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GASTRO-INTESTINAL-SPECIFIC ANTIGEN: AN IMMUNOHISTOLOGICAL AND SEROLOGICAL STUDY

BY

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Fine histochemical differences between the tissues of the body may be demonstrated by immunohistological methods. Thus immunofluorescence offers a means of studying microscopically certain characteristics which distinguish one organ from another, so-called organ-specificity, and the technique has already been used for this purpose by several workers (reviewed by Nairn, 1962). The present investigation is concerned with such a demonstration of an antigenic component specific for gastro-intestinal mucosa; the antibody employed for its detection was produced by immunizing rabbits with microsomal material from human colon mucosa (Nairn *et al.*, 1961).

Methods

The same general methods of cell fractionation, anti-serum preparation, and immunological study were used as in previous investigations of organ-specific antigens in human skin, rat liver, and hamster kidney (Nairn *et al.*, 1960). The antigen was obtained from mucosa scraped from normal areas of fresh specimens of human colon removed at operation for carcinoma. The scrapings were suspended in chilled buffered physiological saline (0.01 M phosphate, pH 7.1) and homogenized for four minutes at about 10,000 r.p.m. in an M.S.E. homogenizer; further cell disruption was obtained by two to three minutes' treatment with a 60-watt M.S.E.-Mullard ultrasonic disintegrator. Sucrose was added to the suspension to make a 0.25 M solution, and the cell components were separated by differential centrifugation. The washed microsomal fraction, resuspended in buffered saline at a concentration of 4 mg. of nitrogen per ml., was used as antigen throughout.

Antiserum was prepared in three 2.5-kg. rabbits by intravenous injection of 0.5 ml. of antigen at intervals of two or three days for three weeks. The antigen, which was toxic, caused *in vivo* coagulation of the blood and was capable of killing a rabbit at the dosage used for immunization; to avoid this hazard it was mixed with heparin (500 I.U./ml. antigen) and given slowly. The total dose of antigen N given to each rabbit was 20 mg. The animals were bled six days after the last injection and each produced antibody of high complement-fixing titre: 1/600 in two and 1/1,200 in the third, which was used in the main experimental study. Complement fixation was carried out against 8 µg. of antigen N with 3 minimum haemolytic doses (M.H.D.) of complement in the test and 2 M.H.D. in the controls. Fixation was carried out at 4° C. for one and a half hours followed by one hour at 37° C.; the haemolytic system was then added and the results

The Research Defence Society helps in various ways to combat attacks by antivivisectionists against medical and veterinary research workers. It has recently produced a pamphlet entitled "The Use of Vaccines in Controlling Disease in Domestic Animals." By means of simple questions and answers this describes for the layman the reasons why domestic animals need to be vaccinated, the diseases against which they need protection, and the methods of vaccination. It is obtainable (6d. net) from the Society at 11 Chandos Street, Cavendish Square, London W.1.

were read after half an hour at 37° C. and after standing overnight at room temperature.

The antiserum was diluted 1 in 5 for immunological absorptions and used at this dilution subsequently. Microsomal material from human kidney, colon carcinoma, and colon mucosa were employed for the absorptions at a concentration of 3 mg. N per ml. of original serum. Absorption by carcinoma microsome material, used for the demonstration of loss of the specific antigen in malignancy (Nairn *et al.*, 1962), was valuable here because of its richness in colonic antigens other than the specific component being investigated. The effect of successive absorptions was studied by complement fixation, gel diffusion, and immunofluorescence staining of tissue sections.

For fluorescent antibody study, frozen sections from snap-frozen tissue blocks stored at -20° C. were cut at 5 μ in a cryostat, stained by the sandwich method using goat anti-rabbit-globulin labelled with lissamine rhodamine B (RB 200), and examined by fluorescence microscopy. The antiglobulin conjugate was absorbed successively with human liver powder, colon powder, and colon homogenate to eliminate non-specific staining. The human tissues examined comprised gastro-intestinal tract, especially colon, and several other organs, and included foetal tissues; comparative studies were made in rodents, cats, dogs, sheep, and cattle.

The identity of the specific gastro-intestinal antigen was investigated by column chromatography on modified cellulose and by conventional histochemical procedures for which the following staining methods for mucopolysaccharides (Curran, 1961) were employed: alcian blue at pH <2.8, colloidal iron, periodic acid-Schiff.

Results

Table I shows the effects of the absorptions and demonstrates the gastro-intestinal specificity of the antiserum. The complement-fixing titre of the serum fell from 1/1,200 to 1/400 after two absorptions with the kidney microsome material. Additional similar absorptions had no further effect on the titre, but four successive parallel absorptions with the colon carcinoma microsomes reduced the titre to 1/50 and then by small steps to 1/25; the corresponding figures after absorptions with the colon mucosa microsomes were 1/25 to less than 1/5 (possibly nil, but this could not be measured because of anticomplementary activity in the absorbed serum). A different pattern of antibody loss was observed in the gel-diffusion tests. The unabsorbed serum gave three precipitin lines against the colon antigen; only two lines were obtained after kidney absorption and no lines after absorption with microsome

TABLE I.—Reactions of Unabsorbed and Absorbed Anti-human-colon Rabbit Serum

Absorptions with human microsome material:	Anti-colon Serum				Normal (Pre-immunization) Serum	
	Nil	Kidney +			Nil	Kidney
		Kidney	Colon Cancer	Colon Mucosa		
Complement-fixing titre against colon antigen ..	1/1,200	1/400	1/25	<1/5	0	..
Gel-diffusion lines at 48 hours against colon antigen ..	3	2	0	0	0	0
Staining of human tissues with fluorescent antibody:						
Gastro-intestinal mucosa	+++	++	++	—	(+)	—
Other tissues and mucosae ..	+	—	—	—	(+)	—

(+) = Weak unsystematic staining.

material from colon carcinoma or mucosa. Thus the difference in complement-fixing titre between these last two serum samples is attributable to a non-precipitating antigen-antibody reaction, and this was capable of causing immunofluorescence staining.

Fluorescent Staining

The antiserum consistently gave intense staining of gastro-intestinal mucosa, and the reaction could be inhibited by absorption with colon mucosa microsome material but not with any of the other absorbing agents tried. It is an interesting practical point that a freshly washed microsomal fraction was an inefficient absorbing agent, and absorption was most satisfactory with material that had been allowed to stand at room temperature for some hours, presumably to release soluble antigen from a previously inaccessible situation in the cytoplasmic membranes. Other human tissues, including liver, kidney, various glandular structures, and connective tissues, gave little and unsystematic fluorescent staining with unabsorbed serum and no staining after absorption of the serum with human material. Control tests with normal (pre-immunization) serum, which gave negative serological results, showed that the usual slight fluorescent staining by unabsorbed serum was not obtained after absorption with kidney microsomes.

The staining was distributed in the human gastro-intestinal tract as follows: irregularly in the stomach, mainly in the superficial glands but sometimes extending to the deepest part of the mucosa and not limited to any one anatomical region; uniformly in the goblet cells of the small intestine and all the epithelial cells of the colonic mucosa. It was of approximately equal intensity wherever present and ceased abruptly at the gastro-oesophageal and recto-anal junctions (see Fig.). Its intracellular distribution was cytoplasmic, excluding the basal basophilic zone, cell membrane, and nucleus, and corresponded with the secretion-containing part of the cell. It coincided with areas which stained positively for acid mucopolysaccharides, but not all gastro-intestinal cells giving such a positive reaction showed fluorescent staining—for example, some gastric mucosal

Section of human recto-anal junction treated first with rabbit anti-colon serum which had been absorbed with microsome material from human kidney and colon carcinoma, and then with RB200-conjugated goat anti-rabbit-globulin. Bright specific staining in a terminal rectal gland but not in the adjacent anal mucosa below. ($\times 240$.)

cells. Moderate or weak fluorescent staining of extra-cellular secretion was also demonstrable in a proportion of specimens, but some of the secretion in the gland crypts, although apparently acid mucopolysaccharide in nature, failed to stain.

Mucosal and glandular tissues outside the gastro-intestinal tract, many of which also gave strongly positive reactions for acid mucopolysaccharides, were not stained by the anti-colon serum. The tissues tested included pancreas, gall-bladder, salivary gland, bronchus, prostate, cervix, endometrium, pseudomucinous ovarian cystadenoma, and mucoid carcinoma of breast. Fluorescent staining was also unobtainable with polysaccharide-containing mesenchymal tissues, including cartilage and umbilical cord. In tissues from three human foetuses, aged 9 to 14 weeks, specific staining was obtained in intestinal mucosa but not in stomach or elsewhere in the body.

These staining reactions in human tissues were compared with those in other animal species, including sheep, cattle, dogs, cats, rabbits, rats, mice, and guinea-pigs (Table II). In the ruminants, specific staining was obtained in the colonic mucosa and there was sometimes a suggestion of staining in the small intestine and none in the rumen. The distribution was different in the

TABLE II.—Immunofluorescence Staining of Human and Animal Tissues by Anti-human-colon Serum

	Human	Ruminant ¹	Carnivore ²	Rodent ³
Gastro-intestinal mucosae:				
Stomach	++ ⁴	—	(+)	—
Small intestine	++	(—)	+	—
Colon	++	+	(+)	—
Other mucosae and tissues	—	—	—	—

¹ Sheep and cattle. ² Dog and cat. ³ Rabbit, rat, mouse, and guinea-pig.
⁴ Patchy distribution. (+) = Weak reaction (—) = Doubtful slight reaction.

carnivores, which showed obvious mucosal staining in the small intestine and less staining in colon and stomach; as in the human, gastric staining was scattered irregularly in the mucosa. The rodents showed no gastro-intestinal staining, and in none of the animal species was there any staining outside the gastro-intestinal tract. The intensity of staining in animal intestine was less than that in the human.

Nature of Specific Antigen

The histochemical investigation suggests that this is an acid mucopolysaccharide, and preliminary study of antigen separated by chromatography supports this view. A carbohydrate-containing component of low isoelectric point, stainable by alcian blue and capable of neutralizing specific antiserum,⁶ has been demonstrated.

Any immunological relationship of the antigen to blood-group substances is excluded by the fact that staining was independent of the blood groups of the tissues examined and was not inhibited by appropriate red-cell absorptions of the antiserum; the blood-group substances are, moreover, not acid mucopolysaccharides and are not confined to the gastro-intestinal tract. The unlikely possibility that the small quantity of heparin mixed with the immunizing antigen might have stimulated antiheparin formation has been investigated, but no evidence of such antibody was demonstrated by serological tests, and absorption of the antiserum by heparin failed to inhibit gastro-intestinal staining. Moreover, several antisera have been produced against heparin-treated cell fractions from other organs and no anti-gastro-intestinal activity has been detected.

Discussion

This gastro-intestinal-specific antigen resembles other organ-specific components by its occurrence in species other than human against which the antibody was prepared. It does not, however, conform to the usual concept of organ-specific antigens in that it appears to be a cell product rather than a structural cellular component. Its apparent absence from cell membranes suggests that it is not a "marker" antigen, though the possibility of its coating cells after secretion and having important surface effects has not been excluded.

There appears to be little doubt that the antigen located by fluorescent staining is an acid mucopolysaccharide; its histological discrimination from the very many chemically similar substances in the tissues examined is a striking illustration of the remarkable specificity of the immunofluorescence method. The antigenic material against which the gastro-intestinal-specific antibody was prepared consisted of cytoplasmic particles washed to remove soluble material, but whether it was an intracellular mucigen of low solubility and possibly bound in cytoplasmic organelles or soluble mucin trapped in the cytoplasmic membranes has not been decided. Nevertheless, it seems to be a different kind of antigen from that used by Kent (1961) in immunofluorescence studies of bovine submaxillary mucin. Kent prepared antibody against the purified mucin and found that its activity was species-specific as well as organ-specific and was demonstrable with formalin-fixed paraffin-embedded material; such processing resulted in considerable diminution of staining of the gastro-intestinal antigen in our experiments.

The significance of the specific gastro-intestinal mucin in human physiology and pathology cannot at the moment be assessed, but its simple detection by immunofluorescence will provide information about any variations which may occur in alimentary diseases. Such variations in colonic and gastric neoplasia are described in the paper which follows (Nairn *et al.*, 1962).

Summary

An antigenic component specific for gastro-intestinal mucosa has been demonstrated by immunofluorescence and serological methods using antisera from rabbits immunized with a microsomal fraction of normal human colon mucosa.

Similar antigenic material is present in the intestines of sheep, cattle, dogs, and cats but not in rodents and not in any other organ studied in man or animals.

The antigen is found in the secretory region of the mucosal cell and appears to be an acid mucopolysaccharide.

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