IN VITRO STUDIES OF ULCERATIVE COLITIS

I. Reactions of Patients' Serum with Human Fetal Colon Cells in Tissue Cultures*

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Plate 40

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It has previously been shown that sera from patients with ulcerative colitis contain antibodies that react with constituents of sterile human colonic tissue (1, 2). Since the clinical and pathological features of this disease are indicative of an immunologic disorder (3, 4), it is pertinent to establish whether or not such antibodies participate in its pathogenesis.

If the anticolon antibodies in ulcerative colitis were of significance for the disease, they might be expected to damage living colon cells. As it has not been possible so far, in this laboratory, to produce an autoimmune ulcerative colitis in animals, this problem was approached by studying the effect of anti-colon antibodies on human colon cells *in vitro*. As will be reported in this communication, fluorescent antibodies from ulcerative colitis sera were adsorbed onto human colon cells in tissue culture, thus indicating that the cells retained an immunological reactivity. In spite of this, *in vitro* exposure of such cells to patients' sera did not lead to any noticeable cytotoxic damage under various experimental conditions.

Materials and Methods

Colonic Tissue.—Human colonic tissue was obtained from 20- to 24-week-old fetuses (legal abortions, blood group O) delivered by Caesarian section. The material was taken under aseptic conditions immediately at operation.

Sera.—Sera from 13 children suffering from ulcerative colitis (U.C.) were used as antisera in the staining experiments. All sera agglutinated sheep red blood cells coated with antigen prepared by extraction of sterile human colon with phenol-water at 65°C (1). Titers, determined as described earlier, are given in Table I. γ-Globulin was prepared from 6 of these by fractionation on diethylaminoethyl cellulose as described previously (5).

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Twelve sera from healthy children and adults and isolated γ-globulin from 5 of these were used as controls (normal). For the detection of blood group substance on the cells, isolated H agglutinins from eel serum were used (5).

TABLE I

Direct Staining of Tissue Cultures

Conjugate	Type	Hemagglutinating titer* of serum	Staining with	
			Whole serum	γ-Globulin
I.S-n.	U.C.	256	+	+
A.K-n.	"	256	−	−
B.H-t.	"	256	+	+
G.J-n.	"	128	−	−
R.E-n.	"	128	+	+
E.L-n.	"	128	−	
E.P-r.	"	64	+	
El.N-n.	"	64	+	
S.G-n.	"	32	−	
Å.A-l.	"	32	−	
E.N-n.	"	16	−	−
U.A-n.	"	8	−	
K.W-n.	"	—	−	
B.G-e.	Normal	—	−	
S.K-n.	"	—	−	
O.B-r.	"	—	−	−
B.W-l.	"	—	−	
C.W-m.	"	—	−	
S.B-w.	"	—	−	−
H.P-n.	"	—	−	
A.D-n.	"	—	−	−
O.J-n.	"	—	−	−
Å.L-g.	"	—	−	−
J.H-s.	"	—	−	
E.B-m.	"	—	−	

+, positive staining; −, no visible staining.

* Reciprocal of highest dilution yielding macroscopic hemagglutination of red blood cells from sheep, coated with phenol-water extract of human colon (1). All titers refer to determinations made in one series of experiments with aliquots of the same batch of coated red cells.

Preparation of Conjugates.—Commercially available samples of fluorescein isothiocyanate (Sigma Chemical Company, St. Louis) or lissamine rhodamine B200 (ICI) were used for conjugation. Details of the conjugation, which followed standard procedures, and subsequent purification by means of gel filtration have been given earlier (5).

Preparation of Tissue Cultures.—Fetal colonic tissue, taken sterilely at operation, was cut into small pieces, suspended in Eagle's complete medium (6), and trypsinized for 2 hours at 37°C with continuous stirring. The cells were then centrifuged off at low speed (400 *g*, 4

minutes), washed twice in Eagle's medium, and finally distributed to small Carell vessels containing Eagle's medium and human serum in equal parts (total volume 1.5 ml). The serum added to the medium was inactivated by heating to 56°C for 20 minutes. The medium was changed at 3-day intervals. Generally, the cells required 4 to 5 days of cultivation to grow out and attach to the glass.

Staining.—The direct method was used in all experiments. Tissue cultures of colonic cells were incubated with 1 ml of conjugated serum or γ-globulin for 15 minutes at 37°C and then thoroughly washed 5 to 6 times with Eagle's medium while still attached to the glass. Subsequently, the cells were trypsinized (0.25 per cent trypsin, Difco 1:250) and transferred to centrifuge tubes. After centrifugation at 500 *g* for 5 minutes, the cells were again washed several times with Eagle's medium and finally spread onto quartz glass microscope slides.

The specificity of the immunological staining was tested in a series of autoinhibition and cross-inhibition experiments (7). In the autoinhibition experiments, the tissue cultures were incubated 15 minutes at 37°C with an unconjugated sample of antiserum, prior to incubation with a conjugated sample of the same antiserum. In the cross-inhibition experiments, unconjugated samples of various sera were applied first. Then the pretreated cultures were incubated with a conjugated sample of a strongly staining serum (or γ-globulin) either from patients or from eel.

Microscopy.—The sections were inspected by ultraviolet fluorescence microscopy and photographed as described earlier (5).

Cytotoxicity Tests.—In the first series of experiments, 4- to 5-day-old tissue cultures of colonic cells were exposed to fresh sera from 8 patients with ulcerative colitis and from 7 healthy persons. In these experiments, tissue cultures containing approximately 3×10^5 colonic cells were incubated for 12 hours at 37°C with a mixture containing 1 ml of the fresh serum to be tested and 1 ml of Eagle's medium to which had been added 0.2 ml of fresh guinea pig serum (complement). After 12 and 24 hours, the cultures were inspected and the number of cells still attached to the glass was counted.

In the second series of experiments, the finding of several authors, *viz.* that the release of isotopic material can be used as a sensitive indicator of cell damage caused by cytotoxic antibodies, was utilized (8–11). In these experiments, freshly removed fetal colon was cut into small pieces and trypsinized (0.5 per cent trypsin) for 3 hours at 37°C in Eagle's complete medium containing 10 per cent of heat-inactivated human serum. After 3 washings in medium, the cells were transferred to a test tube containing 1.5 ml phosphate-free Eagle's medium to which had been added 100 μc of carrier-free inorganic ^{32}P-orthophosphate (The Radiochemical Centre, Amersham, Buckinghamshire, England). After 2 to 3 hours of incubation at 37°C in an atmosphere of 95 per cent O_2 and 5 per cent CO_2, the cells were washed in Eagle's complete medium (containing unlabeled phosphate) by 3 to 4 centrifugations until the radioactivity of the medium was stable and low. An approximately equal number of viable cells (viability test performed with lissamine green, reference 12) in 0.2 ml was transferred to small test tubes (about 2×10^6 cells/tube). To these were added either 0.6 ml of fresh patients' serum or normal serum, 0.3 ml of fresh guinea pig serum, and Eagle's medium to a final volume of 1.8 ml. The mixtures were incubated at 37°C. After gentle centrifugation, samples of 0.6 ml were taken from the medium at different time intervals. The cells were immediately resuspended and the volume was restored by addition of 0.6 ml of a mixture consisting of Eagle's medium, human serum used in the test, and guinea pig serum in the appropriate proportions. The suspension was then reincubated at 37°C.

After completion of the incubation, cells and medium were separated by centrifugation. From all supernatants recovered during incubation, 0.2 ml was removed for determination of the radioactivity of the total phosphorus. From the rest (0.4 ml), inorganic phosphorus was extracted according to Martin and Doty (13). The radioactivity present in the cells at the end

of the experiments was similarly determined on small aliquots of the final cell suspension. The radioactivity of the organically bound phosphorus of the samples was then obtained as the difference between the total radioactivity and the radioactivity of the inorganic phosphorus. Measurements were made with an automatic scaler and a liquid-counting Geiger-Müller tube. The radioactivity found in the medium after different times of incubation had to be corrected for the radioactivity removed for previous measurements. Since $\frac{1}{8}$ of the total medium was removed for each determination and $\frac{1}{8}$ of this was used for measuring total ^{32}P, the true amounts of the total ^{32}P released after different times could be calculated by multiplying the C.P.M. measured with the correction factor $3^{n+1}/2^{n-1}$ where $n = 1, 2, 3 \cdots$ (samples taken at time 1, 2, 3 $\cdots$). Similarly, since $\frac{7}{8}$ of the volume removed each time was used for measuring inorganic ^{32}P, the true amount of the latter was obtained by multiplying with the correction factor $3^{n+1}/2^{n}$.

For the calculation of the *relative* release of isotope from cells to medium, the sum of the radioactivities recovered in both the medium and the cells at the end of the experiment was used as the basis (= 100 per cent). This method of calculation was adopted when it was found in pilot experiments that (*a*) the radioactivity of the medium at the beginning of the experiment was not significant when compared with that in the cells, and (*b*) the error introduced during incubation and fractionation was much smaller than the sampling error arising because of unequal distribution of labeled cells among the different tubes.

RESULTS

In general, the colonic cells required 4 to 5 days of cultivation to grow out and attach to the glass. All cells were spindle-shaped and of a fibroblast like appearance. However, when such cultures were incubated with conjugated serum or γ-globulin from patients with ulcerative colitis, an adsorption of the fluorescent label onto cells could be observed (Fig. 1). In all, 5 of the 13 patients' sera tested gave positive results, whereas the 12 control sera or γ-globulin fractions were all negative. The results were the same regardless of the fluorescent label used. As noted in the previous paper (5), there was a certain correlation between the staining capacities of the sera and their hemagglutinating titers (Table I). Staining appeared mainly on the cell surfaces and was of variable intensity. The number of stained cells was variable. Staining was observed in approximately 20 to 30 per cent of the cells derived from fresh cultures. Nonspecific staining of presumably dead or injured cells occurred occasionally and was always weaker and of a different appearance. The best results were obtained with tissue cultures which were 4 to 5 days old. Longer cultivation resulted both in weaker staining and a lower incidence of stained cells. Cultures more than 12 days old never showed any positive reactions.

The results indicate that antibodies present in the sera of patients were adsorbed onto fetal colonic cells in tissue culture. The immunological nature of the staining could be confirmed by inhibition tests (Table II). Thus, no staining was obtained with any of the 5 positive sera when the tissue cultures had been preincubated with unconjugated samples of the same sera. It will also be noted that normal human serum, which was present in the culture mediums did not prevent staining with ulcerative colitis serum.

As previously shown (5), the presence of blood group substance of the ABO system could be demonstrated in colon slices by applying fluorescent H agglutinins from eel. Similarly, fluorescein-labeled H agglutinins were also adsorbed onto colonic cells in culture (Fig. 2). The incidence of staining was similar to that obtained with the patients' sera but of lower intensity. Even in this case, the intensity of the staining decreased with increasing age of the cultures, but staining persisted for a longer period (at least 20 days) than after treatment with the patients' sera. The autoinhibition tests recorded in Table II confirm the immunological specificity of the staining. That there is no immunological relationship between the staining antibodies of ulcerative colitis

TABLE II

Inhibition Tests with Sera from Ulcerative Colitis Patients and with H Agglutinins from Eel

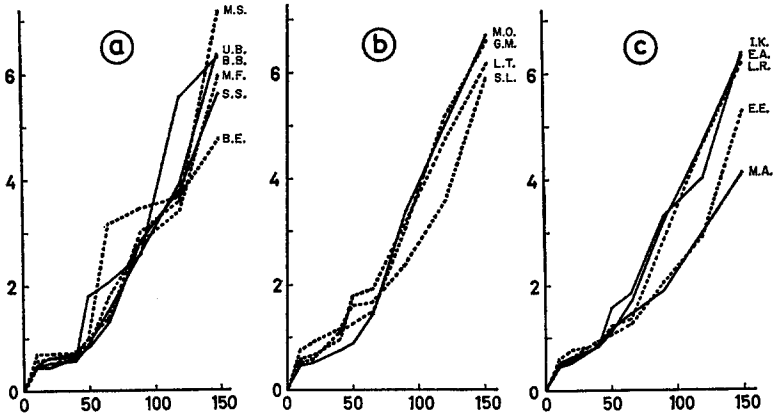
Preincubation with unconjugated sample from	Incubation with conjugated sample from	Staining
I.S-n. (U.C.)	I.S-n. (U.C.)	−
B.H-t. "	B.H-t. "	−
R.E-n. "	R.E-n. "	−
E.P-r. "	E.P-r. "	−
El.N-n. "	El.N-n. "	−
No preincubation	H agglutinins (eel)	+
H agglutinins (eel)	" " "	−
" " "	I.S-n. (U.C.)	+
" " "	B.H-t. "	+
I.S-n. (U.C.)	H agglutinins (eel)	+
B.H-t. "	" " "	+

+, positive staining; −, no visible staining.

sera and the H agglutinins from eels can be concluded from the results of cross-inhibition experiments also recorded in Table II.

The results of the immunofluorescent studies showed that the fetal colonic cells, kept in tissue culture, retained a marked reactivity with antibodies in the sera of the patients for a relatively long period of time. This suggested that they also might constitute useful target cells for the cytotoxic action of ulcerative colitis antibodies. Therefore, tissue cultures of human colon cells were exposed to sera from children with ulcerative colitis, in the presence of fresh guinea pig serum as the source of complement. However, no cytotoxic effect was obtained under the conditions described above. Thus, there was no reduction of the number of living cells in the cultures. Moreover, morphological studies did not reveal any gross changes in the microscopic appearance of the cells. The pseudopodia did not retract and neither vacuolization of the cytoplasm nor swelling of the mitochondria was observed (see reference 14).

Since it could be assumed that a more sensitive method might reveal a possible cytotoxic effect of the patients' sera, a second series of experiments was performed. In this, fresh explants of fetal colon were trypsinized, incubated with radioactive isotope, and finally exposed to the test sera as described above under Materials and Methods. The results of 3 experiments are given in Text-fig. 1. In this series, the sera from 8 different patients and 7 healthy individuals were tested. The colonic explants used in the 3 experiments were also obtained from different fetuses. The patients' sera used in these tests were all of a high hemagglutinating titer.



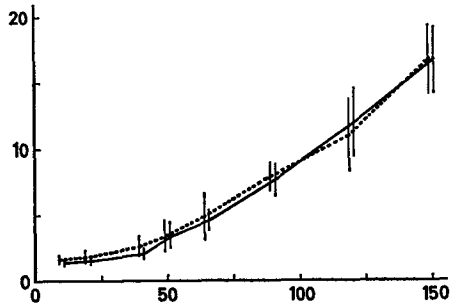
TEXT-FIG. 1. Release of ^{32}P from colon cells upon incubation with serum from patients with ulcerative colitis (dotted lines) or healthy individuals (solid lines). Text-fig. 1 *a* to *c* represent 3 experiments made with colon from different fetuses. The initials of the serum donors are designated by capital letters. For further details see Materials and Methods.

Ordinates: Cumulative values of radioactivity (C.P.M. $\times 10^{-4}$) found in the medium. *Abscissae:* Time of incubation of colon cells with white cells (minutes).

Text-fig. 1 shows that, under the present conditions of incubation, the same amount of total ^{32}P could be recovered from the medium, regardless of the type of serum used. Identical results were obtained when the radioactivity of organically bound ^{32}P was measured in the medium. The radioactivity of this fraction constituted approximately 30 per cent of that present as total ^{32}P. The results were also similar in a few experiments where the antiserum/cell ratio was higher and/or higher concentrations of complement were used.

Since the amount of ^{32}P present in the different incubation mixtures of Text-fig. 1 was variable, the release into the medium was recalculated as the percentage of the amount present in the cells at time 0. (see Materials and Methods.) Text-fig. 2 gives the mean values and standard deviations for this relative release calculated for each time from the release values depicted in Text-fig. 1. When tested with Student's t test, no significant differences

between the effects of normal and patients' sera were found in any case. As will be seen, after 150 minutes of incubation, almost 20 per cent of the isotope was given off by the cells. The reasons for this high non-specific release of isotope are unknown. It can be assumed, however, that it reflects damage done to the cells by trypsinization and repeated centrifugation (15). Moreover, under the present conditions, prolonged incubation led to the death of a large proportion of the cells. Hence, when the incubation time was more than 150 minutes, the percentage of isotope released into the medium increased considerably, making significant measurements impossible.



TEXT-FIG. 2. Relative release of ^{32}P from colon cells upon incubation with serum from patients with ulcerative colitis (dotted line) or healthy individuals (solid line). The curves show the mean values of the relative release calculated from those found in each of the 8 incubations with patients' serum and 7 controls, plotted in Text-fig. 1. The vertical lines are the standard deviations calculated for the measurements at each time. (The SD lines of the patients' samples and controls are drawn side by side to avoid overlapping, although the measurements were made at the same times in all experiments). For further details see Materials and Methods.

Ordinate: Amount of total ^{32}P in the medium given as the percentage of the total ^{32}P present in the cells at the beginning of the experiment. *Abscissa:* Time of incubation of colon cells with serum (minutes).

DISCUSSION

The first part of the present investigation has given evidence that ulcerative colitis sera contain antibodies that react with an antigen (or antigens) present on fetal colon cells in tissue culture. This confirms the previous findings made by Broberger and Perlmann (5) and by Koffler *et al.* (16) that ulcerative colitis sera contain antibodies reacting with an epithelial antigen of colonic mucosa *in situ*. As in the previous study, the incidence of directly staining sera was relatively low and correlated with their hemagglutinating titers. Various types of inhibition experiments with normal sera or γ-globulins proved that the labeling was immunologically specific. The results also indicated that there was no significant non-specific uptake of dye through pinocytosis, as has been observed in experiments with certain types of living cells (17). It could well be that the short period of incubation used here (15 minutes) may have prevented a more extensive non-specific uptake of conjugate (see reference 18).

As indicated in the text above, only a fraction (20 to 30 per cent) of the cells was labeled in this manner. Since there were no safe morphological criteria available, no distinctions could be made between different cell types in the cultures. The reasons for this behavior are unknown. Judging from the previous results obtained with tissue slices, it can be assumed that only certain cells carry (or produce) the staining antigen(s). It should be emphasized, however, that the present, as well as the preceding, experiments were performed with unfixed material which may have led to a loss of antigen from the cells.

As found in the study with colon slices, cultivated colon cells could also be stained with H agglutinins from eel. The pattern and frequency of staining were similar to those obtained with the ulcerative colitis sera. However, the antigens reacting in these cases were immunologically unrelated. As yet, it can not be decided, whether the same cells were carrying H substance and colitis antigen or whether the antigens were located on different cells.

Prolonged cultivation led to a gradual decrease and finally to a complete loss of stainability. This change in immunological behavior of cells in tissue culture has been observed frequently (19, 20). It is not known whether this change was due to a true loss of antigen from the relevant cells or due to a change in the composition of the cell population. Nevertheless, the time of disappearance of the H substance found in this investigation coincides well with that found by Högman using a mixed hemagglutination technique (21).

Although colitis antigen was still present at the time of testing, *in vitro* exposure of the colon cells, in the presence of fresh guinea pig serum, to sera from patients with ulcerative colitis did not cause any measurable injury. Thus, negative results were obtained both with freshly explanted cells and with cells kept in monolayer culture for a short period. This was true whether morphological or more sensitive biochemical criteria were used for measuring cytotoxicity. In this respect, the colon cells behave differently from human thyroid cells exposed to serum from patients with Hashimoto's thyroiditis and certain other thyroid disorders (22–24). Such sera contain a cytotoxic factor which destroys thyroid cells *in vitro* in the presence of complement. This factor is probably an antibody (25, 26). It should be mentioned however, that no cytotoxic effect was obtained with thyroid cells if they were kept in tissue culture for more than 36 hours (22, 26). It will be recalled that the colon cells required a longer time for attachment to the glass and outgrowth (4 to 5 days) and thus may have lost their possible susceptibility to cytotoxic antibodies.

Other reasons may account for the negative results. The localization of the staining antigen on the cells may be such that reactions with antibodies do not lead to cell damage or its concentration on the cell surface may be insufficient for cytotoxicity (27, 26). It is also possible that the antibodies, although reacting with an appropriate antigen, are lacking cytotoxic properties. Finally, although the experimental conditions were varied over a relatively wide range

and were similar to those of other authors who obtained positive results (*e.g.* references 14, 24), perhaps the antibody/cell ratios in the experiments were not high enough or the concentration of complement was too low to effectuate cell damage.

It is evident that no safe conclusions can be drawn from the negative results of the cytotoxicity tests. Nor can it be excluded that colon cells *in vivo* are damaged by the anticolon antibodies occurring in the circulation of patients with ulcerative colitis. However, the possibility remains that the anticolon antibodies of ulcerative colitis are harmless to colon tissue *in vivo*. As yet, the significance of humoral antibodies for tissue damage in human or experimental autoimmune disease has not been established with certainty (28). Instead, it has often been suggested that hypersensitivity reactions of the delayed type could be responsible for tissue destruction (29). Such reactions are commonly held to be mediated by sensitized cells. The results of an experimental approach to this problem will be described in the following paper.

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SUMMARY

By means of immunofluorescent methods it has been shown that sera from children with ulcerative colitis contain antibodies which react with fetal colon cells in tissue culture. 5 out of 13 sera from patients reacted positively when tested for staining antibodies while 12 sera from healthy individuals yielded negative results. The specificity of the staining reactions was confirmed by inhibition experiments. The staining capacity of various sera was correlated to their hemagglutinating titer when tested against phenol-water extracts of human colon.

The presence of blood group substances of the ABO system on fetal colon cells in tissue culture could be demonstrated by application of fluorescent H agglutinins from eel. Cross-inhibition experiments indicated that the H agglutinins stained colon antigens which were different from those reacting with the antibodies of ulcerative colitis sera.

The reactivity of cultured fetal colon cells with the antibodies in ulcerative colitis sera was retained for up to 12 days, with optimal staining at 4 to 5 days. Reactivity with H agglutinins was present for a longer period, sometimes more than 20 days.

Although antigen could be shown to be present on fetal colon cells in tissue culture, exposure of the culture, in the presence of fresh guinea pig serum, to

sera from patients with ulcerative colitis did not lead to any visible cytotoxic damage. In order to investigate the possible cytotoxic effect of the sera with a more sensitive technique, freshly explanted fetal colon was dispersed by trypsinization and the cells labeled with ^{32}P-orthophosphate. Subsequently, these cells were exposed to sera, in a final concentration of 30 per cent, from patients or healthy controls in the presence of fresh guinea pig serum (final concentration 15 per cent). Approximately 20 per cent of the cellular isotope was released into the medium within 150 minutes of incubation, but the release was the same in the samples treated either with patients' sera or normal control sera. Thus, under the present conditions, the patients' sera did not exert any specific cytotoxic action on colon cells.

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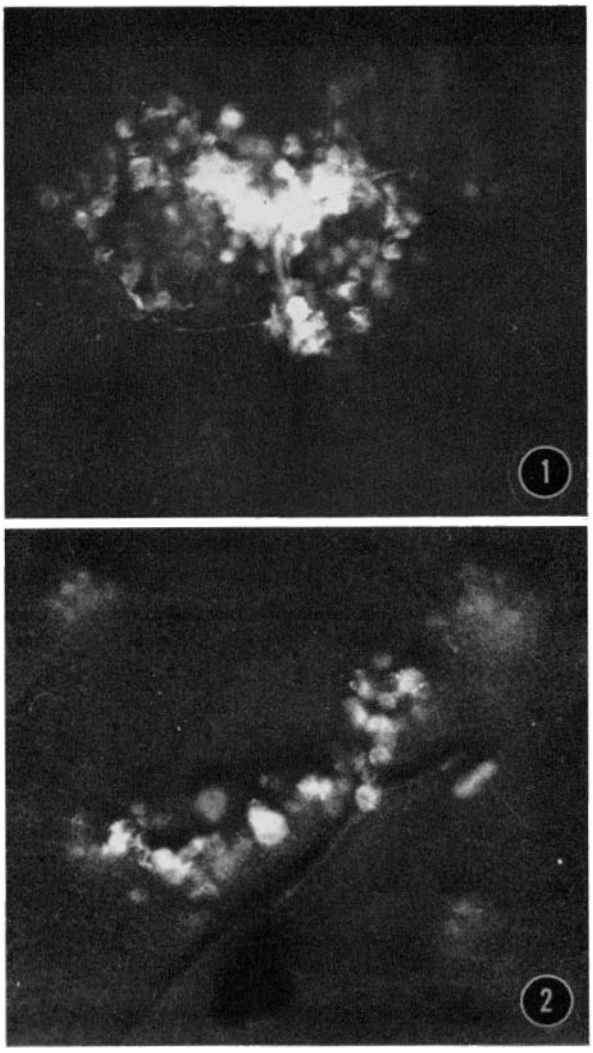
EXPLANATION OF PLATE 40

FIG. 1. Smear of fetal colon cells from a 4 day old culture, showing adsorption of conjugated (fluorescein isothiocyanate) serum from a patient with ulcerative colitis. × 320.

FIG. 2. Smear of fetal colon cells from a 6 day old culture, showing adsorption of conjugated (fluorescein isothiocyanate) H agglutinins from eel. × 320.



(Broberger and Perlmann: Ulcerative colitis. I)