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The Demonstration of Immunoglobulins in Porcine Intestinal Tissue by Immunofluorescence with Observations on the Effect of Fixation

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Summary. The localization of immunoglobulins IgA, IgG and IgM in porcine intestinal tissue by immunofluorescence is described and the influence of three fixatives, ethanol, methanol and acetone on the findings compared. Methanol was found to be the only fixative to give consistent results for IgM; it was also the reagent of choice for the localization of IgA in cells of the lamina propria. However ethanol and acetone were preferred for the demonstration of secretory IgA. Extravascular IgG was shown to be still soluble after fixation and capable of being removed from the tissue. The demonstration of IgM in intestinal crypt epithelium is reported and its possible role as a secretory antibody acting in concert with IgA is discussed.

INTRODUCTION

Since Coons, Creech, Jones and Berliner (1942) showed that antibodies conjugated with fluorescent dyes could be used to locate antigenic components in tissues, the technique has been widely applied to the demonstration of immunoglobulins. The individual immunoglobulins IgA, IgG and IgM have been localized in man, laboratory animals and some domestic animals (for a collected bibliography see Nairn, 1969), but no similar study in the pig has been reported.

The influence of fixatives has received surprisingly little attention in the literature (Nairn, 1969). In studies on human tissues, fixatives used for detection of IgA, IgG and IgM were ethanol (Gelzayd, Kraft and Kirsner, 1968; Rossen, Morgan, Hsu, Butler and Rose, 1968); methanol (Carbonara, Rodhain and Heremans, 1963; Crabbé, Carbonara and Heremans, 1965); and acetone (Paronetto and Koffler, 1965; Rubin, Fauci, Sleisinger and Jeffries, 1965; Tomasi, Tan, Solomon and Prendergast, 1965).

During our investigations of natural immunity in the young pig all three fixatives were used and variations were observed in the appearance and distribution of the immunoglobulins, apparently attributable to the fixatives employed.

The present paper describes a study of the localization of IgA, IgG, and IgM in porcine intestinal tissue and of the influence of fixation on the findings.

MATERIALS AND METHODS

Chromatographic methods

Gel filtration chromatography was carried out on Sephadex G200 columns (45×2·5 cm) Sephadex G150 columns (90×2·5 cm) and Sephadex G100 columns (30×2·0 cm). Elution was carried out with 0·85 per cent NaCl in 0·1 M Tris-HCl buffer pH 7·2.

Anion exchange chromatography was carried out on diethylamino ethyl (DEAE) cellulose as described by Augustin and Hayward (1960).

Isolation of specific immunoglobulins

The isolation of specific porcine immunoglobulins and the preparation of rabbit antisera specific for IgA, IgG and IgM has been described (Porter 1969).

Conjugation of specific rabbit antisera with fluorescein isothiocyanate (FITC)

The method of preparation of the FITC conjugated reagents was based mainly on Goldstein, Slizys and Chase (1961) and Nairn (1969) and aimed to eliminate sources of variability and produces a reagent of consistent quality and specificity. The first step, chromatography of the rabbit antiserum on DEAE cellulose, isolated the rabbit 7S IgG as the necessary precipitating antibody and eliminated the rest of the serum proteins, which may conjugate with the FITC and interfere with the production of a reagent with the precise characteristics required for fluorescent microscopy.

For this purpose 20 ml of specific rabbit anti porcine immunoglobulin serum was prepared for ion exchange chromatography by preliminary gel filtration on a Sephadex G25 column (30×2 cm) equilibrated with 0·1 M sodium phosphate buffer pH 7·6. The protein eluate was applied to a column of DEAE cellulose and antibody was separated in the fall through fraction eluting with this buffer. The eluate was checked for the presence of precipitating antibody to the given immunoglobulin by immunological double diffusion in agar gel.

The antibody was concentrated by dialysis against 30 per cent polyethylene glycol in a carbonate buffer pH 9·0 consisting of $NaHCO_3$ 3·7g/100 ml Na_2CO_3 0·6 g/100ml; (Nairn 1969). The volume of antibody solution was reduced to approximately 3 ml, and 2 ml of FITC solution (50 mg/100 ml) in carbonate buffer pH 9·0 was added slowly thus giving a ratio of FITC/protein of approximately 5 mg/1 g. Conjugation was allowed to take place at 4° overnight with continuous stirring. The conjugated protein was separated from free FITC by chromatography on Sephadex G100 in 0·1 M phosphate buffer pH 7·6, and was subjected to further chromatography on DEAE cellulose. The fraction eluted with 0·15 M NaCl in 0·1 M phosphate buffer was selected as the fluorescent reagent for immunohistological detection of porcine immunoglobulins. This step eliminates unconjugated antibody which in tissue sections may reduce the sensitivity of the reagent by blocking the reactions of fluorescent antibody at receptor sites. Also antibody with a high level of binding of FITC is retained on the anion exchange cellulose under the conditions of elution and is eliminated as a cause of non specific staining due to the acidophilic characteristics of the dye (Mayersbach, 1959).

The conjugated antibody in this eluate was concentrated by use of a polycrylamide hydrogel (Lyphogel*) and adjusted to a precipitin endpoint detectable in agar gel diffusion against a normal pig serum. (The conjugated antiserum was freed of all residual particulate matter by passage through a millipore filter.)

Conjugated reagents were prepared in this way for specific identification of porcine IgG, IgA and IgM.

* Gelman Instrument Co. packed by Hawksley & Sons., Lancing, Sussex.

Preparation of tissue

Tissue obtained at post mortem (within 5 minutes of death) from pigs of 3–4 weeks of age was cut into slices 4 mm thick using a double bladed knife (W.W. Kirkby, personal communication) and immediately quenched in isopentane cooled with liquid nitrogen. The blocks of tissue were stored in liquid nitrogen until required for use. Three levels of the small intestine (duodenum, jejunum and ileum) were examined.

Replicate sections 3–5 μ thick were cut on a Bright–Pearse cryostat at $-15°$ and fixed immediately in one of the fixatives mentioned below, at room temperature.

1. Acetone —10 minutes
2. 70 per cent ethanol —30 minutes
3. 95 per cent ethanol —30 minutes
4. Methanol —20 minutes

Following fixation the sections were allowed to dry thoroughly in air before being stained with the conjugated antisera.

Staining procedure

The sections were incubated with the conjugated antisera in a moist chamber at room temperature (20–22°) for 20 minutes.

They were individually rinsed in phosphate buffered saline pH 7·1 (PBS) before being washed 3 times (5 minutes each wash) in PBS with continuous gentle agitation. Following washing, they were allowed to dry in air, rinsed in xylol and mounted in 'Uvinert' (G. T. Gurr).

Controls

The specificity of the reactions was controlled by (a) blocking with the unconjugated specific antiserum prior to incubating with the conjugated reagent, (b) absorbing the specific conjugated antiserum with the purified homologous antigen before staining, (c) treating with non-immune rabbit serum before incubating with the specific conjugated reagent, (d) staining with conjugated antisera of a specificity different from that under examination e.g. IgG and IgM antisera as controls for IgA. Tissue from newborn, colostrum deprived piglets (which are agammaglobulinaemic since there is no transplacental passage of γ-globulins in the pig) was stained with the conjugated specific antisera as a further check for non specific reactions. Additionally an unstained section was washed in PBS dried and mounted.

Further treatment of the control sections was identical to that of the test preparations.

Microscopy

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

Colour transparencies were taken on high speed daylight Kodak Ektochrome (160 ASA), black and white photographs on Ilford HP 3 (600 ASA).

After examination, the sections were restained with haematoxylin and eosin and the same fields rephotographed on Ilford FP 4 film to verify the sites at which the immunoglobulins had been located.

RESULTS

IMMUNOGLOBULIN IgA

In preparations of intestine fixed with ethanol and stained by fluorescein conjugated specific anti IgA sera, the apical cytoplasm of the epithelial cells in many of the crypts fluoresced brightly indicating the presence of IgA (Fig. 1). In the intercrypt lamina propria tissue fluorescent cells were seen only occasionally, and appeared less bright than in the crypt reaction. No fluorescent cells were found in the cores of the villi nor was any intercellular staining found in the lamina propria.

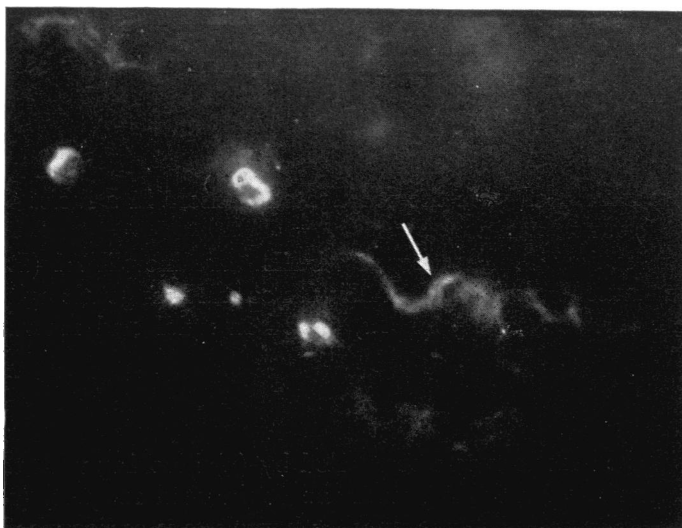

FIG. 1. Pig jejunum fixed in ethanol and stained with fluorescein conjugated antiserum to IgA showing fluorescence in the apical cytoplasm of a crypt (arrowed). The cells of the surrounding lamina propria are poorly demonstrated. (The bright cells are eosinophils.)

Similar preparations fixed with methanol and stained simultaneously, with the same reagent used for the ethanol fixed sections, presented a somewhat different picture. The fluorescence of the crypt epithelium though usually well demonstrated was not as brilliant as that of the ethanol fixed material. This was particularly noticeable in some of the younger animals (Fig. 2).

In contrast, in the lamina propria surrounding the crypts large numbers of stained cells could be seen. These appeared to occur in groups or plaques.

In most of the methanol fixed preparations a faint intercellular fluorescence was seen in the lamina propria around the crypts. This staining was not seen in the lamina of the villous cores nor was it found in the mucosal tissue. The luminal contents of some of the larger blood vessels showed a dull green fluorescence.

Acetone fixed preparations of the same material generally showed a crypt staining having an intensity similar to that of the ethanol fixed material. In the lamina propria, brightly fluorescing cells were seen. Most of these were in the region of the crypts, though

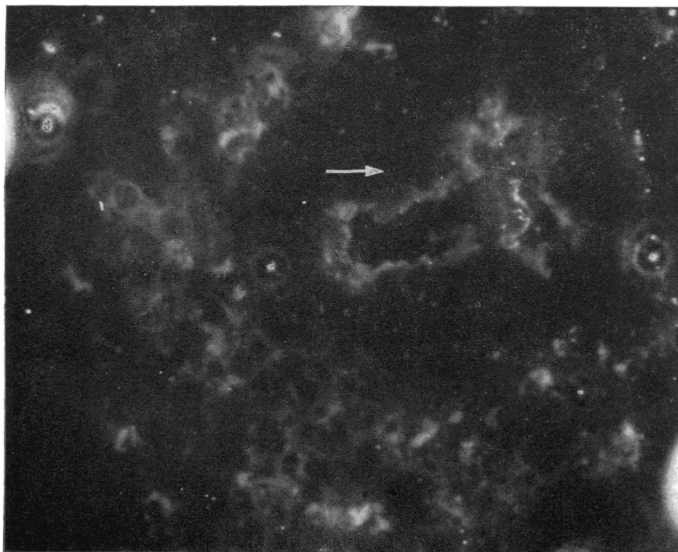

FIG. 2. Pig jejunum fixed in methanol and stained with fluorescein conjugated antiserum to IgA showing fluorescence in the apical cytoplasm of the epithelium of a crypt, cut transversally (arrowed), and in cells of the lamina propria. The brighter cells are eosinophils autofluorescing.

occasionally one or two could be found in the cores of the villi. Unlike the fluorescent laminal cells of the methanol fixed sections these cells occurred as discrete, well defined, bright apple green entities. Occasionally a finely granular deposit was seen in the inter cellular areas.

IMMUNOGLOBULIN IgG

Immunoglobulin IgG which may occur as an extravascular component as well as in the plasma cells was consistently demonstrated in the intercellular tissue of the lamina propria and sub mucosa in the preparations fixed by either ethanol or methanol and stained with conjugated specific rabbit anti pig IgG globulin (Fig. 3). Acetone fixation was less reliable, the globulin being shown in some preparations but not in others.

In all cases in which the immunoglobulin was demonstrated, it was seen as a particulate green fluorescent material. The size of the particle appeared to be dependent upon both the fixative used and the amount of antigen occurring in the tissue. Methanol fixation produced a coarser deposit than ethanol although one which was finer than that of the acetone fixed material. 95 per cent ethanol produced a coarser deposit than 70 per cent ethanol. The size of the particles also appeared to be largest in that part of a section where the fluorescence was strongest, showing the presence of a greater concentration of immunoglobulin. Furthermore, the finer the particle the better the localization of the globulin appeared to be.

If fixed sections were washed in PBS before incubating with the conjugated reagent the amount of IgG demonstrated was either considerably reduced or eliminated completely.

Very occasionally plasma cells which fluoresced were seen in the lamina propria.

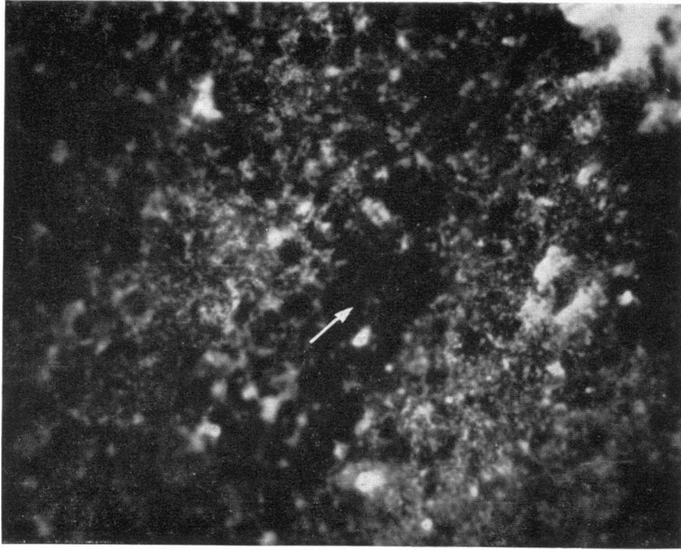

FIG. 3. Pig jejunum fixed in ethanol and stained with fluorescein conjugated antiserum to IgG showing the extra vascular distribution of the immunoglobulin in the lamina propria of the crypt area. The crypt epithelium (arrowed) is unstained.

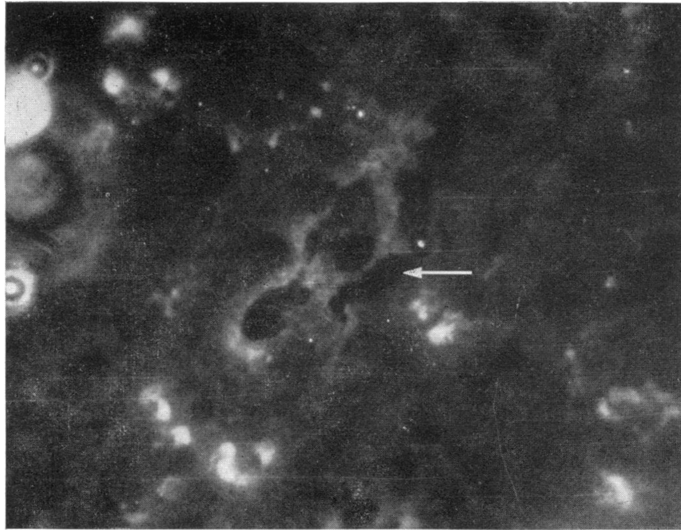

FIG. 4. Pig jejunum fixed in methanol and stained with fluorescein conjugated antiserum to IgM showing fluorescence in the apical cytoplasm of the epithelium of a crypt (arrowed). The brightly fluorescing cells in the surrounding lamina propria are eosinophils autofluorescing.

IMMUNOGLOBULIN IgM

In intestinal tissue, fixed in methanol and stained with conjugated specific rabbit anti-pig IgM serum, the location of the immunoglobulin was similar to that described for IgA immunoglobulin. In all the specimens examined fluorescent staining of the cytoplasm of the epithelial cells of some of the crypts was seen (Fig. 4). However, this was not as marked as in the crypts which showed fluorescence with the reagent for IgA. Plaques of fluorescent cells could be seen in the lamina propria. In one or two specimens a dull fluorescence was seen in the lumen of the larger blood vessels in the sub-mucosa.

Only very occasionally were any of the findings reported above seen in the preparations fixed with either ethanol or acetone.

In general there was little or no variation in the findings, with any of the antisera, at the different levels of the intestine from one animal. No non-specific staining of either the eosinophils, which are numerous in pig lamina propria, the muscularis or basement membrane occurred with any of the three conjugates used.

DISCUSSION

The reliability of observations made with immunofluorescent protein tracing techniques is determined both by the quality of the reagents and by the method of fixation.

Reagents prepared by the techniques described label tissue sections characteristically, without interference from non specific staining due to isoelectric phenomena (Myersbach, 1967). Any conjugate which showed non specific staining of either eosinophils, muscle fibres or basement membrane was considered unacceptable for the present studies.

The method of fixation for any new immunological system can only be determined by trial and may be critical, as is shown by the variability of results observed with the different immunoglobulins subjected to different fixation techniques. Whilst one method of fixation may be successful for a particular antigen it does not necessarily follow that it will be equally satisfactory for a related antigen in the same system.

Immunoglobulin IgG occurs as an extravascular component in porcine intestinal tissue and may be detected in the intercellular matrix as well as in the plasma cells in which it is synthesised. Our results indicate that fixation does not necessarily render this extravascular component completely insoluble; soluble antigen leaches out of the fixed tissue into the antiserum, where it apparently complexes, the resultant precipitate being deposited on the section.

For the localization of IgM in porcine intestinal tissue, methanol was the only fixative giving consistent results. Fixation of piglet small intestine in methanol was also the method of choice for the demonstration of IgA located in the cells of the lamina propria. However the reaction of the secretory IgA in the crypt epithelium was better shown in tissue fixed either in ethanol or acetone.

The demonstration of IgM in porcine crypt epithelium is a new finding and deserves comment in the light of recent observations relating to secretory immunoglobulins in man. The presence of both IgA and IgM in crypt epithelium suggests that these two immunoglobulins may have a complementary role as antibodies in intestinal secretions. Evidence in support of this thesis is provided by our previous studies on external secretions of the pig. In porcine milk, IgA is the predominant immunoglobulin, but IgM is also present and is an additional source of E. coli antibody (Porter, Noakes and Allen, 1970). Furthermore,

Brandtzaeg (1968) has suggested that a common secretory mechanism for both IgA and IgM may be operative in man and in cases of ataxia telangiectasia, where there is an IgA deficiency, the protective role in external secretions is adopted by IgM (Stobo and Tomasi, 1967; Eidelman and Davies, 1968).

The function of IgA and IgM as secretory immunoglobulins in porcine intestine is being studied further.

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