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IMMUNOLOGICAL STUDIES IN ULCERATIVE COLITIS

VII. ANTI-COLON ANTIBODIES OF DIFFERENT IMMUNOGLOBULIN CLASSES

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SUMMARY

Ten out of sixty-three sera from patients with ulcerative colitis gave positive staining of goblet cells in the indirect immunofluorescence test, applied to acetone–ethanol fixed sections of colon from germ-free rats. Nine of these sera were also positive in triple layer tests in which the second layer consisted of rabbit antibodies to human immunoglobulin chains and the third layer of fluorescein conjugated sheep anti-rabbit immunoglobulin. All sera contained anti-colon antibodies having light chains of both κ- and λ-type. Heavy chains of both γ-, μ- and α-type occurred at almost equal frequency and only three of the nine positive sera contained antibodies of a single immunoglobulin class. One out of four tested sera which contained antibody of the IgA-class also gave positive reactions for secretory-piece determinants. The staining pattern obtained with antibodies of different classes was similar. Anti-colon antibodies belonging to the IgD or IgE classes were not detected. The triple layer test was negative when the patients' sera were replaced by normal human sera.

INTRODUCTION

Sera from patients with ulcerative colitis contain antibodies which react with antigen present in sterile human colon tissue (Broberger & Perlmann, 1959, 1962). An antigen that cross reacts with these human antibodies is also present in colon and faeces of germ-free rats (Perlmann *et al.*, 1965). The antigen is organ-specific for gastro-intestinal tissue but not exclusively confined to the large intestine. It is a polysaccharide, chemically related to the blood group substances of the ABH system (Hammarström *et al.*, 1965). However, the immunological specificity of the determinant(s) with which the patients' antibodies react is distinct from any known blood group specificity of the ABH system.

Indirect haemagglutination of antigen-coated sheep erythrocytes, precipitation in agar

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gel and immunofluorescence of colon sections have all been used to test for anti-colon antibodies in the patients' sera. With a few exceptions the results obtained with these different tests and with human or rat antigen are in good agreement (for references, see Perlmann & Broberger, 1969). With the rat (Lagercrantz *et al.*, 1966; Deodhar, Michener & Farmer, 1969; Thayer *et al.*, 1969) but not with the human antigen (Harrison, 1965; Wright & Truelove, 1966) elevated antibody titres to colon have also been observed in sera of patients with Crohn's disease.

The nature of the anti-colon antibodies in ulcerative colitis is not as yet well established. Although the antibodies which give rise to indirect haemagglutination are primarily of the IgM class, antibodies belonging to the IgG class are also present (Perlmann *et al.*, 1965; Harrison, 1965; McGiven, Ghose & Nairn, 1967). This study represents an attempt to throw further light on this question. Better knowledge of the antibody class can be expected to provide some clues as to their origin and possible pathogenic significance.

MATERIAL AND METHODS

Sera were from patients with clinically, rectoscopically and roentgenologically verified ulcerative colitis. They were obtained from the Karolinska Hospital and from St Erik's Hospital, Stockholm. Some of them have been described in a previous report (Lagercrantz *et al.*, 1968). Control sera were from healthy blood donors and laboratory personnel. All sera were heat inactivated, absorbed with sheep- and human A_1-erythrocytes and kept in small vials at $-20°C$ until use. For determination of their haemagglutination titre, they were reacted with sheep erythrocytes, coated with phenol–water extracted antigen from germ-free rat faeces. Details have been given previously (Hammarström *et al.*, 1965; Lagercrantz *et al.*, 1968).

Rabbit antisera to IgG, IgM and IgA, specific for γ-, μ- and α-chains, respectively, were purchased from Behringwerke AG, Marburg, Western Germany. Antisera to IgD and IgE from single rabbits were a gift from Dr G. Johansson, Uppsala, Sweden. Rabbit antisera to κ- and λ-light chains were from Behringwerke AG, and from Brostex A/S, Copenhagen, Denmark. An IgA-deficient serum for absorption of the rabbit anti-IgA was donated by Dr L. Å. Hansson, Gothenburg, Sweden. All sera were absorbed until specific and tested for specificity by double diffusion in agar gel and immunoelectrophoresis. The antiserum to secretory piece of IgA was also a gift from Dr Hansson. It was obtained by injecting a rabbit with colostral IgA from a woman deficient in serum IgA and was absorbed with normal human serum and lactoferrin (Hansson & Johansson, 1970). All rabbit antisera and normal rabbit serum for control were heat inactivated.

For immunofluorescence, pieces of colon or control tissue from germ-free rats, starved for 24 hr, were snap frozen in liquid nitrogen. Cryostat sections of 4–5 μ thickness were cut in a cryostat and fixed in ethanol–acetone for 10 min.

A polyvalent sheep anti-human immunoglobulin, conjugated to fluorescein isothiocyanate, was purchased from the State Bacteriological Laboratory, Stockholm. The purified conjugate had a fluorescein-protein (F:P) ratio of $3{\cdot}6 \times 10^{-3}$ (Nairn, 1969).

Sheep anti-rabbit immunoglobulin was the globulin fraction, obtained by precipitation with ammonium sulphate, of the serum from an animal immunized with purified rabbit IgG. The conjugate with fluorescein isothiocyanate (Baltimore Biological Laboratories, Balti-

more, Md, USA) was prepared according to Clark & Shepard (1963). The conjugate was purified by gel filtration through Sephadex G-25 (Pharmacia AB, Uppsala, Sweden) and absorption with mouse liver powder (100 mg/ml). The conjugate used had a F:P ratio of $3{\cdot}5 \times 10^{-3}$. For control, the globulin of a normal sheep serum was conjugated and purified as described.

For immunofluorescent staining, the human sera were screened by the indirect fluorescent antibody test with the conjugated sheep anti-human immunoglobulin. The conjugate was applied at a dilution corresponding to 4 units/ml (Beutner, Holborow & Johnson, 1965). The procedures have been described previously (Hammarström *et al.*, 1965; Lagercrantz *et al.*, 1966). The ten sera which showed positive colon staining were chosen for further examination of the immunoglobulin class of the anti-colon antibody. The triple layer procedure used was similar to that described by Barnett *et al.* (1964). In brief, the tissue sections were first incubated with human serum applied undiluted or diluted from 1:4 to 1:32. After washing, this was followed by incubation with rabbit antiserum to human immunoglobulin, diluted 1:8 or 1:16 with buffered saline. In the last step, the conjugated sheep anti-rabbit immunoglobulin was added at a dilution of 1:8–1:16 (1–2 mg protein/ml). After staining, the sections were processed and examined in the fluorescent microscope (Zetopan, Reichert, Vienna, Austria) and photographed as described previously (Lagercrantz *et al.*, 1966).

The specificity of the staining was controlled as follows. (1) Normal human serum instead of ulcerative colitis serum was applied to the section in the first step; (2) human serum was omitted; (3) the rabbit anti-immunoglobulin serum was replaced by normal rabbit serum; (4) rabbit serum was omitted; (5) the sheep anti-rabbit globulin conjugate was replaced by conjugated globulin from normal sheep serum; (6) sections of liver and muscle from the same germ free rat were subjected to the triple layer procedure.

RESULTS

In the tests with conjugated sheep anti-human globulin (indirect staining), fluorescence of goblet cells was considered a positive reaction, regardless of the number of stained cells. Sections which only showed staining of mucosal epithelial lining and smooth muscle layers were regarded as negative. An example of positive staining is shown in Fig. 1. Based on these criteria, ten out of sixty-three patients' sera gave positive results. The ten positive sera were subjected to testing with the triple layer techniques. Each serum was tested with two different lots of the rabbit anti-human immunoglobulin reagents. The results of three–six experiments, each comprising tests with all or most anti-human globulin reagents, together with the haemagglutination titres of the sera, have been summarized in Table 1. Four sera contained antibodies of IgG, IgM, and IgA class, one contained antibodies of IgG and IgA class and four had antibodies of either IgG or IgM class. In three out of five cases the specificity of the IgA staining was ascertained by absorption of the anti-IgA-reagents with IgA-deficient serum (see Material and Methods). One serum which was positive in the indirect test was negative in the triple layer test with all reagents. The supply of this serum was limited and the reasons for the negative results are unknown. None of the sera stained for δ- or ε-chains. In contrast, all sera studied were positive when tested for both κ- and λ-light chains. One out of four sera which contained anti-colon antibody of the IgA class, also gave positive reactions for secretory-piece determinants.

The colon staining obtained with the triple layer techniques was similar to that of the indirect test. An example of a reaction positive for IgG is shown in Fig. 2. No difference in type of staining or localization was seen with antisera to the other heavy chains or the different light chains. The sensitivity of the triple layer techniques was slightly less than that

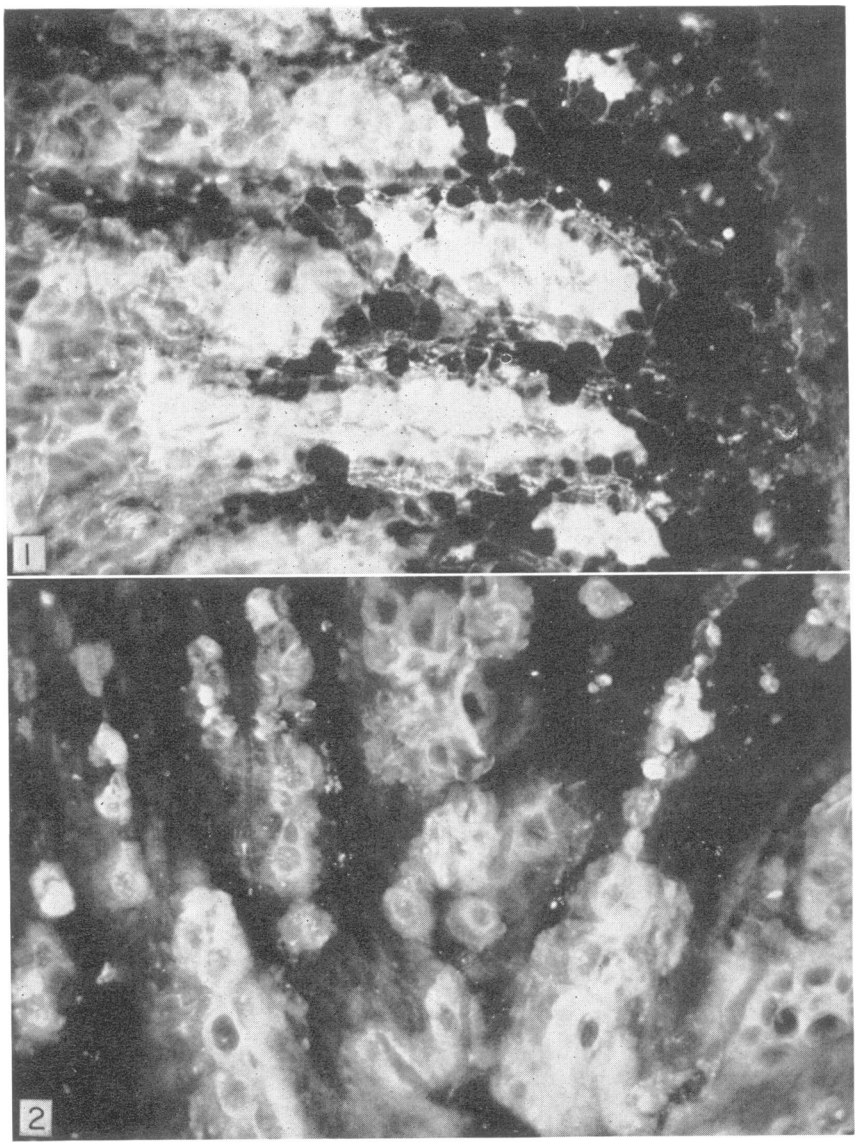

FIG. 1. Section of germ-free rat colon treated with ulcerative colitis serum, followed by sheep anti-human γ-globulin conjugate. Note distinct fluorescence of goblet cells. $\times$ 200.

FIG. 2. Section as above, treated with ulcerative colitis serum, rabbit anti-human IgG, and conjugated sheep anti-rabbit immunoglobulin. Many goblet cells show bright fluorescence. $\times$ 200.

of the conventional indirect method. All patients' sera in the first layer were always applied undiluted and in doubling dilutions up to 1:32. No prozone effect was observed. Only one serum (no. 1) was still positive for IgG and IgA but not for IgM when diluted 1:32. Three sera lost all activity when tested at dilutions >1:8. The rest were only positive when applied undiluted or at dilutions up to 1:4. All positive reactions shown in Table 1 were obtained with both rabbit anti-human globulin in the second layer and the conjugate in the third layer diluted 1:8 or 1:16. Under these conditions all control reactions (see Material and

TABLE 1. Immunofluorescence of ulcerative colitis sera tested for different immunoglobulin classes, light chains and secretory piece of anti-colon antibodies

Ulcerative colitis sera no.	IgG	IgM	IgA	IgD	IgE	Secretory piece	Kappa	Lambda	HA*
1	+	+	+	−	−	−	+	+	1:64
2	−	+	−	−	−	−	nt	nt	1:512
3	−	−	−	−	−	−	nt	nt	1:32
4	+	+	+	−	−	+	+	+	1:32
5	−	+	−	−	−	−	+	+	1:512
6	+	−	−	−	−	nt	+	+	1:2
7	+	+	+	−	−	nt	+	+	1:16
8	+	−	+	−	−	−	+	+	1:128
9	−	+	−	−	−	nt	+	+	1:64
10	+	+	+	−	−	−	+	+	1:4

* Haemagglutination titre in indirect haemagglutination of sheep erythrocytes coated with colon antigen.
nt, Not tested.

Methods) were negative. This was also the case when the patients' sera were replaced by normal human serum. The sera of sixteen control subjects were tested for anti-colon antibodies with both the indirect and the triple layer techniques. One of these gave a weak staining of goblet cells when tested undiluted in the indirect test. However, in the triple layer assay, this serum was negative with all anti-immunoglobulin sera.

DISCUSSION

The incidence of positive reactions was similar to that obtained previously by other authors who used human colon (Harrison, 1965; Wright & Truelove, 1966) or conventional rat colon (McGiven *et al.*, 1967). It was, however, slightly lower than that obtained in our previous studies of germ-free rat colon. The more rigorous application of the various criteria for positive staining probably accounts for this (Lagercrantz *et al.*, 1966). All nine positive sera subjected to the investigations summarized in Table 1 contained anti-colon antibodies having light chains of both κ- and λ-type. Moreover, heavy chains of both γ-, μ- and α-type occurred at almost equal frequency and only three of nine positive sera contained antibodies of a single immunoglobulin class. The findings speak against a monoclonal origin of anti-colon antibodies in ulcerative colitis. Antibodies of different classes seemed to react with the same antigen since their staining pattern was similar. However, blocking tests with

unconjugated rabbit antisera to human immunoglobulin chains and subsequent staining with conjugated sera against the other chains are needed to clarify this point.

The finding of antibodies belonging to the IgM and IgG classes confirms previous observations (Perlmann *et al.*, 1965; Harrison, 1965; McGiven *et al.*, 1967). Anti-colon antibodies belonging to the IgA class have not been described previously. Some authors have recently reported an elevated concentration of IgA in the serum of a post-colectomy group of patients with ulcerative colitis and regional enterocolitis, and in patients with ulcerative colitis of long standing (Kraft *et al.*, 1968). However, none of the sera included in Table 1 had an IgA concentration above normal (unpublished data obtained in collaboration with Dr G. Johansson, Uppsala). It is noticeable that one of four sera which had antibodies of the IgA class also gave a positive reaction when tested for secretory piece determinants. An increased occurrence of secretory IgA in the serum of patients with ulcerative colitis and other gastrointestinal disorders has recently been described. It was stated that this may reflect a reabsorption of intraluminal IgA through damaged intestinal epithelium, or a transfer of secretory IgA from the lamina propria of the bowel wall (Thompson, Asquith & Cooke, 1969). It is possible that more of locally secreted anti-colon antibody of IgA class may be found in the colon tissue of ulcerative colitis patients (see also Gelzayd *et al.*, 1968).

None of the sera gave a positive reaction when tested for anti-colon antibodies of IgD or IgE class. Two sera which had an exceptionally high concentration of IgE (1063 and 4300 ng/ml, respectively; unpublished data, in collaboration with Dr G. Johansson) were subjected to the triple layer test for anti-colon antibody of IgE class but the results were also negative. These sera were also negative when tested with the other anti-immunoglobulin reagents or with conjugated sheep anti-human immunoglobulin.

A certain correlation between haemagglutination titre and immunofluorescence has previously been noted (Lagercrantz *et al.*, 1966) and was also seen in this study. However, this correlation is not an absolute one. Some of the basis for exceptions from the rule are found in Table 1. It will be noted that the two sera whose anti-colon antibodies were IgM alone had the highest haemagglutination titres. Conversely, the serum which had the lowest haemagglutination titre had anti-colon antibodies of the IgG class only.

Antibodies of different immunoglobulin classes have different biological properties and this will be of importance for their possible pathogenic role in autoimmune diseases. Whether anti-colon antibodies of different immunoglobulin classes are of significance for the pathogenesis of ulcerative colitis requires further studies.

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