Immunology, 1973, **24**, 493.

The Relative Distribution of IgM and IgA Cells in Intestinal Mucosa and Lymphoid Tissues of the Young Unweaned Pig and their Significance in Ontogenesis of Secretory Immunity

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(*Received* 15*th July* 1972; *accepted for publication* 8*th August* 1972)

Summary. A comparative study of cells synthesizing immunoglobulins IgA and IgM in intestinal mucosa and various lymphoid tissues of unweaned piglets has been made by immunofluorescence.

The lamina propria of the small intestine contained as many cells synthesizing IgM as those producing IgA. In all other lymphoid organs examined, including Peyer's patches, the number of IgM cells was significantly higher.

The largest population of intestinal immunocytes occurred in the lamina propria of the duodenum where the counts were ten times greater than in the jejunum or ileum.

The relevance of these findings to the earliest stages of development of secretory immunity in the young pig are considered.

INTRODUCTION

The intestinal secretory immune system has been a focus of attention in numerous species. However, there is little or no information in the literature concerning its development as a potential defence mechanism in the young mammal. Although considerable emphasis has been devoted to the role of IgA because of its relative abundance in alimentary tract secretions, other immunoglobulins which can be also demonstrated in the lymphoid cells and secretions of the intestine have been comparatively neglected.

An intestinal-secretory IgA system, analogous to that in the human has been described in the pig (Porter and Allen, 1970; Porter, Noakes and Allen, 1970) and immunofluorescent studies of intestinal tissues from young suckling pigs have indicated that secretion of IgA commences earlier than the fifteenth day of age. In the first week of neonatal life, maternal antibodies in the colostrum and milk provide the only protection. Thereafter, active local synthesis in the alimentary tract becomes increasingly significant.

The presence of immunoglobulin IgM, in addition to IgA, in the apical cytoplasm of intestinal crypt epithelial cells of young pigs was reported in an earlier communication, when it was suggested that these two immunoglobulins may have complementary roles as intestinal antibodies (Allen and Porter, 1970).

The intestinal secretory system must assume paramount importance in the immune

defence of the pig during the immediate post-weaning period when the animal is most susceptible to *Escherichia coli*-associated enteric syndromes. Thus we felt that is was necessary to establish the nature of the immunoglobulin response in the intestine of the young pig before normal early weaning.

The present investigations provide information on the distribution of cells containing and presumably synthesizing immunoglobulins IgA and IgM in various lymphoid tissues of unweaned pigs. The relatively high number of cells containing IgM in intestinal mucosa suggests that this immunoglobulin is also of particular importance in early local protection of the alimentary tract.

MATERIALS AND METHODS

Isolation of specific immunoglobulins and conjugation of specific antisera with fluorescent dyes

The techniques for the isolation of specific immunoglobulins and preparation of antisera conjugated with fluorescein isothiocynate (FITC) have been described previously (Allen and Porter, 1970). Conjugation of rabbit antisera with rhodamine isothiocynate (Baltimore Biological Laboratory Ltd) was achieved by a similar method to that used for FITC

Preparation of tissues

Tissues were obtained at post-mortem from nine unweaned 4-week-old piglets and fixed immediately in chilled absolute ethanol. Paraffin blocks were prepared according to the method of Saint Marie (1962). The following tissues were examined: duodenum, jejunum, ileum, anterior Peyer's patch, i.e. the first macroscopically recognizable patch after the pyloric junction, posterior Peyer's patch, anterior mesenteric node, posterior mesenteric node, intercostal node, spleen and thymus. The anterior mesenteric node was taken from the chain of mesenteric lymph nodes serving the duodenum; the posterior node was taken from the ileocaecal junction.

Staining procedure

Following the removal of wax with chilled xylol and rehydration in chilled ethanol the sections were rinsed in cold phosphate buffered saline (PBS) pH 7·1 and stained overnight at 4° in a humidity chamber to prevent drying. After staining they were washed three times (20 minutes each wash) in PBS with continuous gentle agitation, dried in air and stored unmounted in the dark at 4° until required. They were mounted in buffered glycerin pH 8·5 immediately prior to microscopical examination.

Controls

The tests used to control the specificity of the reactions have been described previously (Allen and Porter, 1970).

Microscopy

The stained preparations were examined by dark ground microscopy on a Reichert Zetopan microscope using an HBO 200 light source with a UG 1 exciter filter and either a GG 13 plus a Wratten 2 B or a GG 9 barrier filter.

This latter filter is particularly useful for counteracting the intense blue autofluorescence

in sections prepared by the Saint Marie technique. It is also an ideal filter for observing specimens double stained with rhodamine and fluorescein (Johnson, 1970).

Counts were made of stained cells present in twenty fields selected at random using a ×40 0·65 NA objective and a ×8 eyepiece.

Colour transparencies were taken on high speed daylight Kodak Ektachrome (160 A.S.A.); black and white photographs, on Ilford FP4.

After examination selected sections were restained with haemotoxylin and eosin and the same fields rephotographed to identify the cells in which the immunoglobulins had been located.

RESULTS

ALIMENTARY TRACT

Cells reacting with fluorescein conjugated rabbit anti-porcine IgA sera were found in the lamina propria of the small intestine at all three levels examined. They were located, almost without exception, in the tissues surrounding the crypts (Fig. 1.) but only very occasionally was a cell containing IgA seen in the core of a villus.

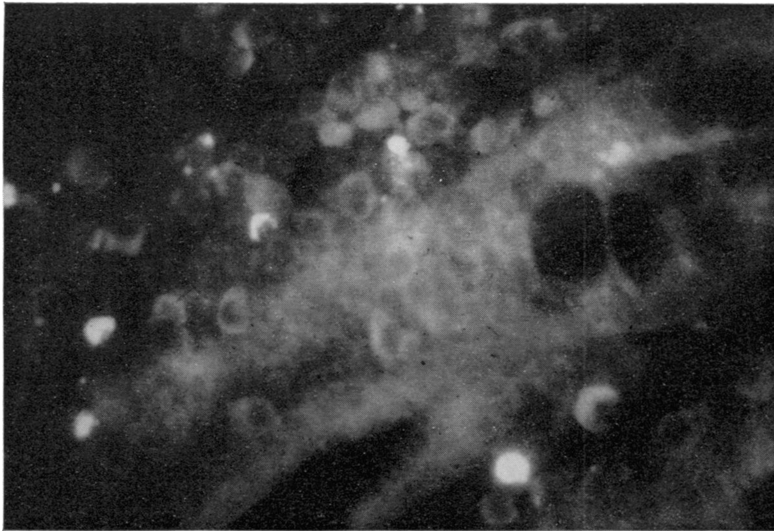

FIG. 1. Pig duodenum stained with fluorescein conjugated antiserum to IgA showing stained cells, close to the crypt epithelium. (×960.)

IgM containing cells were generally similar in distribution to IgA cells, (Fig. 2.) being mainly located in the lamina propria surrounding the crypts. However, they also occurred in small numbers in the cores of some villi.

Both IgA and IgM immunoglobulins were demonstrated in the apical cytoplasm of crypt epithelial cells.

Comparative counts of the two types of cell revealed that in the small intestine of most animals, the number of cells containing IgM exceeded those containing IgA (Table 1). In only one animal (P.M.508) was the number of IgA cells greater than IgM cells at all three levels.

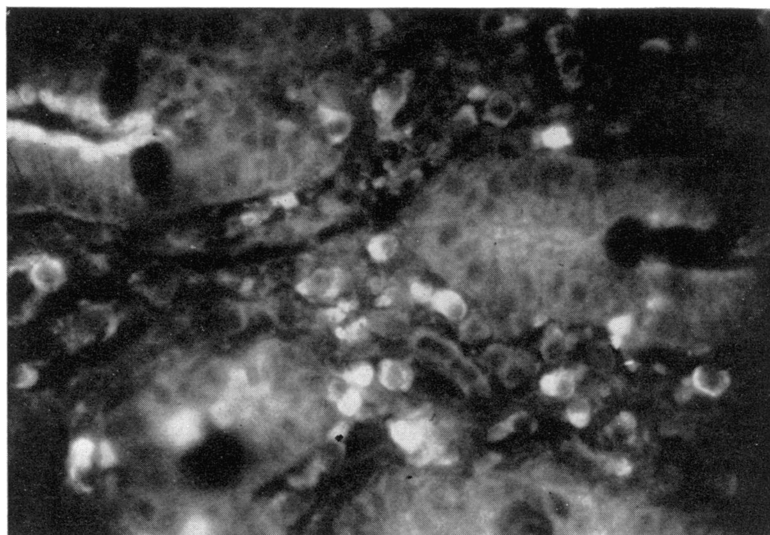

Fig. 2. Pig duodenum stained with fluorescein conjugated antiserum to IgM showing stained cells in the lamina propria surrounding the lower crypts. (×960.)

In all animals the largest populations of immunoglobulin containing cells were found in the duodenum, counts being about ten times greater than in jejunal tissue. The smallest number of cells occurred in ileum where populations were about half those in jejunum. The relative distribution of IgA and IgM containing cells throughout the gut was similar.

Table 1

Number of cells synthesizing IgA or IgM in lamina propria of small intestine of 4-week-old suckling pigs

Organ	Ig	Piglet No 507	508	509	510	511	512	513	514	515
Duodenum	A	516	1250	1458	919	1241	1528	1060	639	741
	M	1710	1053	1769	1335	2078	2010	1065	1077	1780
Jejunum	A	197	151	348	60	44	90	105	170	136
	M	217	56	132	208	182	265	276	231	301
Ileum	A	80	140	99	132	32	117	22	114	87
	M	96	51	82	86	99	145	91	240	99

Counts expressed as number of cells/twenty fields (×40 objective).

Sections of intestinal tissue stained sequentially with fluorescein-conjugated rabbit anti-IgM followed by rhodamine-conjugated rabbit anti-IgA demonstrated that IgA and IgM containing cells comprised two separate populations in the main. Counts of each cell type made on dual stained tissue gave results similar to those obtained from sections treated solely with one or other fluorescein-labelled antiglobulin. Additionally there were a few cells which appeared to be dual stained. Their fluorescence was yellow in colour rather than the green or orange of the majority of cells. A similar yellow fluorescence was observed in many crypts, clearly demonstrating that this colour was characteristic of the joint presence of both IgA and IgM.

Peyer's patches

Counts of both types of cells were higher in the posterior Peyer's patches than in the anterior ones. Proportionally there were also rather more IgA cells in the posterior patches. The ratio of IgM to IgA cells was 5:1 in the anterior and 4:1 in the posterior patches (Table 2).

IgM containing cells occurred in two main areas of the tissue. Many cells could be seen in the lamina propria of the papillae, frequently close to the epithelial border (Fig. 3); a second concentration was found towards the base of the patch.

Table 2

Number of cells synthesizing IgA and IgM in Peyer's patches of 4-week-old suckling pigs

Organ	Ig	Piglet No 507	508	509	510	511	512	513	514	515
Anterior Peyer's patch	A	NA	66	0	22	8	36	17	34	44
	M	128	144	160	92	94	134	218	95	229
Posterior Peyer's patch	A	99	76	67	60	20	44	27	48	10
	M	188	113	259	155	252	168	152	117	328

Counts expressed as number of cells twenty fields (× 40 objective).
NA = Not assessed.

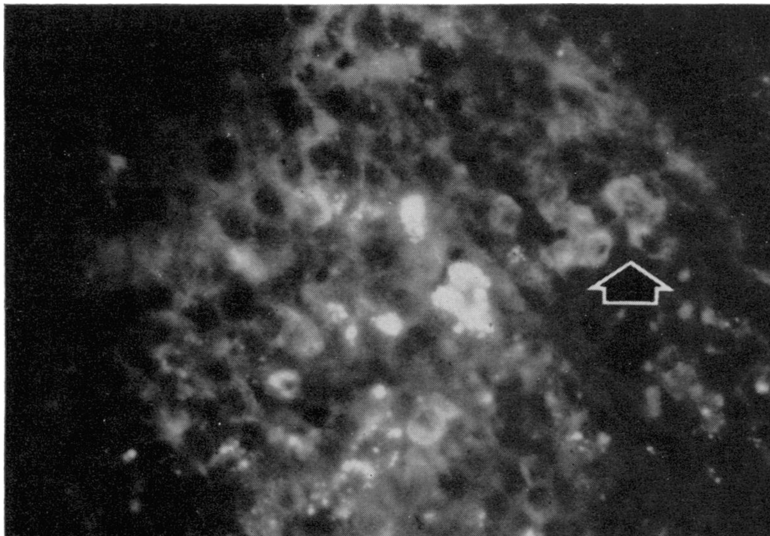

Fig. 3. Pig Peyer's patch stained with fluorescein conjugated antiserum to IgM, showing stained cells (arrowed) in the tip of papilla close to the epithelium. The bright cells are eosinophils autofluorescing. (× 960.)

IgA cells were less often found in the cores of papillae, being principally located in the deeper regions of the tissues.

Lymphoid tissues

The results of comparative counts of cells containing immunoglobulins IgM and IgA made on a variety of other lymphoid tissues are given in Table 3.

TABLE 3

NUMBER OF CELLS SYNTHESIZING IgA AND IgM IN LYMPHOID TISSUES OF 4-WEEK-OLD SUCKLING PIGS

Organ	PM No: Ig	507	508	509	510	511	512	513	514	515
		Piglet No								
Anterior Node	A	22	68	47	10	27	41	14	41	70
	M	112	79	31	97	51	111	90	155	105
Posterior Node	A	25	76	41	70	86	17	11	0	84
	M	66	93	107	120	145	36	38	32	88
Intercostal Node	A	0	0	1	0	0	1	0	0	0
	M	8	12	8	8	22	15	11	16	3
Spleen	A	7	0	2	2	3	0	13	1	0
	M	545	295	371	557	645	399	616	615	534
Thymus	A	0	11	4	7	5	0	0	0	0
	M	2	1	6	1	0	0	0	1	0

Counts expressed as number of cells/twenty fields (×40 objective).

The numbers of IgM containing cells exceeded those of IgA containing cells almost without exception.

In mesenteric nodes, cells reacting with anti-IgA reagent were dispersed throughout the cortical tissue. They were mostly large, with a well stained cytoplasm and a clearly defined nucleus, often eccentrically placed. Very occasionally small foci of larger fluorescent cells, surrounded by a cuff of small ones were seen. These were presumably centres of active immunoglobulin synthesis. Similar foci of IgM containing cells were more numerous. They also showed a network of intercellular fluorescence, probably extravascular IgM. These foci were frequently surrounded by large stained cells occurring singly. Many of these cells had large nuclei surrounded by a thin rim of fluorescing cytoplasm.

In the intercostal nodes, a few cells containing IgM were seen. Some were grouped together into small clones. These were regarded as probably being actually involved in

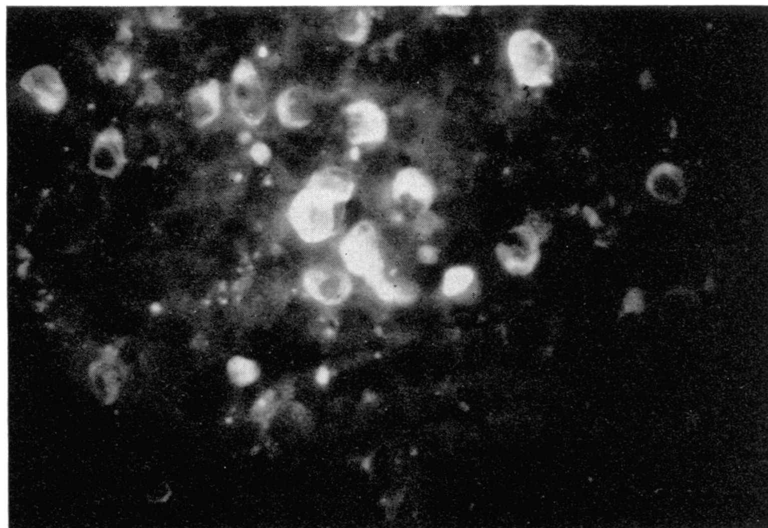

FIG. 4. Pig spleen stained with fluorescein conjugated antiserum to IgM. (×960.)

antibody synthesis. The occasional IgA containing cell was most likely a circulating cell 'in transit' rather than one actively synthesizing immunoglobulin *in situ*.

A similar interpretation was made to account for the IgA containing cells found in the spleen, all of which occurred singly and in a random manner.

On the other hand, the large numbers of IgM containing cells found in the red pulp (Fig. 4.) indicated the importance of this organ as a source of this immunoglobulin.

The few cells containing IgM or IgA found in some thymuses were not considered to be locally derived but rather entrapped circulating cells similar to those seen in spleen and intercostal nodes.

DISCUSSION

The alimentary tract comes into intimate contact with a greater variety of potential antigens than any other organ of the body. In the young pig it is probably the first organ to experience major antigenic challenge and is, therefore, of particular importance in the development of the animal's early immune defences. This is reflected in the profound changes occurring in the gastrointestinal tract soon after birth. In a newborn animal there are very few lymphoid cells in the lamina propria of the intestine and the lymphoid follicles are poorly defined. However, after birth these structures gradually develop and the lamina propria becomes infiltrated with lymphocytes and plasma cells. This infiltration and lymphoid development is almost certainly a response to bacterial challenge as no similar development is found in germ free pigs (Kenworthy, 1970).

Many of the cells infiltrating the gastric mucosa of the young pig are involved in producing immunoglobulin IgM. In fact, the numbers of IgM cells were in excess of those containing IgA at all three levels of the small intestine. These findings are at variance with those of similar studies made on other species (Vaerman and Heremans, 1969; Crabbé, Bazin, Eyssen and Heremans 1968; Crabbé, Carbonara and Heremans, 1965) in which IgA cells predominated.

A possible explanation for the discrepancy may be that the animals used in these investigations were in the earliest stages of active immunoglobulin synthesis whereas those used by other authors could be regarded as immunologically mature. In this respect it is interesting to note that the only immunoglobulin containing cells found by Vaerman and Heremans in the intestinal mucosa of a 6-day-old dog were of the IgM type, although a 3-week-old litter-mate possessed numerous IgA cells in its mucosa (Vaerman and Heremans, 1969).

Clearly, a very different immunological picture must obtain in the adult pig since Bourne, Pickup and Honour (1971) recorded only trace amounts of IgM in extracts of intestinal tissues of the sow. Thus the current findings present a very interesting problem relating to the definition of the primary immune response to antigen in the intestinal lamina.

Studies of the ontogenesis of the intestinal immune response have not yet been recorded in the literature. However, reports on serological changes of immunoglobulins in the neonate possibly substantiate the present observation that the local immune response may commence predominantly with IgM synthesis. In both the human infant (Rothberg, Kraft, Farr, Kriebel and Goldberg, 1969) and the pig (Porter and Hill, 1970) IgM appears in the serum during the first week of life whilst IgA is not detected till several days later. In the former study administration of BSA in the diet resulted in IgM antibodies in the

serum, thus providing further evidence that antigenic challenge in the alimentary tract of the neonate probably gives rise to a primary IgM response.

Papermaster (1967) postulates that the sequential synthesis of IgM followed by IgG in mouse spleen after primary antigenic stimulation is the result of a switching in synthesis. Antigen-sensitive target cells, derived originally from bone marrow stem cells, localize in the spleen and differentiate into IgM clones in response to antigenic stimulation. Subsequently daughter cells from these clones switch to produce 7S IgG. A similar system may be envisaged as operating in the local immune response of the intestinal lamina with daughter cells from the primary IgM clones switching to produce predominantly IgA. The direction of the switching mechanisms may be determined by local tissue hormones which differ between intestine and spleen. A defect in the maturation of this immune system would obviously lead to a deficiency in IgA with a predominance of IgM cells in the intestinal lamina as seen in ataxia telangiectasia (Eidelman and Davis, 1968; Stobo and Tomasi, 1967).

In studies of intestinal secretion from fistulated pigs of a similar age to those in the present studies, IgM formed only a small portion of the secreted immunoglobulins (Porter *et al.*, 1970). Possibly the large molecular size of IgM interferes with its transport across the intestinal epithelium. Certainly its size ensures that the immunoglobulin in circulation is mainly confined to the vascular system (Tate, Douglas, Braude and Wells, 1966). However, this should not detract from the effectiveness of IgM in local defence, since although it may not equate quantitatively with IgA in the external secretion, it might provide an important second line defence in the tissues behind the epithelium. This view is supported by the fact that IgM is potently effective in bacteriolysis in the presence of complement (Michael and Rosen, 1963) and affords protection against the lethal effect of endotoxins (Kim and Watson, 1965).

The majority of authors following the initial concepts of Heremans, Crabbé and Masson (1966) and Tomasi (1967) have emphasised the role of IgA in external defence mainly because of its apparent quantitative dominance. Brandtzaeg (1968) suggested that a common secretory mechanism may operate for IgA and IgM and we have previously suggested that the two immunoglobulins may have a complementary role in the defence of the intestinal mucosa (Allen and Porter, 1970). Thus in *E. coli* associated enteritis in the young weaned pig the protective role of IgM deserves serious consideration.

The major antigenic stimulus for development of IgA synthesising cells in the lamina propria appears to be the enteric flora (Crabbé, Bazin, Eyssen and Heremans, 1968). However, a consideration of the relative distribution of immunoglobulin containing cells at various levels of the small intestine raises certain difficulties in relation to this concept. The present studies and reports of other authors (Crabbé, Bazin, Eyssen and Heremans, 1968) show the occurrence of much greater numbers of immunocytes in the lamina propria of the duodenum than in the jejunum and ileum. However, it is known that in the young pig the population of enteric bacteria is much more abundant in the lower jejunum and ileum than in the duodenum (Kenworthy and Crabbé, 1963). One might, therefore, expect a different distribution of lymphoid cells to arise in the small intestinal tissues. In fact, if the cellular distribution were to correlate with the level of bacterial challenge exactly the reverse observation would be anticipated.

The Peyer's patches are more numerous in the distal small intestine than in the proximal region region and, therefore, apparently correlate with the increasing enteric flora. However, some dispute exists in the literature over the ability of Peyer's patch tissue to

respond to intraluminal antigen. Cooper and Turner (1969) suggest that antigenic stimulus of the Peyer's patches results in a continual supply of IgM cells which rapidly migrate to other lymphoid tissues. These studies were carried out by injection into the patch but in orally immunized animals Bienenstock and Dolezel (1971) failed to find any evidence of antigenic stimulation of Peyer's patches. The present studies indicate that the lymphoid cells of the Peyer's patches are predominantly of the IgM type. In view of the controversy, it will be interesting to determine the response of these tissues to the intestinal microflora and their role in furnishing antigen stimulated cells for other lymphatic tissues, in particular the proximal region of the small intestine.

ACKNOWLEDGMENT

The authors wish to express their appreciation to Mr M. E. Prior for his skilful technical assistance.

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