

IMMUNOLOGICAL STUDIES IN ULCERATIVE COLITIS

II. "COLON" ANTIGEN AND HUMAN BLOOD GROUP A- AND H-LIKE ANTIGENS IN GERMFREE RATS

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Sera from children and adults with ulcerative colitis contain antibodies against antigen present in phenol-water extracts of sterile human colon (1-4). The antigen is heat stable and most likely of polysaccharide nature (5). It seems to be tissue specific and is present in the epithelial cells of the colon mucosa and probably also in the mucus (4).

Sterile (*i.e.* fetal or newborn) human colon contains only small amounts of antigen, insufficient for detailed studies. Large intestine from adults cannot be used because of its inherent contamination with bacterial antigens, against which antibodies are present in most human sera. Attempts were therefore made to use antigen from germfree rats (6). Such animals were considered suitable not only because of the absence of an intestinal flora, but also because of their strongly hypertrophic cecum and the abundance of water soluble intestinal mucus (7). It was found that extracts from the intestine of these rats contained antigen which reacted with antibodies in the patient's sera in indirect hemagglutination and agar precipitation experiments. The antigen was tissue specific and similar to the human colon antigen described earlier. It could be obtained in good yields from wall and content of colon and cecum of the rats, and did not originate from the diet (6). This antigen was therefore used for further immunochemical studies.

Human colonic extracts only rarely reacted with sera of healthy control subjects or those of patients with unrelated diseases, when tested by the sensitive indirect hemagglutination technique. In contrast, most human sera had low or moderate titers against the rat extracts, when tested by the same method.

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Knowledge of the specificity for ulcerative colitis of the colon reactive antibodies in the sera of these patients is essential for an appreciation of the possible autoimmune etiology of this disease. Therefore, the nature of this background reactivity in normal human sera had to be investigated. In previous experiments with human antigen, extracts were made from colon of newborn subjects belonging to blood group O, and it was also observed that the presence of anti-A or anti-B isohemagglutinins did not influence the titers against the tissue antigen. However, human blood group-like antigens are well known to occur in animals (8). It could therefore be assumed that the background activity in normal sera, when tested with rat extracts, was due to antibodies to such antigens. In this paper, the immunological relationship of the colon-specific antigen from germfree rats to blood group-specific antigens of the human ABO system will be described.

Material and Methods

Sera.—The ulcerative colitis sera were from children and young adults (10 to 25 years old), most of them with the disease in a chronic phase. Sera from healthy persons of the same age distribution, and from persons with unrelated diseases served as controls. The eel serum was a pool from 10 animals. Before use it was freed from lipids by centrifugation at 10,000 *g*. All sera were inactivated at 56°C for 30 minutes. The human sera were absorbed once for 30 minutes at room temperature with an equal volume of packed and washed sheep erythrocytes. The eel serum was absorbed with human erythrocytes of blood group A₁B. All sera were stored in small phials at -20°C until use.

Antigen.—Antigen was extracted from germfree rats of the inbred Swedish strain, reared according to the technique of Gustafsson (9, 10). The rats, which were of the 18th inbred generation, were fed the semisynthetic diet D7 (10) and water *ad libitum*. The diet was sterilized by autoclaving at 121°C for 20 minutes. Cecum, colon (wall and contents), small intestine, stomach, liver, and kidney were dissected out aseptically from animals of both sexes. A pool of each of these organs taken from 10 animals as well as individual samples of cecum from 2 additional animals were homogenized separately in a Virtis 45 homogenizer in ice cold distilled water and extracted twice for 15 minutes with 90 per cent phenol at 65°C according to Westphal *et al.* (11). Extraction was begun within 3 hours of the animals leaving the germfree environment. The water phases, separated from the phenol by centrifugation, were combined, dialyzed for 2 days against running tap water and against three changes of distilled water for an additional 2 days. The dialysates were then concentrated to $\frac{1}{10}$ the original volume by vacuum distillation and were finally precipitated with 6 volumes of 96 per cent ethanol (containing traces of sodium acetate) under constant stirring. After standing at 4°C overnight, the precipitates were collected, dissolved in distilled water, centrifuged if necessary, and finally lyophilized. Feces from the germfree rats were extracted in the same way. The rats were kept on raised screens and feces were collected from the stainless steel trays on the bottom of the cages. This type of collection does not exclude some contamination with diet, urine, and hair. Extract of the diet (D7) and of lungs from 20 conventional rats (Sprague Dawley) was also used in some experiments.

Erythrocytes.—The human erythrocytes were from healthy blood donors of blood group A₁, B, A₁B, or O (Rh +). The cells were stored at 4°C in sterile citric acid dextrose (ACD) solution and used within 5 weeks. Sheep erythrocytes were collected aseptically in one volume of sterile Alsever's solution (12) and kept at 4°C until use.

Hemagglutination.—For sensitization, 4 ml of saline, containing 2.0 mg antigen/ml, were added to 0.10 ml of packed and washed sheep erythrocytes. Before use, the antigen solution was heated to 100°C for 1 hour. After incubation at 37°C for 1 hour, the sensitized erythrocytes were washed 3 times and finally diluted to 20 ml with phosphate-buffered saline. Serial twofold dilutions of serum were prepared with the buffered saline in perspex trays (2). To each well containing 0.1 ml of serum dilution, an equal volume of the erythrocyte suspension was added. The trays were kept at room temperature for 18 hours and shaken occasionally. Sensitized erythrocytes in saline only, and serum with unsensitized erythrocytes, were used as controls in each experiment. Isohemagglutinin titers with human red cells instead of sensitized sheep erythrocytes were determined in the same way.

Hemagglutination Inhibition.—In most experiments, a dilution series of antigen, used as inhibitor, was added to the appropriately diluted sera. The serum dilutions varied from 4 to 8 hemagglutinating units (HU) (4 to 8 HU = serum diluted 4 to 8 times less than highest dilution causing hemagglutination). The inhibitor was serially diluted in twofold dilution steps with antigen concentrations ranging from 8 to 1000 $\mu\text{g}/\text{ml}$. 0.1 ml of each antigen dilution was added to equal volumes of the appropriate serum dilution. After 30 minutes at room temperature, 0.1 ml sensitized sheep red blood cells or human erythrocytes were added to each mixture. Hemagglutination was read as above after 3 to 4 hours' incubation at room temperature. The hemagglutinating titers of serum without inhibitor were determined in each experiment. Homologous inhibitions with the antigen used for sensitization, or with the appropriate human red blood cells (*cf.* below), were always performed. In addition to the controls mentioned in the previous section, a control containing the inhibitor in its highest concentration, 4 to 8 HU of serum, and unsensitized sheep erythrocytes was also set up.

In some experiments, a constant amount of inhibitor was added to a dilution series of serum, made up in twofold dilution steps. To 0.1 ml of each serum dilution was added 0.1 ml of inhibitor, containing 2.00 mg/ml. After 30 minutes 0.1 ml of the test erythrocytes was added and hemagglutination was read as described.

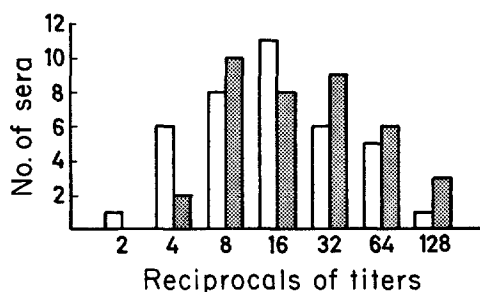
Absorption.—Packed and washed human erythrocytes of proper ABO types were mixed with equal volumes of undiluted serum and the mixtures were incubated for 30 minutes at room temperature. In most cases one absorption was sufficient to remove the corresponding isohemagglutinins. In a few cases absorption had to be repeated once or twice. The completeness of the absorptions was always controlled. In the experiments in which antibody titers were compared before and after absorption sheep erythrocytes were added to the samples called "before absorption" in order to compensate for the dilution.

Fluorescent Antibody Staining.—The indirect method was used throughout (13). Ethanol fixed and dried cryostat sections (5 μ) of freshly dissected proximal colon and distal cecum from 3 germfree rats were incubated for 30 minutes with undiluted or diluted serum. After washing the sections with buffered saline and drying in a stream of cold air, fluorescein-conjugated rabbit anti-human gamma globulin was added. The human gamma globulin was a commercial Cohn fraction II preparation, further purified by chromatography and gel filtration. Conjugation was performed with fluorescein isothiocyanate on celite (Microbiological Associates, Bethesda, Maryland), according to Rinderknecht (14), using the ammonium sulfate-precipitated gamma globulin fraction of a hyperimmune rabbit serum. The fluorescein/protein ratio was 3.2×10^{-3} (15). The sections were incubated with the conjugate at 37°C for 40 minutes. After washing they were inspected in a fluorescence microscope (Reichert Zetopan, Vienna, Austria, equipped with a Osram HB-200 Hg-burner). For control, tissue sections were incubated with the conjugate alone, or with twofold serial dilutions of serum, first absorbed either with germfree rat cecum (2.0 mg/ml) or with human erythrocytes (blood group A₁). Microphotographs were taken on Kodak tri-X-pan emulsion and an automatic photometer.

RESULTS

Human sera could be expected to hemagglutinate sheep erythrocytes, sensitized with cecum extracts from germfree rats, if these extracts contained human blood group-like antigens. In a first series of experiments, the effect of absorption with human A₁ erythrocytes on this hemagglutination was examined. Text-fig. 1 shows the results of an experiment in which 38 unselected ulcerative colitis sera were tested. As can be seen, this absorption had little effect on the antibody titers against the cecum extract. Only in a few sera were the antibody titers reduced significantly (≥ 2 dilution steps). All these sera also had a measurable titer when tested with human A₁ erythrocytes (before absorption).

In order to investigate the significance of this finding, individual sera were



TEXT-FIG. 1. Hemagglutination of rat cecum-sensitized sheep erythrocytes with ulcerative colitis sera before and after absorption with human A₁ erythrocytes. *Ordinate*, No. of sera. *Abscissa*, reciprocals of titers. *Stippled bars*, titers before absorption. *Empty bars*, titers after absorption.

selected for closer examination. These were taken from a sample of 38 ulcerative colitis sera and another of 56 unselected controls, comprising healthy persons and patients with unrelated diseases. Only those sera were chosen which (a) contained anti-A isohemagglutinins, and (b) had a hemagglutination titer $\geq \frac{1}{8}$ against sheep red cells sensitized with rat cecum. The percentage of sera with anti-A isohemagglutinins was 45 and 52 per cent respectively (difference statistically not significant) and there was no difference in their anti-A titers. In the ulcerative colitis group, 16 out of 38 sera fulfilled the criteria given. In the control group, this number was only 11 due to the fact that the anticecum titers were $\leq \frac{1}{4}$ in several sera which contained anti-A isohemagglutinins. The results of A₁ erythrocyte absorptions on the hemagglutinating titers against cecum-sensitized sheep red cells are shown in Table I. It is evident that absorption led to titer reductions in both groups. However, in only 6 out of 16 cases in the ulcerative colitis group was this reduction ≥ 2 dilution steps. In the control group, a corresponding reduction was found in 9 out of the 11 sera. This difference between the groups was statistically highly significant. ($P =$

0.02 when tested by the χ^2 test). Moreover, the highest titer reduction in the ulcerative colitis group was 3 dilution steps encountered in only one serum, while it was 3 or more dilution steps in 4 of the control sera.

The possible occurrence of other antigens of the ABO system was studied in similar experiments. When 25 ulcerative colitis sera containing anti-B isohemagglutinins, were absorbed with human B erythrocytes, no reduction of the anticecum titers ensued. The same was found in the controls (9 out of 40 containing both anti-B antibodies and anticecum titer $> \frac{1}{8}$). The anticecum titers of sera containing both anti-A and anti-B also remained unchanged when absorbed with B erythrocytes after previous absorption with A_1 erythrocytes. Absorption with human O erythrocytes of 15 ulcerative colitis sera and 14 control sera, which had anticecum titers $> \frac{1}{8}$ did not affect hemagglutination of

TABLE I
Hemagglutination Titers against Cecum-Sensitized Sheep Erythrocytes in Sera of Patients with Ulcerative Colitis and in Controls before and after Absorption with Human A_1 Erythrocytes

Type of serum	Absorption*	Reciprocal of hemagglutination titers							
		0	2	4	8	16	32	64	128
UC	-	-	-	-	2	3	4	4	3
	+	-	-	1	4	5	3	2	1
Controls	-	-	-	-	4	3	1	3	-
	+	2	-	5	2	2	-	-	-

* -, before absorption; +, after absorption.

sensitized sheep erythrocytes. None of these sera agglutinated human type O cells.

Further evidence of the presence of blood group A-like antigen in the extract of germfree rat cecum was obtained by studying its inhibiting action on the hemagglutination of human red blood cells. Table II shows the typical results of a series of hemagglutination inhibition experiments. As may be seen, rat cecum significantly inhibited the reaction between anti-A isohemagglutinins and blood cells of blood group A_1 or A_1B , while it did not affect the B/anti-B reaction.

It was of interest to establish whether the rat extract also contained antigen similar to the H substance of the human ABO system. This was studied by means of eel serum, known to contain anti-H agglutinins (8). Table III gives the results of experiments with such a serum. In this experiment, extract of feces from the germfree rats was used for sensitization of the sheep erythrocytes. As can be seen, eel serum agglutinated both sheep erythrocytes, sensi-

tized with rat fecal extract, and human type O erythrocytes. In both cases, hemagglutination could be inhibited completely with human O erythrocytes. Table IV shows that both the rat feces and cecum also inhibited eel serum when tested with human O erythrocytes.

TABLE II
Hemagglutination of Human Erythrocytes with Normal Human Serum before and after Inhibition with Cecum Extract of Germfree Rats

Test erythrocytes	Treatment of serum*	Serum No.†			
		1	2	3	4
A ₁	—	64	256	—	32
	+	1	2	—	1
B	—	16	128	128	—
	+	16	64	128	—
A ₁ B	—	64	128	64	16
	+	8	8	64	1

* —, no inhibitor added. The numbers are reciprocal values of hemagglutination titers; +, inhibitor added. The numbers are reciprocal values of the lowest serum dilution completely inhibited by 2.00 mg/ml cecum extract.

† 1, normal human serum, blood group O; 2, normal human serum, blood group O; 3, normal human serum, blood group A; 4, normal human serum, blood group B.

TABLE III
Hemagglutination of Feces-Sensitized Sheep Erythrocytes and of Human Erythrocytes with Eel Serum before and after Absorption with Human Group O Erythrocytes*

Absorption	Reciprocal values of hemagglutination titers against				
	Rat feces	O erythrocytes	A ₁ erythrocytes	B erythrocytes	A ₁ B erythrocytes
—	256	512	<8	8	<8
Once	8	<8	—	—	—
Twice	<8	<8	—	—	—

* Eel serum absorbed twice with human A₁B erythrocytes before use.

These experiments provided good evidence that the cecum extract of germ-free rats contained human blood group-specific antigens (A and H but not B), as well as a "colon"-specific antigen, recognized by its reaction with antibodies in the ulcerative colitis sera. In order to study the distribution of these antigens in different rat organs, hemagglutination inhibition experiments were performed with various rat extracts and a number of different sera. The colon-

specific antigen was assayed with the serum of a person of blood group A, tested with feces-sensitized sheep erythrocytes (titer 1/32). The A antigen was similarly assayed with serum of a person of blood group O and type A₁ erythrocytes (titer 1/128), and the B antigen with another group O serum and type B erythrocytes (titer 1/32). Finally, the H antigen was assayed with eel serum and type O erythrocytes (titer 1/4096). As can be seen from Table IV none of the rat specimens used for inhibition contained blood group B-like antigen detectable by this method. In contrast, both feces and extracts from various parts of

TABLE IV
Hemagglutination of Feces-Sensitized Sheep Erythrocytes and of Human Erythrocytes with Different Human Sera after Inhibition with Different Rat Extracts

Inhibiting extracts	Serum No.* and test antigens			
	1 Rat feces	2 A ₁ erythrocytes	3 B erythrocytes	4 O erythrocytes
Feces	≤8	≤8	>1000	≤8
Cecum	8	≤8	>1000	≤8
Small intestine	125	64	>1000	≤8
Ventricle	64	<8	>1000	≤8
Liver	>1000	>1000	>1000	500
Kidney	125	>1000	>1000	250
Lung	125	>1000	>1000	500

The numbers in the table give the minimal quantities of antigen necessary for complete inhibition of the hemagglutinating units (HU) indicated for each serum.

* 1, 4 HU of ulcerative colitis serum, blood group A; 2, 8 HU of ulcerative colitis serum, blood group O; 3, 4 HU of ulcerative colitis serum, blood group O; 4, 4 HU of eel serum (not absorbed with A₁B erythrocytes).

the gastrointestinal tract contained A as well as H antigens. As shown earlier (6), the colon-specific antigen was predominant in cecum and also in feces. It may be mentioned that the semisynthetic diet given to the rats has earlier been shown to be free from the colon-specific antigen (6). When tested in the same way as shown in Table IV, no blood group antigens were found.

Since the colon antigen and the blood group A antigen were the most important antigens in the hemagglutination tests, their localization in the rat large intestine was studied by means of fluorescent antibody staining. Figs. 1 and 2 show the staining obtained with the sera of 2 patients, one of blood group A, the other of blood group O, (absorbed with human A₁ erythrocytes). Fig. 3 shows the typical results of a similar experiment with the serum of a healthy person of high anti-A titer. As can be seen, an identical cytoplasmic staining of colon mucosa was obtained in all cases. Most conspicuous was the staining of goblet cells in the crypts. Staining of the mucus, when present, was also

seen, whereas the submucosal layers remained unstained. The staining obtained with the ulcerative colitis sera could be blocked completely by absorption with rat feces. Absorption with A₁ erythrocytes entirely removed the staining obtained with 5 normal sera containing anti-A isohemagglutinins. Normal sera from blood group A individuals gave no staining.

DISCUSSION

The results presented in this paper indicate that organs of the strain of germ-free rats used here contain antigen similar to human blood group A antigen, but none similar to blood group B antigen. In this respect, our findings confirm those published recently by Halpern *et al.* (16). Using fluorescent antibody staining, these authors found blood group A-like antigen in conventional rat colon, but not in a number of other organs, including rat stomach. This is similar to our results with the hemagglutination inhibition technique, except that we also found large amounts of this antigen in rat stomach, small intestine, and feces. In their rats, Halpern *et al.* also noted that the A antigen was present in all individual animals tested, in contrast to what was seen in dogs. Our limited experience points in the same direction, since cecum from 3 individual rats could be stained with human anti-A isohemagglutinin, and pooled extracts from 10 animals in hemagglutination inhibition experiments had the same inhibiting capacity as extracts made from individual animals.

The blood group A-like antigen was present in phenol-water extracts of the rat organs. These extracts also contained a H-like antigen, typical for the human ABO system. Both antigens had a similar, although not quite identical, organ distribution and were present also in the same extracts from individual rats.

The experiments with ulcerative colitis sera demonstrated conclusively that the extracts contain a "colon"-specific antigen, immunologically distinct from the blood group A and H antigens (as well as from the B antigen). While absorption with human type A₁ erythrocytes reduced the hemagglutination titer with rat antigen slightly, and only in some ulcerative colitis sera, this reduction was strong in the controls. In fact, the majority of the elevated titers among the controls was due to the reaction between anti-A isohemagglutinins and the A antigen in the rat extracts. Since such hemagglutinins are present in about 50 per cent of the sera in a Swedish population, this explains most of the "background" reactivity noted earlier in the controls. Routine absorption with type A₁ erythrocytes sharpens the difference between ulcerative colitis patients and controls, when assayed with rat antigen in the hemagglutination test. No conclusion can as yet be drawn as to the nature of the antibodies which could not be removed from the control sera by absorption with human erythrocytes. However, these hemagglutination titers were low and of little significance. Since no such titers were encountered in our previous

experiments with human antigen, it may perhaps be assumed that the antigen involved was different from the colon antigen demonstrable with the ulcerative colitis sera.

Earlier experiments showed, that the colon antigen of the rat is similar to that found in colonic extracts from humans (6). Since only about 40 per cent of the patient's sera, after absorption with A₁ erythrocytes, had a significantly elevated hemagglutination titer with the rat antigen, while this figure was 90 per cent in similarly selected sera when tested with the human antigen (1, 2), it may be that the rat colon antigen lacks some of the determinants present in the human colon antigen. Further work is required to clarify this point. The data presented here also confirm and extend our earlier results (6) which showed that this antigen is tissue specific in so far as it is most abundant in the lower parts of the gastrointestinal tract. The finding of large amounts of antigen in the feces of the germfree animals and the results of the fluorescent antibody staining both suggest that the antigen is part of the mucus.

Unpublished chemical data, indicate that the colon antigen as well as the A- and H-like antigens are all of mucopolysaccharide nature. However, the extracts were physicochemically heterogenous. When studied by means of fractional precipitation and electrophoresis, the blood group antigens and the organ-specific colon antigen were found to be enriched in the same fractions. Fluorescent antibody staining also suggests that these antigens are related in some way, since they are localized to the same sites of the colon tissue. It is interesting to speculate whether we are dealing with 2 or 3 types of molecules of different immunological specificities, or with a common carrier molecule possessing both colon, A- and H-specific determinants. However, the data available so far are insufficient to clarify this problem. It must also be emphasized that the situation in man may be different from that in the rat. In regard to the A antigen, previous experiments with human material give no clues, since both immunochemical experiments and fluorescent antibody staining were performed with specimens from individuals of blood group O (1, 4, 5). On the other hand, in regard to the H antigen, our earlier experiments suggested that extracts of human colon contained both colon antigen and some H antigen. Moreover, the H antigen and the immunologically distinct colon antigen also had a similar distribution in human colon mucosa (4).

SUMMARY

Sera from patients with ulcerative colitis contain antibodies which hemagglutinate sheep red cells, sensitized with phenol-water extracts from colon, cecum, or feces of germfree rats. Minor concentrations of such antibodies are also present in a certain fraction of normal human sera. Hemagglutination and hemagglutination inhibition experiments with human erythrocytes and with the rat extracts showed that the latter contained an antigen similar to human

blood group A antigen. In contrast, a blood group B-like antigen could not be detected in these extracts. However, experiments with eel serum indicated that these extracts also contained an antigen similar to the H antigen of the human ABO system.

Absorption of ulcerative colitis sera with human A₁ erythrocytes but not that with B or O erythrocytes gave, in a few cases, a slight reduction of the hemagglutinating titers against rat cecum-sensitized sheep erythrocytes. In contrast, this treatment considerably reduced such titers when found in sera from healthy persons or from patients with unrelated diseases. It could be concluded that the rat extracts also contained a "colon" antigen, detected with antibodies, present at elevated titers, in the sera of ulcerative colitis patients, but not in those of the controls. This colon antigen is immunologically distinct from the blood group antigens studied.

Hemagglutination inhibition experiments indicated that A, H and colon antigen were widely distributed throughout the gastrointestinal tract of the germfree rats. The colon antigen was found to be enriched in the extracts from colon, cecum, and feces. Fluorescent antibody staining provided evidence that both the colon antigen and the A antigen were present in similar sites of the colon and cecum mucosa, particularly in goblet cells of the crypts, and in the mucus.

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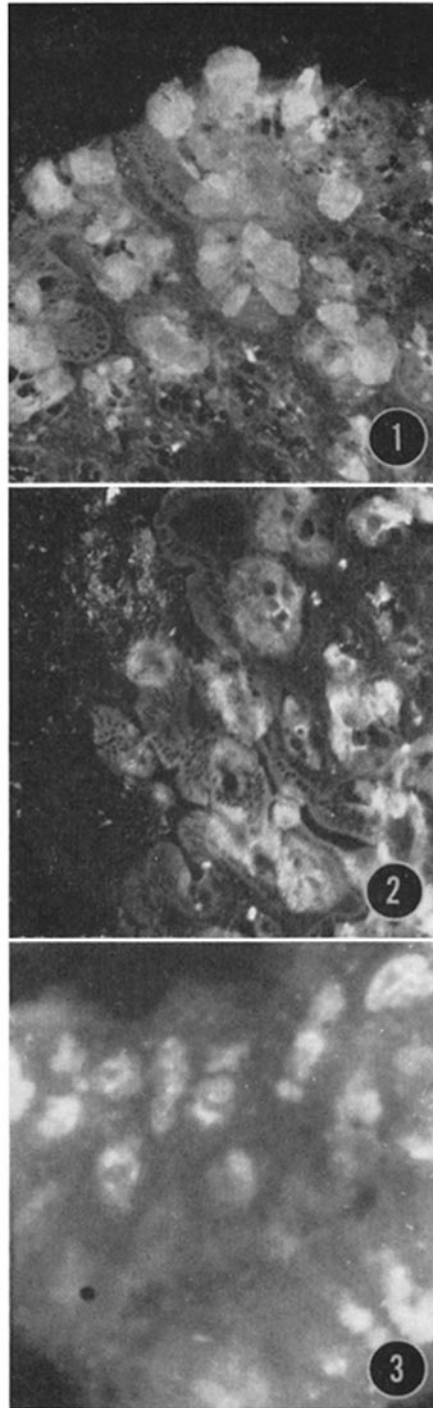
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EXPLANATION OF PLATE 65

FIG. 1. Section of germfree rat colon mucosa stained by the indirect method showing absorption of antibodies from serum of an ulcerative colitis patient of blood group A onto the goblet cells in the crypts. $\times 300$.

FIG. 2. Section of germfree rat cecum mucosa stained by the indirect method showing absorption of antibodies from serum of an ulcerative colitis patient of blood group O (serum previously absorbed with human A₁ erythrocytes) onto the goblet cells in the crypts. $\times 300$.

FIG. 3. Section of germfree rat colon mucosa stained by the indirect method showing absorption of antibodies from serum of a healthy person of blood group O onto the goblet cells in the crypts. This serum contains A agglutinins in high titer. $\times 300$.



(Hammarström *et al.*: Immunological studies in ulcerative colitis. II)