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Ontogeny of the Bovine Immune Response[1]

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The ontogenesis of the bovine immune response was studied in three embryos (<40 days) and 106 fetuses of various ages. In the absence of overt antigenic stimulation, fetuses had lymphoid development of the thymus at 42 days of gestation, the spleen was structurally present at 55 days, and certain peripheral lymph nodes were present at 60 days. Mesenteric lymph nodes were structurally present by 100 days of gestation, and lymphoid tissue of the gastrointestinal tract, particularly the lower ileum, was observed in histologic sections of a 175-day fetus with a bacterial infection. Pyroninophilic cells, plasma cells, and germinal centers were present in lymph node sections of antigenically stimulated fetuses. Lymphoid tissue developed more rapidly in fetuses with bacteria, viral antigens, or apparent maternal red-blood-cell antigens than in the normal fetus. Thymic and splenic indices reached maximal values in the 205- to 220-day fetal age group. Immunoglobulin M (IgM)-containing cells were first observed, by immunofluorescence, in a single fetus at 59 days of gestation. Immunoglobulin G (IgG)-containing cells were observed at 145 days of gestation in one fetus with a bacterial and viral infection. IgM-containing cells were observed in 36 fetuses and IgM and IgG cells were present in seven fetuses. Spleen, lymph nodes, thymus, bone marrow, and liver of one fetus from a dam with lymphosarcoma had immunoglobulin-containing cells. Hemal lymph nodes, blood (buffy coat), Peyer patches, and heart and lung sections from fetuses with immunoglobulin-containing cells in spleen or lymph node did not have immunoglobulin-containing cells.

Antigens of the virus of bovine virus diarrhea-mucosal disease (BVD) were detected in one fetus, and antigens of infectious bovine rhinotracheitis (IBR) virus were detected in three fetuses; however, viruses were not isolated in primary bovine embryonic kidney cells. Two of the three fetuses with IBR virus antigens had neutralizing serum antibody titers to IBR virus. Bacteria including *Escherichia coli*, *Lactobacillus* sp. and *Mima polymorpha* var. *oxidans* were isolated from four fetuses. Antibodies that caused the agglutination of maternal red blood cells were present in 8 of 20 bovine fetal serum samples. The antibodies were 2-mercaptoethanol sensitive and partially heat resistant (56 C for 30 min). The ontogeny of the bovine immune response and human immune response were compared, and it was suggested that the similarities were primarily due to the two species having the same approximate gestation period of 280 days.

The ontogeny of the immune response of several mammalian species was studied, and the results were reported in excellent review articles (9, 25). It is generally accepted that some mammalian fetuses become immunologically competent to certain antigens early in gestation and that competence to additional antigens increases with fetal age (22). The reason for the development of immunological competence to certain antigens at different times in gestation is not known; however, several explanations have been presented (24).

Previous studies on the development of the immune response were concerned with an antibody response to overt antigenic stimulation, and few reports considered the effects of natural antigenic stimulation. The potential for natural antigenic stimulation of the fetus is present in the form of fetal, maternal, and microbial antigens.

The present report is concerned with the

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development of the immune response in the bovine fetus. Information on lymphoid tissue development, occurrence of immunoglobulin M (IgM)- and immunoglobulin G (IgG)-containing cells, serum immunoglobulins, and possible sources of antigenic stimulation are presented. The *Bovidae* have an average gestation period of 280 days, the same as the gestation period for man. This similarity may make it possible to compare certain data obtained from the bovine and human fetuses.

MATERIALS AND METHODS

Experimental fetuses. Fetuses are identified in the text by the letter F, a group of numbers which refer to age, and occasionally a number in brackets (i.e., F-150 [4] is the fourth fetus 150 days old that was studied).

Fetuses were obtained from three sources. The first group, of three embryos (<40 days) and 57 fetuses, was obtained from the Dairy Breeding Research Center. Three embryos and 54 fetuses were from dams used in an experiment on the role of progesterone in pregnancy (27). Briefly, the experimental procedures included removal of all or part of the corpus luteum at a selected time in gestation, and in most animals exogeneous progesterone was substituted at a level estimated to be sufficient to maintain pregnancy (27). Three fetuses, (F-59, F-59 [2], and F-74) were from an experiment on superfetation (27). No manipulation of the fetus occurred in either experiment. The procedure for obtaining the fetus was to kill the dam and remove the uterus. The length of time the fetus remained in the excised uterus after death of the dam was from 6 to 8 h. Fetal ages were determined from artificial insemination (AI) records.

The second group of 36 fetuses was from an abattoir. These fetuses were obtained to determine whether removal of the corpus luteum, injection of progesterone, or the period of time the fetus remained in utero (variables in the procedures described above) affected the development of the fetal immune response. The ages of the fetuses were estimated from a table based on the relationship of age to weight measurements and crown-rump length compiled by T. Y. Tanabe (26). Fetuses in this group were removed from the uterus and examined within a period of 2 to 4 h after death of the dam.

The third group of seven fetuses was obtained from the Animal Disease Laboratory. Uteri were removed from pregnant animals that had been dead for fewer than 12 h. In addition, serum samples were obtained from six 270- to 275-day-old fetuses removed by Caesarean section from the dam. The age of the fetuses was determined from AI records.

Samples from a total of three embryos and 106 fetuses were studied. The five major dairy breeds and one beef breed were represented as follows: 70 Holstein, 19 Guernsey, 3 Brown Swiss, 3 Jersey, 3 Ayrshire, and 8 Hereford fetuses. There were 51 females and 50 males. The sex of five fetuses, 40 days of age, and the three embryos was not determined.

Immunoglobulin and anti-immunoglobulin preparation. IgM was prepared by initial precipitation of pooled bovine serum with ethanol according to the first step in a procedure described by Hess and Deutsch (10). The immunoglobulin precipitate was purified on Sephadex G-200 by repeated gel filtration and concentration cycles (7). IgG was prepared by initial precipitation with 33% $(NH_4)_2SO_4$ and final purification on A-50 diethylaminoethel-Sephadex (6).

IgM and IgG, respectively, were injected into four rabbits intramuscularly (i.m.) at weekly intervals for two months in complete Freund adjuvant. Rabbits were bled, at intervals for up to a year, to obtain antisera. Rabbit antibovine IgM sera was absorbed with 1:20 (vol/vol) gamma globulin-free, fetal bovine serum and 1:40 (vol/vol) bovine IgG. Rabbit antibovine IgG was absorbed with 1:40 (vol/vol) purified bovine IgM. Rabbit antibovine IgM sera and IgG sera were used for radial immunodiffusion, immunoelectrophoresis, and immunofluorescence studies.

Immunofluorescence. Monospecific rabbit antibovine IgM sera and rabbit antibovine IgG sera were conjugated with fluorescein isothiocyanate (FITC) by the method of Coons and Kaplan (3), with modifications. The FITC globulin solution was prepared by dissolving 0.03 mg of dye per mg of protein in a volume of carbonate-bicarbonate buffer (pH 9.0) equal to 10% of the volume of globulin fraction. The globulin solution and FITC were mixed and allowed to react for 18 h. The free dye was removed by gel filtration with Sephadex G-25. To insure that rabbit antibovine IgM and IgG sera had not been affected by conjugation, the conjugated globulin preparations were reacted with bovine serum by immunoelectrophoresis (20). The immunoelectropherograms were viewed by using an ultraviolet lamp as has been described by Hiramoto and Hamlin (12). It was found that neither the electrophoretic character nor the antibody activity was altered by conjugation with FITC. The fluorescent precipitin lines were identical to the precipitin lines observed before conjugation. Immunofluorescent controls included conjugated, nonimmune, preinoculation rabbit gamma-globulin solution and an inhibition test with unconjugated, rabbit antibovine immunoglobulin preparations (30).

Tissue imprints of spleen, thymus (thoracic and cervical), various lymph nodes, liver, "buffy coat" cells, and bone marrow were made at the time of their removal from the fetus. Cryostat sections were made of tonsil, spleen, lymph nodes, and Peyer patches (sections of the lower ileum). All tissue imprints and sections were fixed for 15 min at −15 to −10 C in acetic acid-ethanol (5% glacial acetic acid in 95% ethanol), and duplicate spleen sections were fixed in absolute methanol. The slides were washed for 1 to 2 h in phosphate-buffered saline (PBS) at 4 C, and then were rinsed with distilled water and allowed to air-dry. The tissue slides were reacted with conjugated antiserum for 1 h at 37 C and washed for an additional 1 h in PBS. Cover slips were mounted with buffered glycerol, and the slides were examined with a Leitz Ortholux microscope equipped with a mercury light source and filters for fluorescence microscopy. Similar procedures for the preparation of human tissue and immunofluorescence have been described in detail by others (11).

Viral and bacterial assay. The presence of viral antigens in fetal tissue was assayed by the immunofluorescence technique. Antiviral serum conjugates (from the National Animal Disease Laboratory, Ames, Iowa) of the virus of bovine virus diarrhea-mucosal disease (BVD), infectious bovine rhinotracheitis (IBR) virus, and parainfluenza 3 (PI3) were reacted with spleen and liver sections of all fetuses and with lymph node, tonsil, and kidney sections of certain fetuses. Virus isolation from pooled fetal organ homogenates was attempted in primary fetal bovine (FBK) and newborn bovine (NBK) kidney cells according to standard procedures. The presence of virus in the kidney cells was determined by cytopathogenic effects (CPE), and also be reacting antiviral conjugates with cells on cover slips, which were removed from latent tubes at 22 and 44 h after fetal tissue inoculation. Sera of fetuses suspected of viral infection were checked for neutralizing activity to IBR virus.

In the bacterial assay, the major body organs (heart, intestine, kidney, liver, lung, and spleen) were cultured on blood agar (5% sheep blood). Kidney, contents of the small intestine, and cecum occasionally were cultured on violet-red bile agar. The plates were incubated aerobically and under approximately 5% CO_2 in a candle jar at 37 C.

Serological tests. A hemagglutination procedure, 2-mercaptoethanol treatment, radial immunodiffusion, and immunoelectrophoresis were previously described (21).

Histological and cytological studies. Tissue sections were fixed in 10% Formalin for staining with hematoxylin and eosin and Giemsa stain (29). Sections for methyl green-pyronin staining were fixed in Carnoy fluid (29). Splenic and thymic indexes were determined by using the following formula: wet weight of the organ (g) $\times$ 100/weight of animal (g) = organ index.

RESULTS

Development of fetal lymphoid tissues was determined by microscope examination of histological sections and by gross examination at necropsy. Developing follicles of the lymphoid thymus were first observed microscopically at 42 days of gestation (Fig. 1), and Hassel corpuscles were observed regularly after first appearing at 65 days (Fig. 2). The spleen appeared at about 55 days of gestation, and the predominant cell type was the reticulocyte (Fig. 3). Red and white pulp were partially differentiated between 80 and 100 days. The prescapular and prefemoral lymph nodes were present at 60 days, and the predominant cells were reticular and mesenchymal cells. There was no indication of cortical and medullary structure at this age. Popliteal nodes were not observed until 65 days of gestation. The supramammary lymph node was observed on gross examination at about 90 days. The mesenteric lymph nodes were observed at 100 days, and on microscope examination the mesenteric nodes were highly congested in fetuses of all ages. By 100 to 130 days of gestation, cervical nodes, mediastinal nodes, and most of the other major lymph nodes were observed on gross examination. Hemal lymph nodes were present at 220 days of gestation in some fetuses.

The thymic and splenic indexes of fetuses are presented according to age groups in Table 1. Both the thymic and splenic indexes reached maximum values in the group of fetuses 205 to 220 days of age.

The results of immunofluorescent studies to detect immunoglobulin-containing cells are presented in Table 2. Fetal age, immunoglobulin presence, organs in which the cells appeared, intensity of the fluorescence within the cell, and approximate percentage of positive cells are listed, as well as the number of animals with positive cells and the total number of fetuses in the age group. The approximate percentage of positive cells was an estimate based on the total number of positive cells per 1,000 cells, and this percentage was estimated from the organ showing the highest number of fluorescent cells. The tissues were examined by immunofluorescence for immunoglobulin-containing cells as well as the percentage of tissues with positive cells (Table 3). The youngest fetus with IgM-containing cells was 59 days of age (F-59 [2]), and the youngest fetus with IgG-containing cells was 145 days of age (F-145). Thirty-six fetuses had IgM-containing cells and seven fetuses had both IgM- and IgG-containing cells. Immunoglobulin-containing cells were found most often in the spleen and lymph nodes of fetuses.

No apparent differences existed with regard to breed or sex of the fetus and the number having immunoglobulin-containing cells, for 38% of the females and 40% of the males had Ig-containing cells. Slight, but not significant, differences were found when the source of the fetus was considered. The fetuses of dams from the Animal Disease Diagnostic Laboratory had a 43% incidence of immunoglobulin-producing cells, 37% of the fetuses from the Dairy Breeding Research Center had positive cells, and 32% of the fetuses from the local abattoir had immunoglobulin-containing cells.

Three typical cell types were observed to be positive in the immunofluorescence test for IgM: blast cells, typical plasma cells, and small lymphocyte-like cells (Fig. 4). Cells positive for IgG generally were the typical plasma cell and, occasionally, the small lymphocyte-like cell (Fig. 5). Spleen sections that were reacted with the conjugated nonimmune rabbit serum were negative for all fetuses. The inhibition test (unconjugated serum) used on 25% of the positive tissue sections reduced or completely elimi-

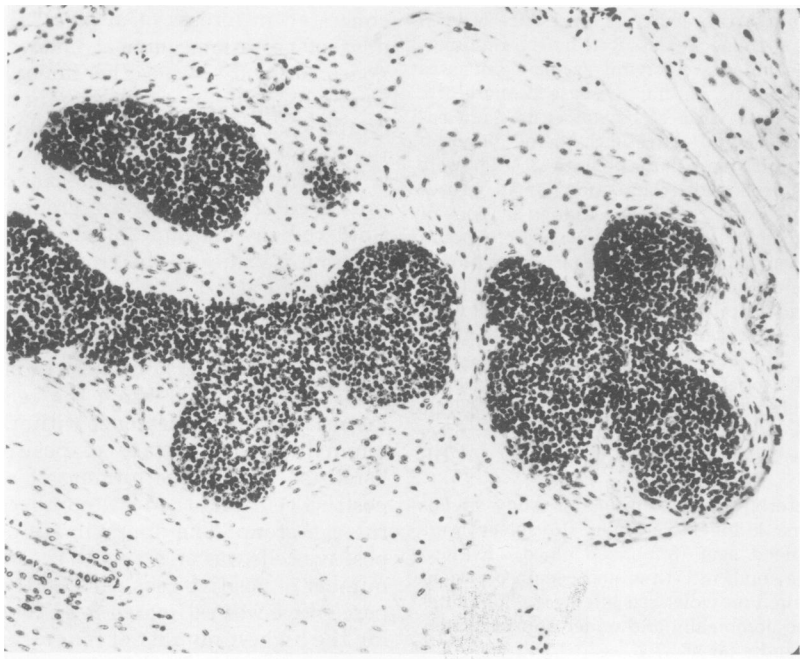

FIG. 1. *Developing lymphoid lobules in the thymus from a 42-day-old fetus, ×140 (H & E stain).*

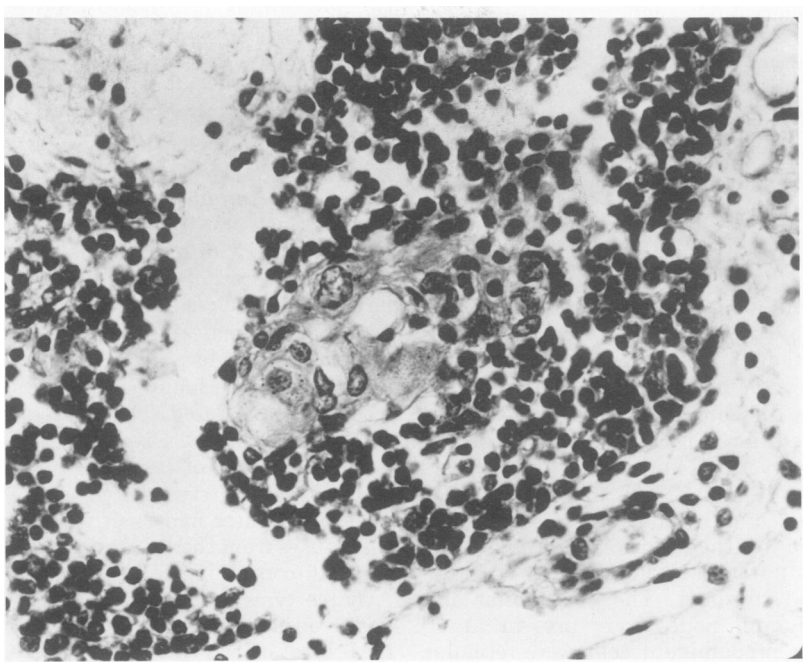

FIG. 2. *Hassel corpuscles clearly identified in the thymus of a 65-day fetus, ×450 (hematoxylin and eosin stain).*

nated positive fluorescence for IgM and IgG.

Methyl green-pyronin staining of spleen, thymus, and lymph node sections demonstrated the presence of pyroninophilic cells in organs of fetuses with immunoglobulin-containing cells and in the organs of certain fetuses without immunoglobulin-containing cells. Typical Marschalko-type plasma cells were regularly ob-

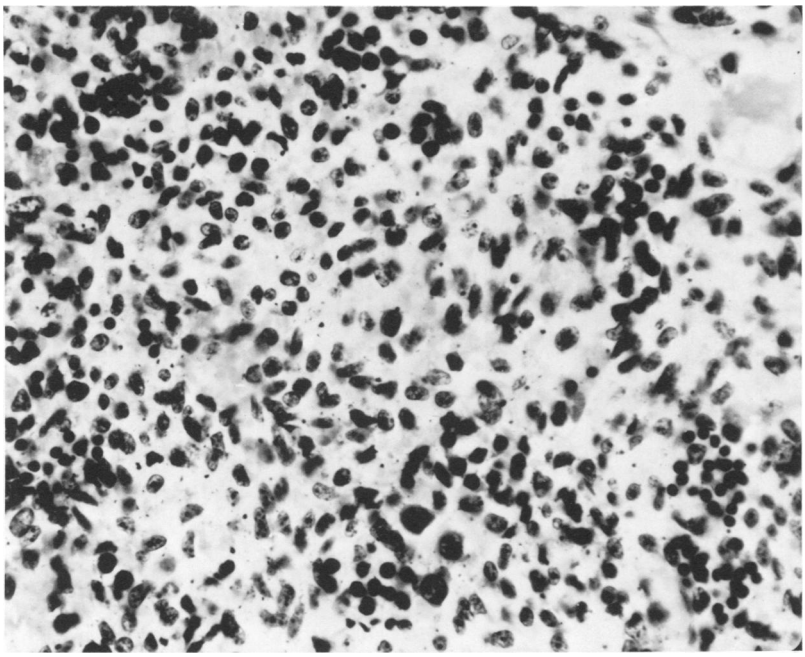

FIG. 3. *Spleen section from 55-day-old fetus composed predominantly of reticulocytes. Hematopoiesis is visible in the form of clusters of small, dark, round nuclei with very little cytoplasm, ×450 (hematoxylin and eosin strain).*

TABLE 1. *Splenic and thymic indexes of fetuses*

Age (days)	Animals (no.)	Splenic index	Thymic index
Embryos:			
20–35	3	NP[a]	NP
Fetuses:			
40–50	9	NP	P[b]
55–65	4	0.04	0.08
70–80	9	0.04	0.10
85–100	8	0.16	0.16
104–120	12	0.24	0.20
125–135	9	0.28	0.28
140–155	7	0.32	0.41
160–170	7	0.35	0.45
175–190	7	0.39	0.62
205–220[c]	7	0.40	0.88
235–250	10	0.33	0.68
265–270	2	0.33	0.62

[a] Not present.
[b] Present (histopathology).
[c] Group with highest indexes.

served in fetuses with immunoglobulin-containing cells, particularly in those producing IgG in addition to IgM. Plasma cells were present most often in spleen and lymph node sections, but they were also found in thymus sections.

The possible sources of antigenic stimuli evaluated were viruses, bacteria, and maternal red blood cell (RBC) antigens. The results of the immunofluorescence assay for BVD, IBR, and PI3 viruses are listed in Table 4. Spleen and kidney cells of one of the fetuses were positive when reacted with BVD-conjugated antiserum. The liver and spleen cells of three fetuses reacted positively with IBR-conjugated antiserum. None of the fetal tissues was positive with the PI3 conjugate. The remaining fetuses assayed were negative for the three viruses. Attempts to isolate virus from fetal tissue in FBK or NBK cells were negative for CPE and for immunofluorescent reactions with conjugated antiserum to BVD, IBR, and PI3 viruses. Serum neutralization tests for IBR virus antibodies were positive at a 1:2 dilution of serum for two of the three fetuses found to be positive for IBR virus by immunofluorescence assay (Table 4). Seven other samples, one positive for BVD and all positive for serum immunoglobulin, were negative in the neutralization test for IBR virus.

In the bacterial assay of the fetuses, *Escherichia coli* was isolated from two fetuses, and *Mima polymorpha var. oxidans* and a *Lactobacillus sp.* each were isolated from one fetus (Table 4).

Twenty of the fetal serum samples were checked for antibodies to maternal RBC. Eight of the 20 fetal sera, at a 1:1 dilution, agglutinated the RBC of the dam. The agglutina-

TABLE 2. *Detection of immunoglobulin (Ig)-containing cells in fetuses by immunofluorescence assay*

Age (days)	Fetus	No. positive/total	Class of Ig (intensity of fluorescence)	Percentage[a] of Ig cells	Tissues with[b] Ig cells
20–54	None	0/12	Negative	0	none
55–65	F-59 [2]	1/9	IgM (+2)	0.1	spl
70–80	F-75	1/9	IgM (+3)	0.05	thy, ln
85–100	F-90	2/8	IgM (+2)	0.1	spl, thy, bm
	F-100		IgM (+2)	0.05	spl, thy, ln, bm
101–120	F-104	2/12	IgM (+2)	0.1	spl
	F-120		IgM (+2)	0.05	spl, thy, ln, bm
125–135	F-122[c]	5/10	IgM (+2)	0.1	spl, thy, ln
	F-130		IgM (+2)	0.05	spl, thy, bm
	F-130 [2]		IgM (+2)	0.05	spl, ln
	F-130 [4]		IgM (+3)	0.5	spl
	F-135 [2]		IgM (+2)	0.5	spl, thy, ln
140–155	F-145[d]	4/8	IgM (+3) + IgG (+3)	1.0	spl, thy, ln, bm
	F-150		IgM (+2)	0.1	spl, ln, bm
	F-155		IgM (+2)	0.05	spl
	F-155 [3][d]		IgM (+2)	0.05	spl
160–170	F-160 [4][e]	3/9	IgM (+3) + IgG (+1)	0.5	spl, liv
	F-165 [2][d]		IgM (+3)	0.1	spl, ln
	F-170		IgM (+3)	0.05	spl, ln
175–190	F-175[d]	5/7	IgM (+3)	0.05	spl, thy, ln
	F-175 [2][d]		IgM (+3) + IgG (+2)	0.1	spl, ln
	F-180[d]		IgM (+3)	0.1	spl, bm
	F-185		IgM (+3)	0.1	thy, ln, bm
	F-190		IgM (+2)	0.1	thy, ln
205–220	F-205	4/7	IgM (+3)	0.5	spl, ln
	F-205 [2]		IgM (+2)	0.05	spl
	F-210		IgM (+3)	0.5	spl, ln
	F-220 [2][d]		IgM (+2)	0.5	spl, ln, bm
235–250	F-235	7/10	IgM (+3)	0.5	spl, ln
	F-235 [2][d]		IgM (+3) + IgG (+3)	1.0	spl, ln
	F-235 [3]		IgM (+3)	0.5	spl, ln
	F-240		IgM (+3)	0.1	spl, ln
	F-250		IgM (+3) + IgG (+3)	5.0	spl, thy, ln
	F-250 [2][d]		IgM (+2)	0.05	spl, ln
	F-250 [5][d]		IgM (+3) + IgG (+3)	5.0	spl, thy, ln
265–270	F-266	2/2	IgM (+2)	1.0	spl, ln
	F-270		IgM (+3) + IgG (+3)	5.0	spl, ln

[a] Estimated from the organ with the highest number of immunoglobulin-containing cells.
[b] Spleen (spl), thymus (thy), lymph node (ln), bone marrow (bm), liver (liv).
[c] Aborted fetus.
[d] Source of apparent antigenic stimuli was determined.
[e] Fetus from a dam with generalized lymphosarcoma.

tion reactions were not abolished by heat (56 C, 30 min), but were abolished by 2-mercaptoethanol treatment. The serum of the dam or fetus did not agglutinate the fetal RBC. The youngest fetal serum assayed that was found to agglutinate maternal RBC was from a 155-day-old fetus (F-155 [3]).

A summary of lymphoid tissue development, occurrence of immunoglobulin-containing cells, and time of production of antibody to maternal RBC antigens in gestation is presented in Fig. 6.

DISCUSSION

This study provided results on the ontogenesis of the bovine immune response. Unlike studies on the ontogeny of the immune response reported previously (25), in which specific antigens were inoculated at various times during gestation, this study provided values from fetuses without intentional antigenic stimulation. Although the fetuses in this study were not inoculated, the results suggest that antigenic stimulation was necessary for the induction of both the functional and the morphological immunological activity observed in certain fetuses.

IgM was first detected in spleen cells of one fetus at 59 days of gestation. Spermatozoa, or components of the media in which they were suspended, may have been a possible source of

antigenic stimulus for this fetus (F-59 [2]). The spermatozoa were presumably inoculated into the uterus, between the endometrium of the uterus and the allantoic membrane, four days prior to removal of the fetus for immunological assay (26). The detection of IgM-containing cells in this fetus was about 10 days earlier than the earliest period in gestation in which a fetal lamb (age adjusted to account for difference in gestation period) was shown to produce IgM antibodies to ϕX-174 and a human fetal spleen culture produced IgM (8, 23). Silverstein has suggested that the fetal lamb will produce antibodies to ϕX-174 prior to 41 days of gestation if it is technically possible to inoculate and bleed the fetus earlier. IgG-containing cells were not observed in a bovine fetus until 145 days of gestation. This fetus, F-145, had a positive fluorescent reaction with BVD-MD antisera, and *E. coli* was also present. This gestational age is similar to the results of Thorbecke and Van Furth, who reported that human, fetal-spleen cultures first synthesized IgG at about 140 days of gestation (28). However, Gitlin and Biasucci reported that a human fetal liver and gastrointestinal-tract culture produced IgG as early as 84 days and that fetal-spleen cultures synthesized IgG at about 119 days of gestation (8). The period of time between detection of IgM- and IgG-containing cells in this study presumably resulted from the inability to examine the 59-day-old fetus at a time later than 4 days after possible antigenic stimulation. It is not suggested that a period of 85 days is required for the appearance of IgG after IgM is first detectable. Morphologically the cell types producing immunoglobulins were heterogenous and included lymphocyte-like cells of all sizes, blast cells, and plasma cells. These results are in agreement with those of Cunningham (5), numerous morphological cell types were observed to produce antibody.

Previously reported results for immunoelectrophoresis and radial immunodiffusion of bovine fetal serum samples indicated that IgM was not detectable in the serum until 130 days

TABLE 3. *Fetal tissues assayed for immunoglobulin (Ig)-containing cells*

Tissue	Fetuses assayed (no.)	Percentage positive[a]
Spleen	90	31
Lymph nodes	85	29
Tonsil	8	25
Thymus[b]	95	14
Bone marrow	80	8
Liver	95	1
Hemal lymph nodes	4	0
Blood (buffy coat)	5	0
Peyer patches	1	0
Heart	4	0
Lung	6	0

[a] Approximate percentage of those tissues checked which had immunoglobulin-containing cells.

[b] Cervical and thoracic thymus.

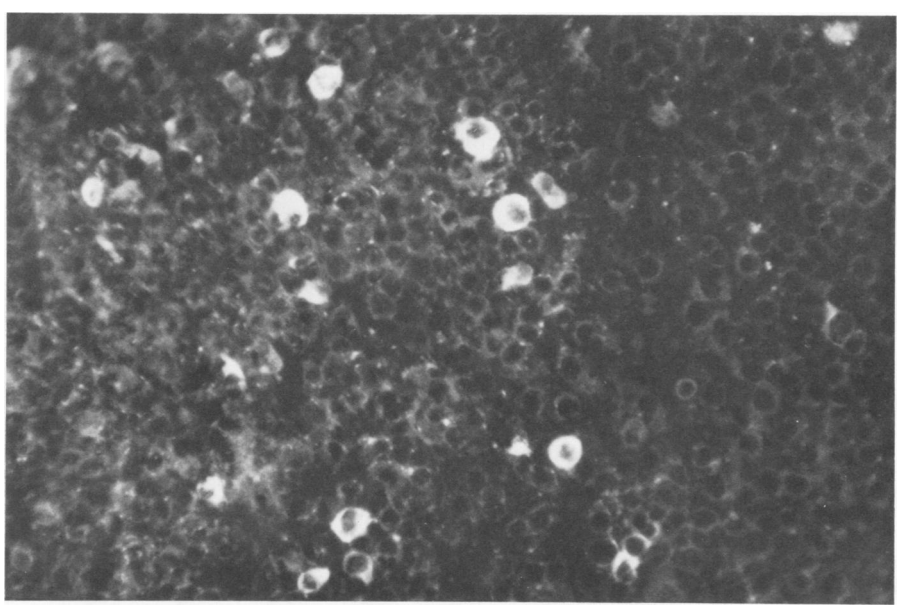

FIG. 4. *Immunoglobulin M-containing cells detected by the immunofluorescence test in the spleen of a 145-day-old fetus, ×500.*

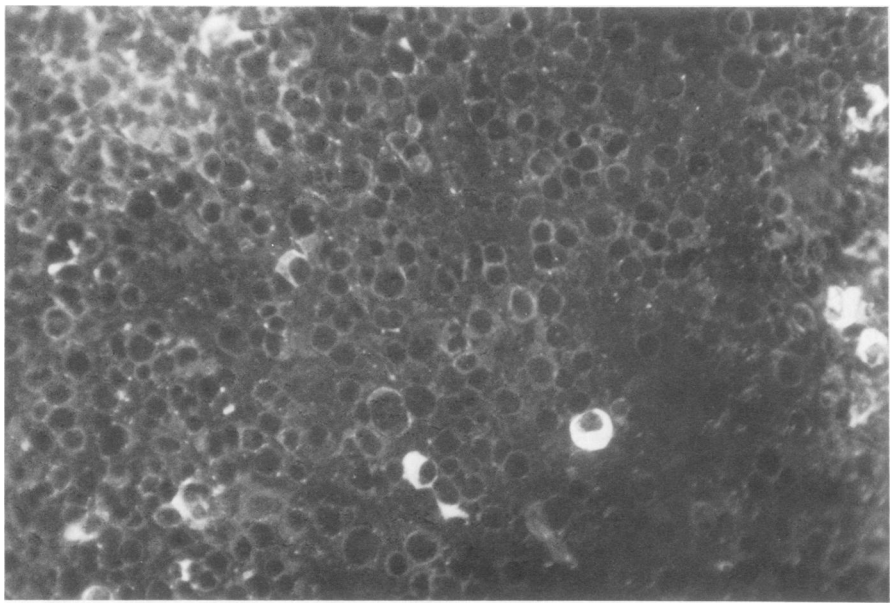

FIG. 5. *A typical immunoglobulin G-containing plasma cell detected by the immunofluorescence test in spleen of a 145-day-old fetus, ×500.*

TABLE 4. *Virological and bacteriological assay of fetuses*

Virological				Bacteriological	
Fetus	FA[a]	Isolation[b]	Neutral[c]	Bacteria isolated	Source of isolate[d]
F-145	BVD	Neg	Neg	*Escherichia coli*	Lung, liv spl
F-165	IBR	Neg	Neg	Neg	Neg
F-175	IBR	Neg	1:2	Neg	Neg
F-175 [2]	Neg	Neg	Neg	*Mima polymorpha* var. *oxidans*	Int, kid, lung, liv
F-180	IBR	Neg	1:2	Neg	Neg
F-235	Neg	Neg	Neg	*Lactobacillus* sp.	Kid, int
F-270	Neg	Neg	Neg	*E. coli*	All

[a] Immunofluorescence (FA) of tissue sections reacted with bovine virus diarrhea (BVD), infectious bovine rhinotracheitis (IBR), and parainfluenza 3 (PI3) antibody conjugates; Neg, negative.

[b] Isolation in primary bovine kidney cells; method of detection was cytopathogenic effect and immunofluorescence.

[c] Serum neutralization (neutral) test with IBR virus only.

[d] Abbreviations: heart, intestine (int); kidney (kid); liver (liv); lung and spleen (spl).

(F-130 [4]) of gestation, and IgG was detected at 145 days (F-145) (21). Immunoglobulins may not have been detected in the serum earlier, because fetuses younger than 130 days generally had fewer immunoglobulin-containing cells than the older fetuses; therefore, a lower serum immunoglobulin concentration would be expected. IgM was detected by radial immunodiffusion in a human fetal-serum sample at 120 days of gestation (8).

In agreement with other results, peripheral lymphoid tissues matured slowly in the normal fetus, and plasma cells were observed only infrequently (22). In fetuses stimulated by antigens and which produced immunoglobulins, the lymphoid tissues were more mature than were those in a normal fetus of the same gestational age. Plasma cells, pyroninophilic cells, and germinal centers were regularly observed in fetuses, more than 175 days of age, with immunoglobulins in their sera. The significance of germinal centers to the immune response has been reported by others (13). Maturation of lymphoid tissues during development of the human and sheep fetuses has been described and was similar to the maturation of bovine lymphoid tissue described here (22).

The development of the thymus and the

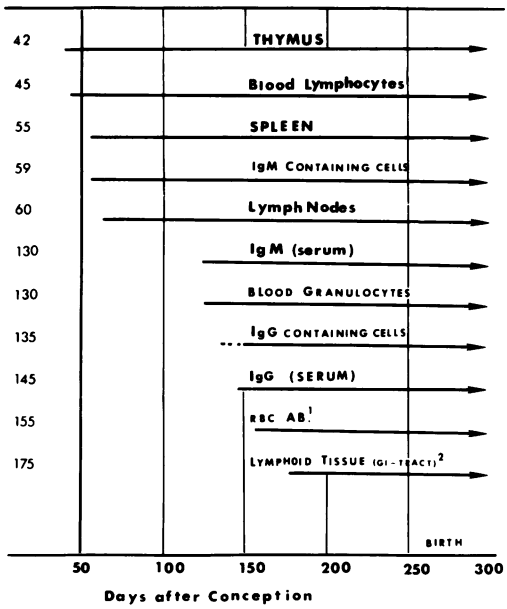

FIG. 6. *Timetable of immunological development of the bovine fetus. 1, Hemagglutination reaction of maternal red blood cells (RBC) by fetal serum. 2, Does not include the mesenteric lymph nodes.*

appearance of lymphocytes in the blood of the bovine fetus occurred at approximately the same time in bovine gestation as that of lymphocytes of the peripheral blood and the lymphoid thymus in the human fetus (15). The thymus of certain bovine fetuses contained pyroninophilic cells and typical plasma cells containing IgM and IgG, especially IgG. This observation was similar to the results reported by others for the human fetal thymus. The occurrence of IgG-containing cells was reported in the calf thymus, as was IgA production by cow thymus (1, 31). Eosinophils were sometimes observed in the fetal thymus. Although the significance of these cells is unknown, they often are present at the site of antigen-antibody reactions. Because the peak thymic index occurred in the 205 to 220 day-old-fetuses, the immunological function of the thymus might have occurred prior to birth; other authors have stated that the immunological function of the thymus in sheep and pig fetuses occurred prior to birth. Furthermore, it has been reported that thymectomy of the newborn calf has no imediate effect on the immune response (2). It is questionable, however, whether the antigens used were thymus dependent.

The spleen, structurally present at 55 days of gestation, contained primarily reticulocytes, as was reported for the fetal spleen of other species (22). However, lymphoid cells (immunoglobulin-containing cells) were present in at least one fetus by 59 days (F-59 [2]). The splenic red and white pulp was partially differentiated in some fetuses at 80 days. Immunoglobulin-containing cells were observed in the thymus and peripheral-lymph-node sections at 75 days; and IgM containing cells were present in the bone marrow at 90 days. IgM was detected in human, fetal thymus cultures at about 120 days, but comparative data were not reported for human, fetal lymph node and bone marrow cells.

Cooper et al. (4) have suggested that the gastrointestinal (GI) lymphoid tissue in rabbits is analagous to the bursa of Fabricius of chickens. Therefore, the lymphoid tissue of the GI tract was a primary lymphoid organ and developed independently of antigenic stimulation, similarly to the maturation of the lymphoid thymus. However, our microscopy and gross examinations of the GI tract, particularly of the ileum, suggested that prior to day 175 there was an absence or scarcity of lymphoid tissue in all of the fetuses with immunoglobulin-producing cells. Furthermore, development of lymphoid tissue of the GI tract in fetuses older than 175 days was minimal. Lymphoid development of the GI tract in fetuses over 250 days old, which had presumed antigenic stimulation of the GI tract, was similar to that of the newborn calf. The mesenteric lymph nodes developed more slowly than the peripheral lymph nodes and remained highly congested until after birth. Therefore, in the bovine fetus the lymphoid tissue of the GI tract probably is not a primary lymphoid tissue, for development was directly related to antigenic stimulation. Kruml et al. (16) reported that in germ-free piglets the lymphoid tissue of the GI tract developed only after antigenic stimulation and was not primary lymphoid tissue (16). Silverstein and Pendergast (24) reported recently that surgical removal of the intestinal tract in utero did not influence the development of the humoral antibody response, and they concluded that the gut-associated lymphoid tissue was not central, but developed only after appropriate stimulus in fetal lambs. The functional significance of the lymphoid tissue of the GI tract of the calf was recently demonstrated to be a principal source of IgA- and IgM-producing cells (19).

The results of studies on the development of immunoglobulins and lymphoid organs in the human fetus reported by a number of investigators (8, 15, 25, 28, 30) were strikingly similar to our results for the bovine fetus. These similarities occurred even though different techniques were used to obtain the results, results which

suggest that the bovine fetus may serve as an animal model for further studies on the development of the immune mechanism.

Results of numerous studies on congenital infections suggested that certain bovine fetuses could contain viral antigens or bacteria. The three viruses, assayed by immunofluorescence, BVD, IBR, and PI3, are three of the most commonly recognized bovine viruses and are widespread in cattle populations. Furthermore, these viruses are believed to cause congenital anomalies or fetal death, or both, in cattle. Although some fetal tissue had viral antigens and IBR-infected fetuses produced low titers of detectable antibody, virus was not isolated in FBK or NBK cells. Kahrs et al. (14) reported that a number of fetal sera from IBR- and BVD-infected dams contained antibody to the respective viruses. Dunne and co-workers have reported that fetal fluids from aborted fetuses had neutralizing antibody to several serotypes of bovine enteroviruses and had hemagglutination inhibition activity for PI-3 virus (manuscript submitted for publication).

The bacteria isolated from fetuses were not considered pathogenic to the adult bovine, and the *E. coli* isolated were not serogroups believed to be pathogenic for the bovine neonate (unpublished data). Therefore, bacterial infection resulted in immunoglobulin formation with no apparent pathological affects. The bacteria isolated from the fetuses are inhabitants of the bovine vagina and other organs of cattle.

Eight of 20 sera assayed for antibody to maternal RBC were positive. The mechanism by which the bovine fetus was exposed to maternal RBC antigens or cross-reacting antigens was not known, nor was it known if this exposure was a normal or a pathological condition. The mechanical extrusion of the corpus luteum as a cause of maternal RBC stimulation has not been excluded. It is known that sheep, which like cows, have an epithelio-chorial cotyledonary-type placenta, maternal hemorrhage at the placentome is a normal occurrence from mid-gestation to parturition. However, it is not known if this type of hemorrhage is a normal occurrence in the *Bovidae*. The production of fetal antibody to maternal RBC would not present a pathological problem for the fetus or dam, unlike the problems that result from the production of maternal antibodies to fetal RBC (18). Antibodies in the bovine fetal sera to the RBC of numerous species have been recently reported (17).

Antigenic stimulation, by viral, bacterial, or maternal antigens, was detected in 15% of the fetuses assayed. Approximately 37% (39 fetuses) had immunoglobulin-containing cells or immunoglobulin, or both, in their sera. These results lend support to the concept that immunoglobulin synthesis detected by the techniques used in this study may have resulted from antigenic stimulation.

The detection of immunoglobulins by radial immunodiffusion and specific antibody activity detectable by numerous serological techniques in the serum of fetuses, stimulated by microbial antigens, would suggest that these procedures could be used routinely to diagnose in utero infection in bovine fetuses. Studies of an immunological diagnostic test for detection of in utero antigenic stimulation are in progress.

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