INFECTION AND IMMUNITY, Apr. 1972, p. 454-460

Vol. 5, No. 4
Printed in U.S.A.

Prenatal Immunization by the Oral Route: Stimulation of *Brucella* Antibody in Fetal Lambs[1]

MARVIS RICHARDSON AND GABEL H. CONNER

Departments of Microbiology and Public Health and Veterinary Surgery and Medicine, Michigan State University, East Lansing, Michigan 48823

Received for publication 5 October 1971

No simple means exists for immunizing infants and large animals in utero. The feasibility of stimulating immune response by the oral route was investigated by using the fetal lamb as experimental model and *Brucella* as prototype antigen. Antigenic stimulation was assessed by serum agglutinin levels. Primary and secondary responses were elicited in fetal lambs by *Brucella* antigen introduced into the amniotic fluid 10 to 50 days prenatally. One lamb born 75 days after primary exposure in utero mounted a secondary response to antigen at 2 days of age. The evidence of immunization by the oral route suggests that local intestinal immunity also might be acquired in fetal life by this route.

Enteric infections are the leading cause of mortality of the newborn. When a high death-to-case ratio exists, the newborn should be actively immunized. However, this is the period when mammals are least responsive to antigenic stimulus. For example, Smith (15) found that only 1 of 58 infants receiving typhoid vaccine at 1 to 30 days of age produced antibody against the somatic antigen. Newborn domestic animals are equally nonresponsive to gram-negative bacterial antigens (8), although many species are immunocompetent in fetal life. Richardson et al. (11) reported that the nonreactivity of lambs to gram-negative *Brucella* could be circumvented by prenatal immunization. Fetal lambs responded to *Brucella* antigen with relatively high levels of antibody synthesis, in contrast to newborn lambs, which responded with little or no antibody. After primary immunization of the fetus, a second antigenic stimulus either in utero or at birth elicited levels of antibody comparable to that of adult sheep (12). Thus, prenatal immunization can overcome the null period in neonatal life.

A simple means does not exist for immunizing the fetus. Antigen might be introduced into amniotic fluid through the intact maternal abdominal wall if the fetal gut could be stimulated by the oral route. Parshall and co-workers (*personal communication*) found that introduction of antigen into the stomach of fetal lambs, through the catheterized ligated esophagus, led to enlarged lymph nodes of the gastrointestinal system. They considered that this might be due either to regional stimulation by antigen or to the lack of amniotic fluid. For some bacterial and viral agents, gut immunization has proved the route of choice. In addition to humoral antibody, local immunity appears to be stimulated. Little is known of intestinal immunity per se, but evidence suggests that it plays a significant role in protection against enteral agents (16), preventing penetration of the organism into the bowel (4, 5). The objective of the work reported here was to determine whether prenatal immunization could be effected by the oral route. It was found that *Brucella* antigen introduced into the amniotic fluid elicited humoral antibody responses in fetal lambs. Laparotomy preceded the injection of antigen to ensure its introduction into amniotic fluid. Now that the efficacy of the oral route has been demonstrated, procedures might be simplified.

MATERIALS AND METHODS

Antigen. *B. abortus* type I 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% CO_2 for 72 hr at 37 C, washed from the surface with 0.85% NaCl in 0.5% phenol, and stored at 4 C. Prior to use, the killed antigen was washed twice and diluted in saline to contain 10^{10} or

[1] Published as journal article no. 5678 from the Michigan State Agricultural Experiment Station.

10^{11} brucellae per ml, as determined by a Libby photronreflectometer.

Immunization of fetal lambs. Laparotomies were performed on crossbred, anesthetized ewes 70 or more days after breeding. The fetus was manipulated into position to permit injection without exteriorizing the uterus. For intracardiac injection, the needle was inserted through the uterine wall and, upon entering the heart, the syringe plunger was slightly retracted to assure proper location before 10^{10} brucellae in 1 ml were injected. For primary oral immunization, 10 or 20 ml of antigen containing 10^{11} brucellae per ml was injected into the amniotic fluid, with the exception of a 70-day-old fetus which received 5 ml (ewe 19). For secondary oral immunization, 20 ml was introduced.

Multiple births are common in sheep. In this species, twin concepti usually develop in separate amniotic sacs, although occasionally two fetuses are found in one. Each twin of the 11 pairs in these experiments was in an individual sac. Antigen was introduced into the amniotic fluid of both twins of two ewes and of one of the other nine pairs. To minimize trauma due to exteriorizing the uterus and the surgical risk of suture of the amniotic sac, the twins were not suture-tagged to identify the fetus exposed to antigen Nor were the fetuses dye-marked, since only "blind" in utero bleedings were anticipated. With two exceptions, the fetal lambs were at 90 to 110 days of the gestation period when first exposed to antigen. The exact breeding date of the ewes was not known; the age at which the fetus was first exposed to antigen was estimated from the size and its development at successive laparotomies.

Determination of agglutinin levels. The procedure for obtaining fetal blood samples was by laparotomy and cardiac puncture through the uterine wall. Blood was drawn slowly into the syringe and expressed with care to prevent hemolysis of the fragile ovine fetal erythrocytes.

Brucella antibody was determined by the standard test tube agglutination method (Tube serum-agglutination test, National Animal Disease Diagnostic Reagents Manual 65D, Agricultural Research Service, USDA, Ames, Iowa). In brief, 0.2 ml of serum was placed in 1.8 ml of USDA strain 1119 *Brucella* antigen and diluted twofold in 1.0-ml amounts of the antigen. After incubation for 48 hr at 37 C, the degree of agglutination was read without shaking the tubes. Marked agglutination and sedimentation with 50% clearing (++) was taken as the end point. Sera that were negative or low by the macromethod were retested by the microagglutination method of Claflin et al. (1).

Immunofluorescence staining. Anti-*Brucella* bovine gamma globulin was labeled with fluorescein isothiocyanate by the method of Riggs et al. (14). Imprints of fresh tissue were fixed in cold acetone for 15 min, covered with antiserum for 30 min at room temperature, prewashed with buffered saline (*p*H 7.4), and then immersed in buffered saline and rotated for 10 min. The imprints were mounted in buffered glycerine, *p*H 7.4 to 7.8. For microscopy, a Zeiss microscope, equipped with apo 40/1.0 oil immersion lens, was used with an HBO-200 lamp, primary BG 12/2 mm filter, and OG-5 barrier filter. Photographs were taken on daylight, high-speed Ektachrome film.

RESULTS

For immunization by the oral route, laparotomies were performed on 17 ewes at approximately 70 to 140 days of the 147-day gestation period. Since no parameters for oral immunization had been established, two approaches were taken: (i) primary immunization by the oral route and (ii) secondary immunization in utero using the oral route for one of the two antigenic stimulations. The principal problem anticipated was that uptake of antigen by mouth might occur slowly, or not at all early in gestation, and that low concentration of antigen in the gut might prime the fetus but not stimulate detectable antibody synthesis. The secondary response was utilized to resolve this possibility.

Primary response. Nine fetal lambs were exposed 10 to 75 days prenatally to *Brucella* antigen in the amniotic fluid to examine the primary response (Table 1).

Ewe 52 lambed naturally 10 days after antigen was introduced into the amniotic fluid of one of the two fetuses. The full-term twin lambs were bled before they nursed. The macroagglutinin titer of one lamb was 1:40; serum from the other was negative. Since *Brucella* antibody is not acquired through the ovine placenta (11), it was concluded that late in pregnancy the fetal lamb could synthesize antibody in response to antigen by the oral route.

Ewe 42 aborted 21 days after the laparotomy; since the fetus was partially absorbed, no blood was available for testing.

A second laparotomy was performed on six ewes to obtain blood samples in utero 21 to 33 days after exposure to antigen. Insofar as retention of the fetuses for exploration of a secondary response seemed paramount, the uterus was not exteriorized to bleed the fetus. By using "blind" cardiac puncture, blood could be obtained only from one each of the twins of ewes 23, 65, and 68. One fetus of the twins of ewes 15, 23, and 29 and the single fetus of ewe 40 responded to antigen by the oral route with a macroagglutinin level of 1:10 and microagglutinin levels of 1:128, 1:64, and 1:32, respectively. No agglutinin was detected in the one serum each obtained from the twin fetuses of ewes 65 and 68. These bloods may have originated from the twin not exposed to antigen. Other evidence indicated that one fetus of ewe 68 had been antigenically stimulated by the oral route. Although blood could not be obtained from one twin at 21 days, a second attempt at cardiac puncture succeeded

TABLE 1. *Primary antibody response of fetal lambs to Brucella antigen by the oral route*

Ewe no.	Fetus			Agglutinin[a]		
	Age[b] (days)	No. present	No. exposed to antigen	Day after antigen	Titer[c] Macro	Titer[c] Micro
15	95	Twins	1	21	Neg	Neg
				21	10	>512
19[d]	70	Single	1		NB[e]	NB
23	110	Twins	1	21	Neg	128
				21	NB	NB
29	95	Twins	1	33	Neg	Neg
				33	Neg	64
40	110	Single	1	23	Neg	32
42[f]	90	Single	1		NB	NB
52[g]	137	Twins	1	10	40	256
					Neg	Neg
65	100	Twins	1	21	Neg	Neg
				21	NB	NB
68[h]	110	Twins	1	21	Neg	Neg
				21	NB	NB

[a] Titers done on serum obtained in utero by cardiac puncture except for the lambs of ewe 52, which were bled at birth.

[b] Age when first exposed to antigen. Estimated from the size and development of the fetus as determined at successive laparotomies and when the fetus was sacrificed.

[c] Reciprocal. Neg = <10 (macro), <2 (micro).

[d] No blood obtained in utero; lamb nursed at birth before blood sample could be obtained.

[e] NB = No blood obtained.

[f] Aborted 21 days after antigen; fetus partially absorbed and no blood could be obtained. .

[g] Blood obtained at birth before nursing.

[h] Antigen was injected into both twins by the intracardiac route at 21 days. The macrotiter of a blood sample obtained from one fetus 7 days later was 1:160; macro- and microtiters of the other fetus were negative.

and antigen was introduced. Therefore, both twins received antigen at this time, but only one had been exposed to antigen by the oral route. When they were bled in utero 7 days later, one serum was negative and one had a macroagglutinin titer of 1:160. The high level of antibody might have resulted either from primary stimulation by the oral route, initiated 28 days earlier, or as a secondary response to the antigen injected 7 days before. Even low macroagglutinin levels of antibody have not been detected before day 8 in the primary response of lambs to *Brucella* antigen (11, 12; *unpublished data*). Thus, it appeared that the response was not due to a primary response to antigen intracardially at 21 days but that it was initiated by the oral route.

The lamb of ewe 19 was born 75 days after antigen was introduced. Unfortunately, the lamb nursed before a blood sample could be obtained for determining the antibody level. This lamb must have been primed in utero, however, as it evidenced a secondary response to antigen injected at 2 days of age (*see below*).

The data obtained with the few ewes and fetuses that survived the vicissitudes of major abdominal surgery in the last third of pregnancy clearly demonstrate that antibody production can be elicited by antigen in the amniotic fluid. Two fetal lambs responded with macroagglutinin levels and three with microagglutinin levels. Only one of each pair of twins was exposed to antigen; *Brucella* antibody was detected only in one of the sera from the twins. Although the response to antigen taken by mouth was unequivocal, due to the small numbers of fetuses and differences in time of bleeding no conclusions can be drawn concerning the extent of the antibody response at different stages of maturation.

Response of primed fetal lambs to antigen by the oral route. A group of fetal lambs was primarily immunized at 90 to 110 days of the gestation period to determine whether an anamnestic response would occur when the fetus was exposed to antigen in the amniotic fluid. The fetus of ewe 4, one of the twins of ewe 8, and both twins of ewe 11 were injected with antigen intracardially; antigen was injected into the amniotic fluid of the fetus of ewe 40. Blood samples were obtained by cardiac puncture in utero 23 to 37 days later, and antigen was introduced into the amniotic fluid of all six fetuses, including the twin which was not immunized originally. We had found with neonatal lambs which were immunized prenatally and stimulated a second time at birth that maxima were attained on days 5 to 7 (12). Therefore, 7 days after the second exposure to antigen, the fetuses were bled in utero to determine the antibody levels.

The data in Table 2 indicate that secondary responses can be stimulated by the oral route. High antibody levels were attained by day 7 in three of five fetuses primed 23 to 35 days earlier. Macroagglutinin titers increased from 1:40 to 1:640; from 1:20 to 1:2,560; and from a negative to 1:320. Slight increases occurred in the other two primed fetuses and none in the fetus first injected 7 days earlier. The fetus that received both the first and second antigenic stimulations by the oral route was one of the three showing a marked response.

We have reported that 10 newborn lambs which first had been stimulated intracardially 33 to 52 days before birth responded to a second stimulus at birth with maximal antibody titers of 1:160 to 1:10,240 by days 5 to 7 (12). In addi-

TABLE 2. *Antibody response of primed fetal lambs to Brucella antigen by the oral route*

Ewe no.	Fetus		Primary response					Secondary response				
	Age[a] (days)	No. present	No. exposed to antigen	Route	Day[b]	Agglutinin titer[c]		No. exposed to antigen	Route	Day[d]	Agglutinin titer	
						Macro	Micro				Macro	Micro
4	100	Single	1	IC	37	40	>512	1	Oral	7	640	>512
8	90	Twins	1	IC	35	Neg	Neg	2	Oral	7	Neg	Neg
						Neg	32				Neg	128
11	100	Twins	2	IC	25	Neg	Neg	2	Oral	7	10	128
						20	256				2560	>512
40	110	Single	1	Oral	23	Neg	32	1	Oral	7	320	>512

[a] Age when fetus first exposed to antigen.
[b] Days after antigen.
[c] Reciprocal. Titers done on serum obtained in utero by cardiac puncture. IC = intracardiac; neg = <10 (macro), <2 (micro).
[d] Days after second exposure to antigen.

tion, four fetuses which were bled at intervals after intracardiac immunization 50 days prenatally evidenced a secondary antibody response in utero. At 39 or 49 days after the first stimulation, when the titers were 1:20 to 1:40, antigen was injected a second time by the same route. Six days later the titers of the four were 1:640 to 1:5,120. Since the antibody levels of the fetal lambs were as high at 6 days and appeared at the same time as those attained in lambs and adult sheep after a second antigenic stimulus, this was considered a secondary response of the fetus. The time and extent of the secondary response contrasts markedly with the primary response of neonates to *Brucella* antigen. We found with a group of 29 lambs which were first injected with antigen at birth, or soon after, that the 14 responding lambs achieved low-level maxima of 1:10 to 1:320 (1:40 for seven lambs) at 11 to 18 days (12). Also, when the response was induced 4 to 10 days prenatally and the antibody levels were determined daily after birth, the time from injection of the fetus to maximal levels in the lamb was 14 to 17 days (11). Whether the primary was induced in utero or at birth, macroagglutinin was not detected until day 8 or after.

The kinetics of the antibody response of the fetus are not readily established. Of the 73 fetal lambs we have immunized by the intracardiac route, only 18 have been bled in utero to determine the antibody level. We have succeeded in bleeding only a few of these two or three times. The limited data indicate that the primary response is relatively low and slow when initiated 50 days prenatally. At 18 to 21 days after antigen (when agglutinin levels late in gestation were maximal), six of nine fetuses had macrotiters of 1:10 to 1:80 and two were positive at the micro level. Four of these were retested at 39 or 49 days after antigen; the titers of three increased from 1:10 or 1:20 to 1:40 and one decreased from 1:80 to 1:20. No curves exist for either primary or secondary responses in utero. Nevertheless, the significant increases in fetal antibody 7 days after second antigenic stimulation provides strong evidence of a secondary response in utero. This is the time when maxima are reached in the secondary response of newborns, as compared to little or no increase in the primary at this time.

Secondary response after birth. One lamb was born 75 days after primary exposure to antigen by the oral route. The udder was not taped prior to lambing and the lamb nursed before a blood sample could be obtained. At 48 hr, the macrotiter was 1:10. Antigen was injected intravenously at this time. Since maternal antibody does not pass the ovine intestinal barrier after 48 hr, any increase in antibody would be due to active synthesis by the lamb. The macroagglutinin levels were 1:20, 1:320, and 1:160 on days 3, 5, and 7, respectively, after the neonate received antigen. On the basis of an antibody level of 1:320 at 5 days, indicating a secondary response, it was considered that the lamb had been primed in utero by antigen injected into the fetal fluid 75 days before birth.

Detection of antigen in the fetal gut. Imprints of ileum tissue were prepared 7 days after the second antigenic stimulation of the fetus of ewe 4 by antigen in the amniotic fluid. The primary stimulus was intracardiac. Figure 1 shows an imprint of the ileum tissue stained with fluorescent *Brucella* antibody. The discrete particles in the macrophage-like cells correspond in appear-

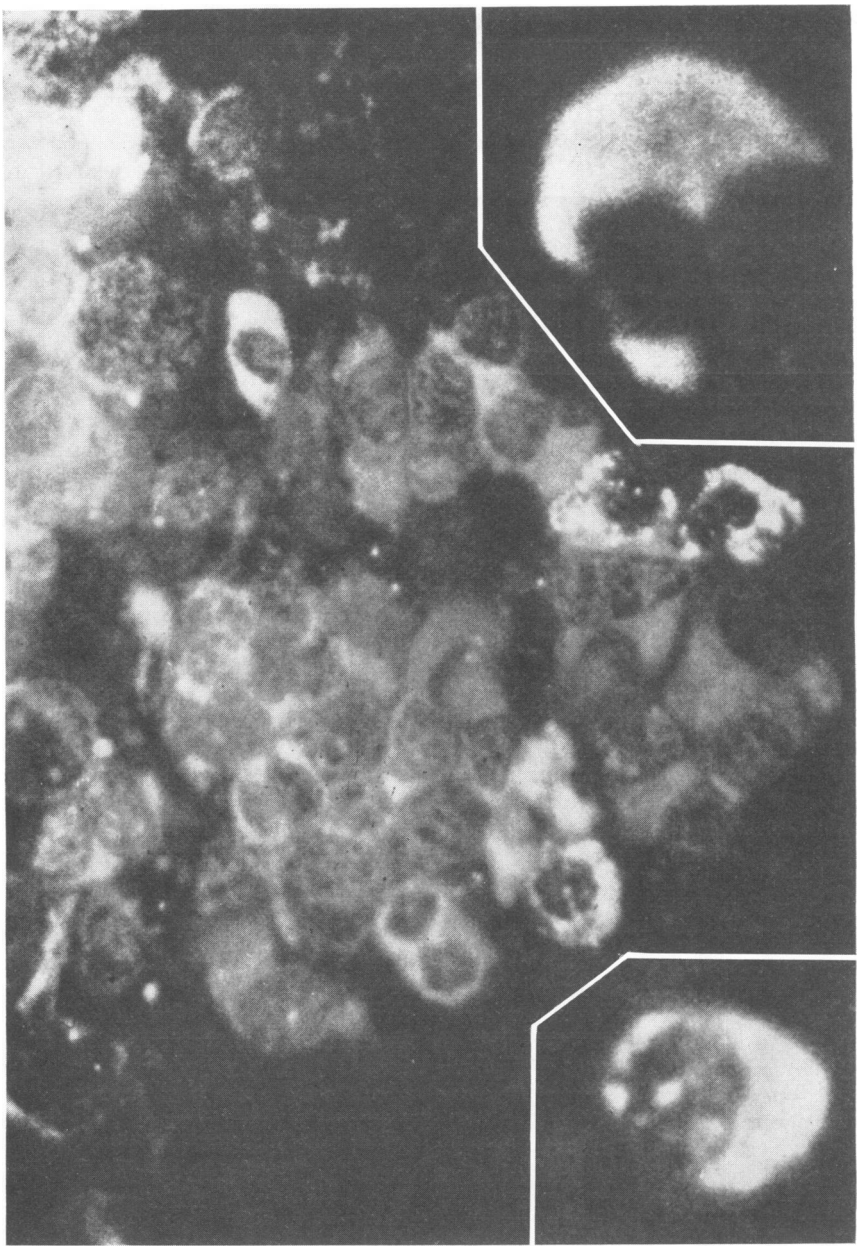

FIG. 1. *Imprint of tissue from the ileum of a fetal lamb; first stimulus with Brucella antigen intravenously at −44 days; second antigenic stimulus at −7 days by the oral route; direct fluorescent antibody staining.*

ance to those observed after uptake of viable brucellae by cultured bovine spleen cells (13). Some cells stained uniformly, suggesting solubilized antigen. Single, extracellular, *Brucella*-sized particles were evident. It was concluded that antigen which had been introduced into the amniotic fluid 7 days previously had reached the ileum and had been phagocytosed.

Little is known of the permeability of the fetal ovine gut. Large molecules are absorbed by the normal lamb during the first day of life before the intestinal barrier closes. Presumably, similar absorption occurs in the fetus. Whether particulate antigen penetrates between the cells of the mucosal surface or is actively phagocytosed to stimulate synthesis of antibody in the lymph

nodes and spleen has not been examined. The evidence of phagocytosis obtained here in the detection of antigen in the gut may occur only after specific immunization. The uptake of labeled brucellae from the gut of the normal and the primed fetus should clarify the point.

In our exploration of the oral route for immunization, no attempt was made to detect local production of antibody per se. If antigen in the gut elicits local secretion of antibody, antibody-synthesizing cells might be identified in ileum tissue by the "sandwich technique" with fluorescein-labeled, anti-*Brucella* antibody, or anti-immunoglobulin A (IgA), or both.

DISCUSSION

The work reported here has demonstrated that antibody responses can be stimulated in fetal lambs by *Brucella* antigen in the amniotic fluid. Evidence of a secondary response was obtained. To our knowledge, this is the first report of a secondary immune response elicited in utero by the oral route. During this investigation, Gay (7) reported a pilot study to immunize calves in utero with *Escherichia coli* antigen. In one of five fetuses, the antigen was injected into the amniotic fluid; at birth, serotype-specific antibody was present. The five calves vaccinated in utero were resistant to colisepticemia; four control calves died. Therefore, Gay has evidence of protection to challenge, besides antibody synthesis, in one calf immunized orally in utero.

Diarrhea disease is a leading cause of infant morbidity and mortality, and enteric infections remain the most important cause of mortality in newborn domestic animals (17). South (16) recently reviewed the accumulating evidence that a local immune system of the mucous membranes exists, that it is separate from the general immune system, and that secretory IgA antibody is stimulated and synthesized locally to cope with antigens in the gut. Oral administration is the route of choice for some vaccines, among them polio and cholera vaccine. Ogra and Karzon (10) found that polio vaccine stimulated local immunity. Freter and Gangarosa (6) determined that a major proportion of intestinal antibody to *Vibrio cholera* antigen by the oral route was locally produced. Freter (4) demonstrated that either serum antibody or intestinal antibody reduced the *V. cholera* population associated with the mucosal surface in vivo. He also obtained evidence of an antibody-mediated bactericidal reaction at the mucosal surface of rabbit ileum in vitro (5). Although other classes of immunoglobulin may be synthesized or transported, or both, to the intestine, IgA appears to function locally and possibly uniquely as a first line of defense. Craig and Cebra (2) found Peyer's patches an enriched source of precursor cells for IgA-producing immunocytes in the rabbit. These cells proved more efficient in seeding the gut of irradiated rabbits than cells from peripheral blood or popliteal lymph nodes.

In the present work with the fetal lamb as an experimental model and *Brucella* as prototype antigen, serum antibody levels have been utilized to assess stimulation by the oral route. Secretion of IgA is known to occur in sheep after local antigenic stimulation (9). Presumably local secretory antibody could be stimulated in utero by the oral route. If a second antigenic stimulus at birth with killed antigen or attenuated viable agents of enteric disease elicited a local secondary response in the gut, the newborn might be protected. Immunization of the fetus by the oral route might provide lasting immunity to many pathogens.

ACKNOWLEDGMENTS

This investigation was supported by a National Science Foundation general research support grant to the College of Veterinary Medicine and by the Michigan Agricultural Experiment Station.

LITERATURE CITED

1. Claflin, A. J., O. Smithies, and R. K. Meyer. 1966. Antibody responses in bursa-deficient chickens. J. Immunol. 97:693–699.
2. Craig, S., and J. J. Cebra. 1971. Peyer's patches: an enriched source of precursors for IgA-producing immunocytes in the rabbit. J. Exp. Med. 134:188–200.
3. Davies, P. A. 1971. Bacterial infection in the fetus and newborn. Arch. Dis. Child. 46:1–27.
4. Freter, R. 1969. Intestinal immunity: studies of the mechanism of action of intestinal antibody in experimental cholera. Texas Rep. Biol. Med. 27:299–316.
5. Freter, R. 1970. Mechanism of action of intestinal antibody in experimental cholera. II. Antibody-mediated antibacterial reaction at the mucosal surface. Infect. Immunity 2:556–562.
6. Freter, R., and E. J. Gangarosa. 1963. Oral immunization and production of coproantibody in human volunteers. J. Immunol. 91:724–729.
7. Gay, C. C. 1971. Problems of immunization in the control of *Escherichia coli* infection. N.Y. Acad. Sci. 176:336–349.
8. Ingram, D. G., and A. N. Smith. 1965. Immunological responses of young animals. I. Review of the literature. Can. Vet. J. 6:194–204.
9. Lascelles, A. K., and W. H. McDowell. 1970. Secretion of IgA in the sheep following antigenic stimulation. Immunology 19:613–620.
10. Ogra, P. L., and D. T. Karzon. 1969. Distribution of poliovirus antibody in serum, nasopharynx and alimentary tract following segmental immunization of lower intestinal tract with poliovaccine. J. Immunol. 102:1423–1430.
11. Richardson, M., C. C. Beck, and D. T. Clark. 1968. Prenatal immunization of the lamb to *Brucella*: dissociation of immunocompetence and reactivity. J. Immunol. 101:1363–1366.
12. Richardson, M., G. H. Conner, C. C. Beck, and D. T. Clark. 1971. Prenatal immunization of the lamb to *Brucella*:

secondary antibody response *in utero* and at birth. Immunology **21**:795–803.
13. Richardson, M., and J. N. Holt. 1964. Multiplication of *Brucella* in cultured lymphoid and nonlymphoid cells. J. Bacteriol. **88**:1163–1168.
14. Riggs, J. L., P. C. Loh, and W. C. Eveland. 1960. A simple fractionation method for preparation of fluorescein-labeled gamma globulin. Proc. Soc. Exp. Biol. Med. **105**:655–658.
15. Smith, R. T. 1960. Response to active immunization of human infants during the neonatal period, p. 348–368. *In* G. E. W. Wolstenholme and M. O'Connor (ed.), Cellular aspects of immunity. Little, Brown and Co., Boston.
16. South, M. A. 1971. IgA in neonatal immunity. N.Y. Acad. Sci. **176**:40–48.
17. Tennant, B. 1971. Neonatal enteric infections caused by *Escherichia coli*. N.Y. Acad. Med. **176**:7–8.