; two (536 and 537) showed rises of only one two-fold dilution in titre; and one (557) did not increase.

Three twins of prenatally immunized lambs did not receive primary antigenic stimulation until birth. Lamb 556 and its immunized twin, 557, acquired high levels of antibody via colostrum; by day 3 the agglutinin levels were 1/80 and 1/160, respectively. Agglutinin did not increase in 556 during the 11–20-day period when primary response occurs. No secondary response was apparent in the twin although a low-level one could have been obscured by the maternal antibody. The agglutinin levels of lambs 560 and 566 did not increase over that of Day 3 whereas their prenatally immunized twins 558 and 567, respectively, attained maxima of 1/640 and 1/1280.

CLASS OF IMMUNOGLOBULIN ELICITED IN THE SECONDARY RESPONSE

The class of immunoglobulin in foetal lamb sera 6 days after secondary stimulation was examined in the four sera available by reduction with 2-mercaptoethanol (2-ME) and

TABLE 2

AGGLUTININ LEVELS OF NEONATAL LAMBS IMMUNIZED WITH KILLED *Brucella abortus*: PRIMARY STIMULUS OF 10^{10} BACTERIA *in utero* AND SECONDARY STIMULUS OF 10^{10} BACTERIA AT BIRTH

Foetal immunization			Reciprocal of agglutinin titre													
Days pre-natal	No. foetuses / No. injected	Lamb No.	0 hour (before nursing)		Days after birth (macroagglutination)											
			Micro	Macro	1–2	3	4	5	6	7–8	9–10	11–12	13–14	15–16	17–18	19–21
44	2/2	536	128	10	80		80		160	160		160	160	80	—*	—
		537	128	20	80		80		160	160		80	20	—	—	—
48	1/1	543	—	—	40		20		40	40		—	—	—	—	—
44	2/2	544†	128	20	20		320		10240	5120		2560	1280	1280	640	640
		545	256	20	80		640		2560	2560		1280	640	640	320	320
33	2/2	554	128	20	80		160		320	320	320	320	160	160	160	40
		555	128	20	40		160		160	160	80	80	80	40	40	—
37	2/1	556	—	—		80		80		80	40	—	—		—	—
		557	256	20		160		160		160	80	80	40		40	20
45	2/1	558	64	10		20		320		640	160	160	160		80	40
		560	—	—		10		—		—	—	—	—		—	—
45	1/1	559	256	20		160		2560		2560	1280	640	640		320	320
37	1/1	561	128	10		160		320		160	80	80	40		20	20
51	1/1	565	64	—		40		640	640	320	320	320		80	80	40
47	2/1	566	—	—		20		—	—	—	—	—		—	—	—
		567	128	10		40		1280	1280	640	320	320		320	320	160
52	1/1	568	64	—	10		80		320	160	160	80	40	20	—	—

* — = < 10 (macro), < 2 (micro).

† Lamb not reared with the ewe; no colostrum; fed a soy-vitamin diet until death at 3 weeks.

by sucrose density gradient centrifugation. The sera contained only 1·5–6·2 per cent agglutinin that was insensitive to 2-ME. With discontinuous sucrose density gradients, over 90 per cent of the agglutinin pelleted under 25 per cent sucrose. Since no activity was detected at the interfaces, one foetal serum (107) was precipitated with 50 per cent ammonium sulphate and the globulin dialysed and concentrated. A band at the 15–20 per cent interface contained agglutinin; the titre of the light fraction was 1/20, as compared to 1/1280 in the fraction pelleted under 25 per cent sucrose. Thus, as indicated by sensitivity to 2-ME, the foetal antibody was predominantly IgM; a small amount of IgG was present.

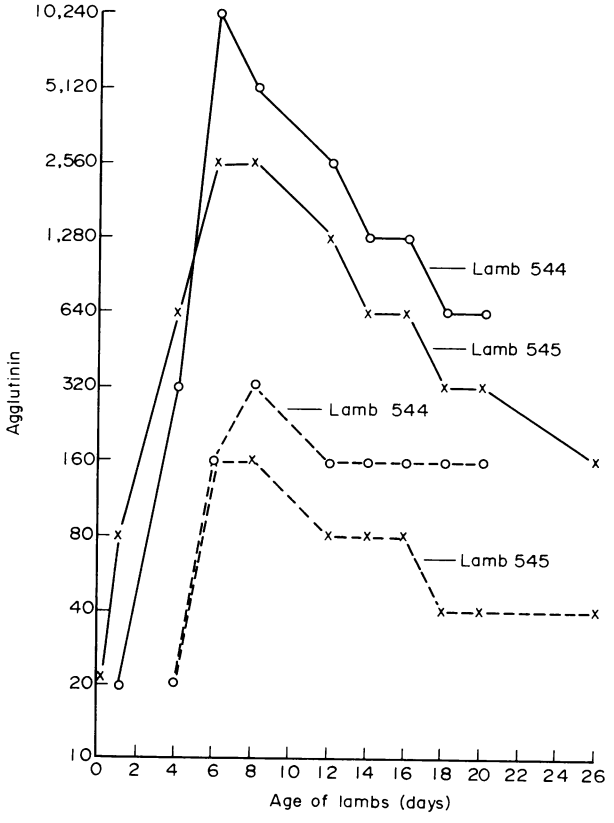

FIG. 3. Effect of 2-mercaptoethanol on the anti-*Brucella* activity of sera from twin neonatal lambs immunized 44 days prenatally and at birth. (○) Lamb 544 separated from the ewe at birth, no colostrum ingested. (×) Lamb 545 reared normally with the ewe. (—) Normal (- - - -) 2-ME.

The class of immunoglobulin synthesized during the secondary response initiated at birth was determined by 2-ME reduction of sera from thirteen lambs. Maternal IgG could not be distinguished from that synthesized by the lamb, with one exception. Lamb 544 was separated from the ewe before nursing. Although lamb 545 acquired an agglutinin level of 1/80 during the first day of nursing and the agglutinin level on day 6 was lower than its twin, 544, the rates of fall of IgM were similar (Fig. 3). As is customarily found with older animals, IgG appeared later and regressed more slowly than IgM.

DISCUSSION

It has been demonstrated that a secondary antibody response comparable to that of adult sheep can be elicited in foetal and newborn lambs by killed *Brucella abortus*. To our knowledge, this is the first report of a secondary response *in utero*. A secondary response of newborn piglets to less complex antigens has been reported. Šterzl *et al.* (1965), although unable to detect any response to *Salmonella paratyphi* B or *Brucella suis* antigens injected 1 month before term, found that antibody to sheep erythrocytes could be demonstrated in sera of some piglets at birth. If haemolysin was not present and a second dose of antigen was given, a typical secondary response ensued. Binns (1967) reported that a secondary response occurred in piglets injected with allogeneic cells *in utero* and stimulated with test skin grafts at 10–12 days of age. Foetal pigs also produced antibody *in utero* to *Salmonella flagellin* and mounted a secondary reaction after birth.

Our investigation was undertaken to determine if the non-reactivity of neonates to somatic bacterial antigens could be overcome by prenatal immunization. To establish the response of the newborn to 10^{10} brucellae, twenty-nine normal lambs received this dose of antigen at birth. The agglutinin levels of sera from fourteen of the lambs increased to average maxima of 1/80 on days 11–18; fifteen lambs did not evidence a response. Since older lambs and adult sheep respond to the same dose of Brucella antigen with average agglutinin levels of 1/1280, the newborn must be considered relatively non-responsive. The present work has shown that the null period in early life can be circumvented by secondary antigenic stimulation at birth.

Foetal lambs immunized 50–65 days before birth responded slowly with low levels of antibody. The high ratio of antigen to immunoreactive cells in the younger foetus may partially paralyse the response. Cotes, Hobbs and Bangham (1966) found that 100 mg, but not 1 mg, of bovine serum albumin in foetal rhesus monkeys at 10–16 weeks gestation induced tolerance in neonates. Binns (1967) reported tolerance in piglets if allogeneic cells were injected into foetal pigs at 60 days gestation but not after 80 days of foetal life.

When this program was initiated, we attempted to immunize 90–100-day old foetal lambs with 10^{3}–10^{8} whole brucellae, cell walls and soluble antigen. Since no agglutinin was detected at birth, we have employed 10^{10} organisms. Lower doses of antigen might elicit higher antibody levels *in utero* than were found with 10^{10} brucellae. Nevertheless, this dose of antigen, administered when surgical intervention is not too hazardous, adequately primed for a pronounced secondary response in four of four foetal lambs and distinct responses in ten of thirteen neonatal lambs. The failure to overcome non-reactivity of some of the lambs may lie in genetic differences, the amount of maternal antibody ingested and/or the stress of parturition.

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