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Local and Systemic Immune Responses following Oral Immunization of Foetal Lambs

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Summary. Foetal lambs were immunized orally 6–15 days before birth by introducing horse spleen ferritin into the amniotic fluid. Immunized and non-immunized lambs were killed at birth, usually before they had suckled, blood and intestinal contents were collected and single cell suspensions were prepared from spleen, mesenteric lymph nodes and jejunum.

Specific antibody was detected in serum and intestinal contents of all immunized lambs which had not suckled. Specific antibody was usually not detected in samples from non-immunized lambs. In immunized lambs antibody activity in serum was associated with IgM and in intestinal contents with IgA and IgM. In agreement with these findings, the levels of IgM and IgA in serum and intestinal contents of immunized lambs were relatively high. Generally, immunoglobulins were not detected in samples from non-immunized lambs.

Relatively high proportions of cells secreting specific antibody were present in the tissues of immunized but not non-immunized lambs. In the spleen most of the cells were secreting IgM antibody, in mesenteric lymph nodes IgM cells predominated and small numbers of IgA cells were detected, and in the jejunum approximately equal numbers of IgA and IgM cells were secreting specific antibody.

INTRODUCTION

Although enteric disease is a frequent cause of death in neonatal domestic animals, procedures for the control of enteric infections rely almost solely on antibiotic therapy and improved husbandry practices. Attempts to immunize the dam and thus establish passive immunity against enteropathogens in the neonate via colostral antibody have generally been unsuccessful (Stevens and Blackburn, 1967; Wilson, 1972a, b; Schipper and Kelling, 1974) and similarly most attempts to actively immunize newborn animals have met with little success because of the characteristic non-reactivity of the neonate to antigenic stimulation (Silverstein, Uhr, Kraner and Lukes, 1963; Ingram and Smith, 1965; Cole and Morris, 1971). A different approach was used by Porter and his colleagues, who reported a reduction in post-weaning enteritis and increased weight gains in newborn piglets after repeated oral immunization with *Escherichia coli* antigens incorporated in

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creep feeds (Porter, Kenworthy, Holme and Horsfield, 1973; Porter, Kenworthy and Allen, 1974).

Despite the unresponsiveness associated with the neonatal period, good responses to a wide range of systemically administered antigens have been reported in foetal animals (Silverstein *et al.*, 1963; Richardson, Beck and Clark, 1968; Richardson, Conner, Beck and Clark, 1971). Thus, the possibility has arisen of oral immunization of the foetus as a means of establishing immunity to enteropathogens encountered after birth. Gay (1971) reported protection against colibacillosis in a calf orally vaccinated *in utero* and Richardson and Conner (1972) demonstrated the appearance of primary and secondary antibody responses in serum to *Brucella abortus* administered orally to foetal lambs. The first serious attempt to protect against neonatal enteritis by prenatal oral immunization was made by Conner, Richardson and Carter (1973) who demonstrated that immunization of foetal lambs and calves with *E. coli* antigens protected them against a lethal dose of challenge organisms at birth. However, this work can be criticized on the basis that the lambs were permitted to suck their mothers after challenge and consequently the protective effect of maternal antibody in colostrum may have confounded the results although the authors considered that this effect would have been minimal. In addition there was no investigation of the mechanisms of immunity other than to observe that protection appeared to be independent of serum antibody.

The present experiments were designed to extend the work of Conner *et al.* (1973) by investigating the immune responses of unsuckled newborn lambs following prenatal oral immunization with horse spleen ferritin. Measurements have been made of the numbers and distribution of immunoglobulin-containing cells as well as cells containing specific antibody in tissues from gut, mesenteric lymph nodes and spleen. In addition levels of each of the classes of immunoglobulin and specific antibody in intestinal contents and peripheral blood have been determined.

MATERIALS AND METHODS

Immunization procedure

Laparotomies were performed on seven cross-bred ewes 6–15 days before lambing and 500 mg of twice crystallized horse spleen ferritin (Nutritional Biochemical Corporation, Cleveland, Ohio), in a volume of 5 ml of 0·9 per cent saline, injected through the uterus into the amniotic fluid. When multiple concepti were present (ewes 13 and 25) antigen was introduced into the fluid of only one foetus which was not identified. In sheep the concepti develop in separate foetal membranes therefore only one foetus would have been immunized.

Sample collection

Lambs were taken from ewes at birth before they had suckled (with the exception of lambs 216 and 260) and a serum sample taken from each lamb and ewe. The lambs were then killed and the small intestine removed. Intestinal contents were washed out using phosphate-buffered 0·9 per cent saline, pH 7·2 (PBS) and heated at 56° for 30 minutes. The final dilution of intestinal contents was approximately 10-fold. Mesenteric lymph nodes, spleen and jejunum were collected into Tris-buffered (pH 7·2) Hanks's balanced salt solution containing 1 per cent gelatine (HBSS) at 4°. Single cell suspensions prepared from these tissues were washed once in HBSS.

Antibody assay

Anti-ferritin antibody in serum and intestinal contents was measured by passive haemagglutination of tanned sheep erythrocytes sensitized with ferritin (Boyden, 1951).

Fractionation of samples

Serum and intestinal washings (2·0-ml samples) were fractionated on Sephadex G·200 (Pharmacia, Uppsala, Sweden) in a 75 × 2·7 cm column. Consecutive fractions of column effluent were pooled, concentrated by dialysis, and their antibody activity determined as described above. The presence of each class of immunoglobulin was determined by double diffusion in agar against monospecific antisera prepared as described by Watson, Brandon and Lascelles (1972).

Antibody-forming cells

Antibody-forming cells were detected using a modification of the haemolytic plaque technique described by Cunningham and Szenberg (1968). Briefly, sheep red cells were sensitized with ferritin using chromic chloride (Perucca, Faulk and Fudenberg, 1969) and 0·1 ml of a 5 per cent (v/v) suspension of these cells was mixed with 0·02 ml of a suspension of washed lymphocytes (5×10^6 cells/ml) prepared from either spleen, mesenteric lymph nodes or the jejunum. For detection of direct plaques 0·05 ml of 1/10 guinea-pig serum was added and for the detection of IgG1, IgG2 and IgA plaques, 0·02 ml of rabbit antiserum monospecific for ovine IgG1, IgG2 or IgA was also added. Coverslips were fixed to microscope slides using double-sided tape and the cell mixture allowed to run between the slide and the coverslip to form a monolayer. After sealing with heated paraffin wax, the slides were incubated at 37° for 30 minutes and plaques counted under a microscope at low magnification.

Immunoglobulin-containing cells

Washed lymphocytes from the various tissues examined were sedimented onto slides using a cytocentrifuge (Doré and Balfour, 1965), fixed in 95 per cent ethanol for 30 minutes and stained for 45 minutes with the appropriate fluorescein-conjugated monospecific anti-immunoglobulin serum or with fluorescein-conjugated ferritin using the conjugation procedure described by Beh and Lascelles (1974). Slides were then washed in PBS, rinsed in distilled water and mounted under a coverslip with phosphate-buffered glycerol. Cells were counted using a Reichert Zetopan microscope with dark field condensor, UG 1/1·5 mm exciter filter and GG 9·1 mm and OG 1/1·5 mm u.v. blue absorption filters and ×10 eyepieces with high power (×100) oil immersion objective. At least 200 cells were counted in each preparation.

Quantification of immunoglobulins

A modification of the single radial immunodiffusion technique of Mancini, Carbonara and Heremans (1965) was used to measure the concentrations of IgG1, IgG2, IgA and IgM (Brandon, Watson and Lascelles, 1971).

RESULTS

The titres of anti-ferritin antibody in ewe and lamb serum and lamb intestinal contents are presented in Table 1. Ewe 13 had triplets, only one of which had been orally immunized.

TABLE 1

ANTI-FERRITIN TITRES IN SERUM AND DILUTED INTESTINAL CONTENTS FROM IMMUNIZED AND NON-IMMUNIZED LAMBS AND IN SERUM FROM RESPECTIVE EWES

	Ewe and lamb number	Time of immunization (days before birth)	Antibody titre		
			Lamb		Ewe serum
			Serum	Intestinal contents*	
Immunized	13 (1)	6	128	32	32
	281	14	2	16	16
	313	15	2	4	32
	25 (2)	6	0	16	4
	156	14	16	64	8
	216	12	32	n.a.	64
	260	12	16	16	64
Non-immunized	296	—	4	8	0
	25 (1)	—	0	0	4
	89	—	0	0	n.a.
	152	—	8	0	n.a.
	13 (2)	—	0	n.a.	32
	13 (3)	—	0	n.a.	32

n.a. = Not assayed.

* Intestinal contents diluted approximately 10-fold.

It was necessary therefore to measure the antibody titres in the sera of all three lambs before suckling had occurred to determine which one to kill for further examination. One of the lambs, 13(1), had a titre of 128 whereas the other two, 13(2) and 13(3), had no detectable antibody. Ewe 25 delivered twins, only one of which had been orally immunized, but both were killed. These lambs provided useful controls in addition to the other three single lambs which were not immunized (89, 152 and 296).

Antibody was detected in serum and intestinal contents of all immunized lambs and when the dilution rate of the intestinal contents is taken into account it is apparent that larger amounts of specific antibody were present in intestinal contents than in respective serum samples. Only two control lambs (152 and 296) had any detectable antibody. It is also of interest to note that antibody was present in the serum of all ewes whose lambs received antigen *in utero*. Thus the finding that lambs 216 and 260 received colostrum suggests that the titres recorded in their serum and intestinal contents would not represent only endogenous antibody. However, it is considered unlikely that maternal antibody, derived from colostrum, would have affected the number of distribution of cells secreting specific antibody or immunoglobulin-containing cells in the short time before the lambs were killed.

Fractionation of samples of sera and intestinal contents from the lambs which had not suckled revealed that all the antibody was associated with IgM or IgA, whereas fractionation of samples from the lambs which had suckled revealed an additional peak of antibody activity associated with IgG. The latter presumably represented passive colostral antibody. Antibody in the serum of each ewe was found to be associated entirely with IgG. A set of fractionation profiles (ewe 281 serum and lamb 281 serum and intestinal contents) is presented in Fig. 1.

With respect to cells secreting specific antibody it is apparent from Table 2 that oral immunization before birth stimulated the appearance, in the tissues examined from

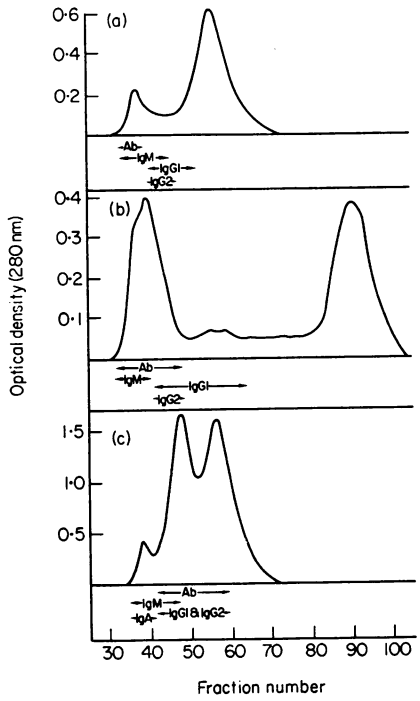

FIG. 1. Optical density profiles of Sephadex G-200 fractionations of samples from ewe and lamb 281. (a) Lamb serum. (b) Lamb intestinal contents diluted approximately 10-fold. (c) Ewe serum. Immunoglobulins and specific antibody activity associated with regions of the profiles are indicated.

immunized lambs, of significant numbers of these cells. This was particularly obvious in the jejunum where a high proportion of lymphocytes produced direct and IgA plaques and in the spleen where a high proportion of direct plaques were observed. A similar situation was observed when cells were stained with fluorescein-conjugated reagents (Table 3). Whereas in control lambs there were no cells staining with ferritin (except for small numbers in the spleen of lamb 152 and the gut of lamb 25(1)) and very few cells containing immunoglobulin, there were substantial proportions of all cell types in the tissues of immunized lambs. In the latter lambs ferritin-positive cells were well represented in all tissues, especially for lambs 13(1) and 156, and immunoglobulin-containing cells of IgA and IgM specificities generally predominated in the jejunum and spleen.

The stimulation of immunoglobulin-containing lymphocytes was reflected by the increased immunoglobulin concentrations in serum and intestinal contents from those immunized lambs which did not receive colostrum (Table 4). All classes of immunoglobulin were detected in the sera of the immunized lambs but IgM and IgA concentrations were particularly elevated and although the intestinal contents had been diluted approximately 10-fold either IgM or IgA was still detectable in each case. In contrast, the control lambs had virtually no immunoglobulins detectable in serum or intestinal contents and indeed the non-immunized lambs from ewes with multiple concepti (13 and 25) also had no detectable serum immunoglobulin. It should be noted however, that

TABLE 2

PLAQUE-FORMING CELLS (EXPRESSED AS PERCENTAGE OF TOTAL LYMPHOID CELLS) IN SINGLE CELL SUSPENSIONS PREPARED FROM SPLEEN, MESENTERIC LYMPH NODES AND JEJUNUM OF IMMUNIZED AND NON-IMMUNIZED LAMBS

Lymphoid cells from:	Type of plaque	Lamb number: Immunized 13 (1)	281	313	25 (2)	156	216*	260*	Mean	Non-immunized 296	25(1)	89	152	Mean
Spleen	Direct	11·5	n.a.	4·7	2·5	3·0	11·5	8·5	7·0	2·3	1·7	0·5	1·5	1·5
	IgG1	n.a.	n.a.	3·5	4·0	2·7	11·5	8·5	6·0	1·7	2·0	1·5	0·5	1·4
	IgG2	n.a.	n.a.	2·5	3·7	2·0	12·0	6·5	5·3	1·7	1·3	0·5	0·5	1·0
	IgA	n.a.	n.a.	2·5	3·3	4·7	11·0	9·0	6·1	1·0	2·3	1·5	2·0	1·7
Mesenteric lymph nodes	Direct	6·0	n.a.	3·6	3·5	4·0	4·5	2·0	3·9	1·0	1·3	1·5	0·5	1·1
	IgG1	n.a.	n.a.	6·0	5·0	2·7	4·0	2·5	4·0	0·5	1·0	4·0	1·5	1·8
	IgG2	n.a.	n.a.	2·7	2·5	3·5	3·5	4·5	3·3	1·0	0·0	1·5	2·0	1·1
	IgA	n.a.	n.a.	4·9	2·7	5·8	9·5	5·0	5·6	1·4	0·0	0·5	1·5	0·9
Jejunum	Direct	n.a.	n.a.	7·5	3·0	3·8	10·5	13·0	7·6	3·0	1·0	0·0	1·0	1·3
	IgG1	n.a.	n.a.	14·3	3·0	3·0	11·5	9·0	8·2	0·5	1·5	n.a.	1·0	0·8
	IgG2	n.a.	n.a.	9·0	2·5	6·0	13·0	8·0	7·7	1·6	2·0	2·0	0·0	1·4
	IgA	n.a.	n.a.	16·5	4·7	12·0	25·0	21·0	15·8	1·0	1·0	3·0	0·0	1·3

n.a. = Not assayed.

* Lambs had suckled.

TABLE 3

CELLS STAINING WITH FLUORESCEIN-LABELLED FERRITIN AND MONOSPECIFIC ANTISERA IN SMEARS OF CELLS FROM SPLEEN, MESENTERIC LYMPH NODES AND JEJUNUM OF IMMUNIZED AND NON-IMMUNIZED LAMBS (RESULTS ARE EXPRESSED AS PERCENTAGE OF TOTAL LYMPHOID CELLS)

Lymphoid cells from:	FITC conjugated to:	Lamb number												
		Immunized								Non-immunized				
		13(1)	281	313	25(2)	156	216*	260*	Mean	296	25(1)	89	152	Mean
Spleen	Ferritin	12·0	7·1	4·4	7·6	25·1	3·3	7·5	9·6	0·0	0·0	0·0	0·7	0·2
	Anti-γ1	n.a.	16·0	1·5	2·8	2·0	6·4	3·8	5·4	0·5	0·0	0·0	1·1	0·4
	Anti-γ2	n.a.	4·8	0·5	2·0	3·2	1·2	3·8	2·6	0·7	0·0	0·0	0·0	0·2
	Anti-μ	n.a.	19·8	6·3	8·6	13·0	7·2	5·0	10·0	0·8	1·0	2·3	0·0	1·0
	Anti-α	n.a.	7·0	16·5	1·0	7·7	14·5	2·0	8·1	0·0	0·0	0·8	1·0	0·5
Mesenteric lymph nodes	Ferritin	20·0	7·8	1·7	4·5	13·9	2·5	8·5	8·4	0·0	0·0	0·0	0·0	0·0
	Anti-γ1	n.a.	7·0	6·5	3·7	3·1	4·0	7·0	5·2	0·3	0·5	1·3	0·3	0·6
	Anti-γ2	n.a.	8·1	3·9	4·0	4·8	1·5	4·7	4·5	0·5	0·0	0·0	0·3	0·2
	Anti-μ	n.a.	4·0	4·8	4·2	10·2	4·2	1·5	4·8	0·8	1·5	0·4	1·0	0·9
	Anti-α	n.a.	4·0	10·5	1·5	4·0	4·3	7·9	5·3	0·0	0·3	0·0	0·0	0·1
Jejunum	Ferritin	38·0	7·3	3·4	5·6	15·7	3·8	9·5	11·9	0·0	0·5	0·0	0·0	0·1
	Anti-γ1	n.a.	4·4	4·9	5·6	1·6	4·3	5·0	4·3	1·0	1·3	0·3	0·3	0·7
	Anti-γ2	n.a.	2·6	0·8	3·6	0·7	1·6	1·1	1·7	0·0	0·0	0·0	0·0	0·0
	Anti-μ	n.a.	3·0	6·3	9·2	10·1	7·1	3·9	6·6	0·5	1·2	1·0	0·0	0·7
	Anti-α	n.a.	3·1	9·5	5·7	6·9	8·1	10·4	7·3	0·0	0·0	0·3	0·7	0·3

n.a. = Not assayed.

* Lambs had suckled.

TABLE 4

CONCENTRATIONS OF IMMUNOGLOBULINS (mg/ml) IN SERUM AND DILUTED INTESTINAL CONTENTS FROM IMMUNIZED AND NON-IMMUNIZED LAMBS WHICH HAD NOT SUCKLED

	Lamb number	Sample*	Immunoglobulin concentration (mg/ml) IgG1	IgG2	IgM	IgA
Immunized	13 (1)	Serum	0·060	0·128	0·192	0·230
		IC	0·008	0·000	0·051	0·000
	281	Serum	0·075	0·036	0·458	0·256
		IC	0·000	0·069	0·000	0·150
	313	Serum	0·052	0·048	0·326	0·278
		IC	0·000	0·000	0·000	0·059
	25 (2)	Serum	0·250	0·232	0·185	0·000
		IC	0·109	0·082	0·002	0·054
	156	Serum	0·265	0·214	0·633	0·000
		IC	0·037	0·043	0·357	0·126
Non-immunized	296	Serum	0·003	0·001	0·000	0·000
		IC	0·000	0·000	0·000	0·000
	25 (1)	Serum	0·018	0·014	0·004	0·000
		IC	0·000	0·000	0·000	0·000
	89	Serum	0·003	0·000	0·000	0·000
		IC	0·000	0·000	0·000	0·000
	152	Serum	0·001	0·000	0·000	0·000
		IC	0·000	0·000	0·000	0·000
	13 (2)	Serum	0·000	0·000	0·000	0·000
	13 (3)	Serum	0·000	0·000	0·000	0·000

* IC = intestinal contents diluted approximately 10-fold.

the total immunoglobulin concentrations in samples from each lamb did not correlate with their respective anti-ferritin titres. For example, lamb 13(1) which exhibited the highest antibody titres did not have the highest immunoglobulin concentrations.

DISCUSSION

While initial studies have demonstrated the ability of foetal animals to respond to oral immunization (Richardson and Conner, 1972; Conner *et al.*, 1973) the details of this response and the relative importance of the various immune mechanisms were incomplete. In this paper the response to oral immunization of the foetal lamb has been evaluated according to several criteria, and it was shown that it is possible to elicit a specific local immune response. The foetal lamb is a convenient experimental model because of the failure of maternal antibody to cross the ovine placenta.

The finding that titres of antibody in intestinal contents were generally higher than those in serum, after accounting for the dilution (Table 1), suggets that most of the antibody in intestinal contents was formed locally. Much of the antibody appearing in serum probably originated from the mesenteric region, although a systemic response to antigen reaching the circulation via the intestine cannot be discounted (compare Rothberg, Kraft and Farr, 1967). The combined evidence that antibody activity in lamb serum and intestinal washings was associated with IgM or IgA (as revealed by Sephadex G-200 fractionation), that there were generally more antibody-forming cells in the jejunum than

in the other tissues studied (Table 2) and that IgM and IgA concentrations were particularly increased in serum and intestinal contents (Table 4) provides further support for the conclusion that the response was predominantly local. Similarly, stimulation of a mucosal surface typically gives rise to an IgA response and there is evidence that IgM-producing cells are the precursors of those producing IgA (Cebra, 1969; Lawton, Asofsky, Hylton and Cooper, 1972). Furthermore Porter, Noakes and Allen (1972) and Porter, Kenworthy, Noakes and Allen (1974) have reported the early appearance of IgM in antibody responses of the secretory immune systems of neonatal pigs and calves.

It could be argued that the increased immunoglobulin concentrations and antibody titres observed in the unsuckled, immunized lambs could have arisen from leakage of small amounts of maternal antibody into the foetal circulation at or before birth. Had this occurred it would be expected that antibody in the lambs would have been IgG rather than IgM or IgA. Moreover, the concentrations of IgG would have been elevated in serum rather than those of IgM or IgA. The appearance of IgG-associated specific antibody in serum of the ewes, presumably resulting from small amounts of antigen reaching the maternal circulation at the site of injections, and its inevitable appearance in colostrum, (compare Brandon *et al.*, 1971), indicates the necessity of carrying out studies in colostrum-deprived animals.

The high proportion of cells staining with fluorescein-conjugated ferritin (Table 3) is a further indication of the specificity of the response produced. It is of interest to note that the high proportion of ferritin-positive cells in the tissues of lambs 13(1) and 156 correlated with the high antibody titres in samples from these lambs. Conversely the small number of ferritin-positive cells in the spleen of the control lamb 152 and the gut of control lamb 296 presumably would account for the low anti-ferritin titres observed in their sera (Table 1). However, there were more immunoglobulin-containing cells observed than could be accounted for by the number of cells positive for ferritin. This may be explained by stimulation of lymphocyte development by the inadvertent introduction of antigens other than ferritin during immunization or by infection of the foetus with virus of material origin which may have crossed the placenta. Either of these occurrences would account for the finding that the immunoglobulin concentrations for lambs did not correlate with their respective anti-ferritin antibody titres. Alternatively it is possible that a proportion of cells staining with anti-immunoglobulin reagents were precursors of cells producing ferritin antibody and it was only mature plasma cells which stained ferritin-positive (compare Miller, Ternynck and Avrameas, 1975).

Nevertheless, the data presented in this paper indicate the possibility of oral immunization of foetal animals as a means of stimulating specific local immunity in the intestine before birth. Future experiments will determine whether this procedure is in fact effective in establishing protection against enteropathogens encountered at birth, as suggested by the work of Conner *et al.* (1973), or whether it is simply a means of overcoming the characteristic neonatal unresponsiveness by priming for a protective response induced by subsequent postnatal exposure.

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