# Autoantibodies to Colon in Rats and Human Ulcerative Colitis: Cross Reactivity with *Escherichia coli O:14* Antigen.* (32253)

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It is reasonably well established that the sera of patients with ulcerative colitis contains autoantibodies which react with colon antigen of both human and animal origin (1-3). The anti-colon antibodies seem to be typical for this disease(4,5). A cross reactive substance can be obtained in good yields from colon, cecum or even feces of germ free rats(6). The colon antigen is a heat stable polysaccharide, located in the mucus producing cells of the colon mucosa and also present in the mucus(7-11). It is chemically similar but immunologically distinct from the blood group antigens of the human ABO system(7,12).

The stimulus giving rise to autoimmunity in ulcerative colitis is unknown. By means of the fluorescent antibody techniques, it has been shown that autoantibodies against gut antigen can be produced in rabbits by injecting them with intestinal antigen from rats. Moreover, autoantibodies could also be demonstrated in a proportion of the rabbits immunized with different bacteria(13,14). These findings suggest that antigens of foreign origin may be of importance for the production of autoantibodies against colon in the human as well. To throw further light on this question, the formation of anti-rat colon antibodies in rats injected with either rat or rabbit colon, free from bacterial contamination, was investigated. The antibodies were then studied in regard to their reactivity with antigens of different animal or bacterial origin and were compared with the anti-colon antibodies in human ulcerative colitis.

*Materials and methods.* Male Sprague-Dawley rats (200 g) were injected with dialyzed and lyophilized tissue homogenates or bacterial extracts, incorporated into complete Freund's adjuvant.† The tissue homogenates were either obtained from colon or lung of germ free rats or from colon of newborn rabbits within one hour after parturition. The germ free rats were of the inbred Swedish strain,‡ fed the semisynthetic diet D:7 and reared according to Gustafsson(15,16). In the first two series, groups of 12 rats for each antigen were injected every second week with 2.5 mg of antigen in 0.4 ml buffer, mixed with an equal volume of the adjuvant. Each rat received 10-12 intraperitoneal injections. Blood samples were taken from all rats by cardiac puncture before the injections, as well as one week after the fourth and one week after the last injection. In a third series, the rats received 8 intraperitoneal and 4 intravenous injections with antigen without adjuvant, following a schedule described by Weigle for production of anti-thyroid autoantibodies in rabbits(17).

Sera from patients with ulcerative colitis were obtained from the Department of Pediatrics, Karolinska Hospital and from the Medical Department (Prof. B. Ihre), St. Erik's Hospital, Stockholm. Collecting and processing of the human sera have previously been described(5).

Antibody titers of the separated sera were determined by means of indirect hemagglutination of antigen coated sheep erythrocytes. The sera were heat-inactivated and absorbed with sheep cells as previously described(12). Test antigens were obtained by extraction of the sterile tissues, feces of germ free rats, or bacteria, with phenol-water (1:1) at 65°C and precipitation from the water phase with

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ethanol(18). All *E. coli* antigens used were from bacteria grown on a synthetic medium for one to 3 passages. (Grown for 18 hours at 37°C in one liter of medium containing 10 g sodium lactate, 1.0 g $NH_4Cl$, 0.7 g $K_2HPO_4$, 0.3 g $KH_2PO_4$, 0.25 g $Na_2SO_4$ (10 $H_2O$) and 0.02 g $MgSO_4$ (7 $H_2O$); pH 7.2) *E. coli O:14* (O14:K 7(6):H —) was kindly given to us by Dr. F. Örskov at the E. coli International Center, Copenhagen, Denmark. The other strains were isolated from patients with urinary tract infections and were typed for O-antigen specificity by Dr. Örskov. *Staphylococcus aureus (S 209)* was obtained from the Dept. of Bacteriology, Karolinska Hospital, Stockholm. *Old Tuberculin* was a commercial preparation from the State Bacteriological Laboratory, Stockholm. Preparation of antigens, sensitization of the sheep erythrocytes (without tannic acid) and titration of serum in 2-fold dilution steps on perspex trays have previously been described(6,12).

For hemagglutination inhibition, 0.1 ml of a proper serum dilution was mixed with 0.1 ml of the antigen dilutions used for inhibition. The inhibitors were serially diluted in 2-fold dilution steps, with antigen concentrations ranging from 8 to 1000 μg/ml or more. After 30 minutes, 0.1 ml sheep erythrocytes ($5 \times 10^7$ cells/ml) sensitized with optimal concentrations of germ free rat colon, were added and hemagglutination was recorded as usual. A detailed description of the procedure and the controls which were included has previously been given(12).

For fluorescent antibody staining, ethanol fixed colon sections of germ free rats were exposed to various rat sera and were subsequently stained with a fluorescein isothiocyanate conjugated γ-globulin of a rabbit anti-rat globulin serum(5).

*Results and discussion.* The antibody response against colon antigen from germ free rats in 4 groups of animals is summarized in Fig. 1. The highest titers appeared in the rats injected with rabbit colon. This curve gives the mean titers of serum samples from 9 rats. All 19 rabbit colon injected rats thus far tested have responded in the same way. Maximal titers were occasionally

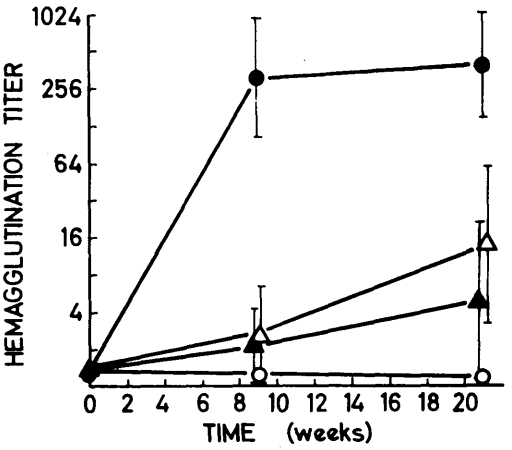


FIG. 1. Formation of anti-rat colon antibodies in rats injected with rabbit colon (filled circles), germ free rat colon (empty triangles), or Freund's adjuvant only (filled triangles). Empty circles: untreated controls. Vertical lines: standard errors of mean titers, calculated from the titers of responding rats (see text). Abscissa: time of immunization. Ordinate: hemagglutinating titers (reciprocals of highest hemagglutinating serum dilution).

reached already after 3 injections. In the rat colon injected group, the response was slower and the final mean titers, calculated from 15 responding rats, were significantly lower. Moreover, the titer variation between the responding animals was more pronounced and of 30 tested rats, only 50% had responded at all. The third curve shows the anti-colon titers of 6 responding rats which were injected with Freund's adjuvant alone. This response was even less pronounced. Moreover, the incidence of responding animals was small and only about 20% of 24 animals tested developed a measurable antibody titer after injection. Fig. 1 finally shows that the untreated controls (24 rats) remained negative throughout (titers $\leq 2$). This was also the case when the animals were injected with antigen without adjuvant. Rats injected with a phenol-water extract of rat lung or of *Staphylococcus aureus (S 209)* in Freund's adjuvant gave the same anti-colon response as the rats injected with adjuvant alone.

The animals of Fig. 1 did not develop hemagglutinating anti-rat lung antibodies when lung extract was the test antigen on the erythrocytes. Immunization with colon did not lead to a significant rise of the anti-

TABLE I. Hemagglutination Inhibition Assay of Organ Specificity of Anti-Rat Colon Reactions. Sheep erythrocytes sensitized with colon extracts from germ free rats. All inhibitors were phenol-water extracted preparations from germ free rats. Hemagglutination titers of the sera and hemagglutinating units (HU) used in the assays given at top of each column (4-8 HU = serum diluted 4-8 times less than highest dilution causing hemagglutination). Numbers in the columns are minimal amounts of inhibitor ($\mu$g/ml) required for inhibition of hemagglutination. >1000 : no inhibition with 1000 $\mu$g/ml of the inhibitor.

| Inhibitor | Sera of 3 rats injected with rabbit colon | | | Serum of ulcerative colitis patient (blood group A) |
|---|---|---|---|---|
| Titers | 512 | 256 | 256 | 64 |
| HU | 8 | 4 | 8 | 4 |
| Feces | 16 | ≤8 | ≤16 | ≤8 |
| Cecum | 16 | ≤8 | — | ≤8 |
| Small intestine | 250 | 32 | 500 | 125 |
| Stomach | 64 | ≤8 | 250 | 64 |
| Liver | >1000 | >1000 | >1000 | >1000 |
| Kidney | >1000 | 1000 | >1000 | 125 |

body titers against unsensitized sheep erythrocytes nor against human erythrocytes of blood group A or O.

In further experiments, the specificity of the anti-rat colon reaction was studied by means of hemagglutination inhibition. Table I shows the organ specificity of the anti-rat colon reaction of three rats injected with rabbit colon. The antigen was specific for the gastrointestinal tract and was particularly abundant in its lower parts and in the germ free feces. Liver and kidney extracts did not inhibit hemagglutination under the experimental conditions applied. Table I also includes the typical results of a similar test performed with the serum of a patient with ulcerative colitis. This serum was free from antibodies against blood group A antigen, which is also present in the rat extracts(12). Obviously, in most cases, the rat and human antibodies reacted with similar antigens.

The organ specificity of this reaction was also brought out by indirect fluorescent antibody staining of colon sections of germ free rats. Sera from rabbit colon injected rats gave a strong staining of mucus producing cells in the colonic crypts. Sera taken from the same rats before immunization were negative. The staining was undistinguishable from that obtained previously with human ulcerative colitis sera(5,12).

The anti-colon antibodies of ulcerative colitis patients have previously been tested for cross reactivity with somatic antigens of different strains of *Escherichia coli* and *Salmonella*(6,19). Thus far, a marked cross reactivity has been found only with phenol water extracts of *E. coli O:14*. This strain was chosen for investigation since it contains a high concentration (or an immunogenically active form) of a "common antigen" which is present in low concentrations (or in a masked form) in most *Enterobacteriaceae* (20-25). An immunological relationship between colon and this "common antigen" would be of great interest, since it implies that the permanent exposure of the human intestine to small amounts of a tissue related bacterial antigen could be an important contributing factor for autoantibody formation in ulcerative colitis(1-3). As seen from the typical results in Table II, the anti-rat colon reactions of the sera from 4 rabbit colon injected rats were easily inhibited by *E. coli O:14*, but not by 3 other common *E. coli* strains. A fourth strain, *E. coli O:8*, was slightly inhibitory in some cases. These data do not exclude that inhibitory antigen was present in low concentrations or in a less active form in all *E. coli* extracts tested. Table II also contains the quite similar results obtained with human antibodies in a patient's serum. Up to now, 8 *E. coli* strains have been tested but all except O:14 were inactive or only of very weak activity. Thus far, this type of cross reactivity has been found in 20-30% of 30 ulcerative colitis sera. In the negative sera, antibodies against cross reacting determinants may also occur, but will escape detection, if antibodies against non-cross reacting colon determinants are presented in higher concentrations.

A comparison of the anti-rat colon reactions exhibited by rats injected with different materials demonstrated that the antibodies in the different sera were directed against determinants of different specificities (Table III). In two typical sera from the rabbit colon injected group, hemagglutination was inhibited by rabbit and rat colon as well

TABLE II. Hemagglutination Inhibition Assay of Cross Reactivity of Anti-Rat Colon Antibodies with Somatic Antigens from *Escherichia coli*. Sheep erythrocytes sensitized with colon extracts from germ free rats. The bacterial antigens were phenol-water extracted preparations from *E. coli* strains, grown on a synthetic medium. For other explanations see Table I.

| Inhibitor | Sera of 4 rats injected with rabbit colon | | | | Serum of ulcerative colitis patient (blood group A) |
|---|---|---|---|---|---|
| Titers | 512 | 512 | 256 | 2048 | 64 |
| HU | 4 | 8 | 4 | 8 | 4 |
| Feces from germ free rats | ≤8 | 32 | 16 | ≤8 | 16 |
| *E. coli* 0:14 | 16 | 250 | 125 | 64 | 125 |
| " 0:2 | — | >1000 | >1000 | >1000 | >1000 |
| " 0:8 | 500 | >1000 | 1000 | 1000 | >1000 |
| " 0:18 | >1000 | >1000 | >1000 | >1000 | >1000 |
| " 0:75 | >1000 | >1000 | >1000 | >1000 | >1000 |

TABLE III. Comparison by Hemagglutination Inhibition Assay of Anti-Rat Colon Antibodies from Rats Injected with Newborn Rabbit Colon, Germ Free Rat Colon or Freund's Adjuvant Alone. Sheep erythrocytes sensitized with colon extract from germ free rats. All inhibitors except *Old Tuberculin* were phenol-water extracted preparations of the sources indicated. For other explanations see Table I.

| Inhibitors | Sera of 6 rats injected with | | | | | |
|---|---|---|---|---|---|---|
| | Rabbit colon | | Rat colon | | Freund's adjuvant | |
| Titers | 64 | 512 | 128 | 16 | 128 | 16 |
| HU | 8 | 8 | 8 | 2 | 8 | 2 |
| Germ free rat colon | 125 | 32 | 64 | 125 | 64 | ≤8 |
| Autologous rat colon (not germ free) | 500 | 64 | — | — | — | ≤8 |
| Newborn rabbit colon | 32 | ≤8 | >1000 | 125 | >1000 | 250 |
| *Escherichia coli* 0:14 | 125 | 125 | >1000 | 500 | >1000 | >1000 |
| *Staphylococcus aureus* S 209 | >1000 | >1000 | >1000 | >1000 | >1000 | >1000 |
| *Old Tuberculin* | >1000 | >1000 | >1000 | — | — | >1000 |

as by *E. coli O:14*. The rabbit colon extract was slightly more inhibitory than the other antigens. It should be noted that the anti-rat colon antibodies were autoantibodies, since colon extracts of the serum donors also inhibited the reaction. Of the 2 typical rat colon injected rats, one reacted in the same manner as the rabbit colon injected animals. In contrast, the second rat had antibodies which only reacted with rat colon. This was usually also the case with those responding rats which were injected with adjuvant only. However, as seen in the last column of Table III, rat sera were occasionally found in which hemagglutination could be inhibited by both rat and rabbit colon, but not by *E. coli O:14*. Thus, at least 3 different determinants seemed to be involved, one common for rabbit colon, rat colon and *E. coli O:14*, one for both colons, and one specific for rat colon. It is not known whether the antibodies in the different cases were directed against different parts (=determinants) of the same antigen, or against different antigenic molecules, all present in the phenol water extracts. It must be stressed that all the sera gave identical staining of rat colon sections when they were tested with the fluorescent antibody techniques.

Table III also shows that the anti-rat colon reactions could not be inhibited by antigen from *Staphylococcus aureus*, nor by *Old Tuberculin*, in spite of the fact that all these sera agglutinated sheep erythrocytes, sensitized with staphylococcal or mycobacterial antigen. Rats injected with phenol water extract of *E. coli O:14* developed an anti-O:14 titer. As was to be expected, in the sera of such rats specific anti-O:14 antibodies predominated and hemagglutination of O:14-sensitized erythrocytes could therefore not be inhibited with colon extracts. This was also true for the anti-staphylococcus reaction of rats injected with *St. aureus*. In a few preliminary experiments with rats injected with rabbit colon, a significant rise of the hemagglutinating titer against O:14 sensitized cells was observed. This reaction could

TABLE IV. Hemagglutination Inhibition Assay of Anti-Rat Colon Antibodies from Patients with Ulcerative Colitis, with Rat- and Rabbit-Colon Antigen. Sheep erythrocytes sensitized with colon extracts from germ free rats. The inhibitors were phenol-water extracted preparations. For other explanations see Table I.

| Inhibitor | Sera from 5 patients with ulcerative colitis (blood group A) | | | | |
|---|---|---|---|---|---|
| Titers | 128 | 32 | 64 | 128 | 64 |
| HU | 8 | 2 | 8 | 8 | 8 |
| Germfree rat feces | 250 | 32 | 32 | 8 | 125 |
| Newborn rabbit colon | 500 | 64 | 250 | 64 | 250 |

be inhibited by rat colon antigen. Finally it should also be mentioned that the anti-rat colon reaction of human ulcerative colitis sera can be inhibited with both rat and human colon(6), as well as with rabbit colon (Table IV).

In conclusion, injection of rats with rabbit colon is an efficient way of producing autoantibodies against colon. Conclusive evidence has been provided that germ free rat and rabbit colon contain one or several immunologically active structures in common with a bacterial polysaccharide which, most likely, is of widespread occurrence in the animal and human intestine. Moreover, depending on the stimulating antigen, the antibodies may be directed against different determinants of the tissue antigen. Thus, in the present case, the antibodies appearing after heterologous stimulation invariably reacted with cross-reacting determinants. In contrast, after homologous or "autologous" stimulation animals were also encountered which only reacted with rat colon specific determinants. Little attention has thus far been focused on this type of heterogeneity, which is not easily detected when autoantibodies are demonstrated by the fluorescent antibody techniques or by other common assay procedures. It can be assumed that further analysis along these lines may provide important clues as to the nature of the stimulating antigen in several different autoimmune situations. The present results indicate that the experimentally induced anti-colon antibodies in rats have a similar pattern of reactivity as the anti-colon antibodies in human ulcerative colitis. It remains to be established whether stimulation by heterologous antigen contributes to autoantibody formation in this disease.

All rats injected with colon + adjuvant developed severe microscopic damage of their colon mucosa. However, adjuvant injected rats also had such lesions. It is possible that the intraperitoneal introduction of Freund's adjuvant was responsible for colon damage as well as for autoantibody production occurring occasionally after injection of adjuvant alone. Hence, for the production of a truly specific colon disease, other routes of injection should be preferable. According to a brief report appearing recently after this study was completed, an experimental colitis has been produced by injecting rats intradermally with extracts of canine colon(26).

*Summary.* Autoantibodies to germ free rat colon were produced in rats by repeated intraperitoneal injections with newborn rabbit colon in Freund's complete adjuvant. Antibodies were assayed by means of indirect hemagglutination of sheep erythrocytes sensitized with a heat stable phenol-water extract of germ free rat colon or by fluorescent antibody staining of rat colon sections. The response of rat colon injected rats was less intense. A few of the rats injected with adjuvant alone also developed an anti-colon titer. Hemagglutination inhibition experiments demonstrated that the antibodies were specific for gastrointestinal antigen and represented autoantibodies in a true sense. They were found to be specific for at least three different colon determinants: one unique for germ free rat colon, one common for rat and rabbit colon, and one common for both colons and a polysaccharide from *Eschericria coli O:14*, grown on synthetic medium. No or only very weak cross reactions appeared with similar antigens extracted from a number of other *E. coli* strains. *Old Tuberculin* and extract of *Staphylococcus aureus* were inactive. The pattern of reactivity of the rat antibodies was similar to that of patients with ulcerative colitis. Stimulation by cross reacting bacterial antigen may be an important contributing factor for autoantibody formation in this disease.

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## Effects of Anti-Thymocyte Serum on Lymphocytic Choriomeningitis (LCM) Virus Infection in Mice. (32254)

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Several studies(1,2,3) have suggested that lethality in acute lymphocytic choriomeningitis (LCM) virus infection of mice is a result of host immunologic response, rather than a direct effect of virus multiplication. Mice inoculated neonatally are protected from lethal effects of the virus, while adult morbidity and mortality can be reduced or delayed by various immunosuppressive methods, including X-irradiation, antimetabolite therapy, and neonatal thymectomy(1,2). In the present study, the role of cellular immunity in LCM pathogenesis was further examined, using rabbit anti-mouse thymocyte (RAMT) serum to inhibit host response.

*Materials and methods.* Three- to four-week-old ICR mice of both sexes were used in all experiments. RAMT serum was prepared as described previously(4), by immunizing rabbits over a several-week period with dispersed suspensions of viable mouse thymus cells. Serum effectiveness was assayed by its ability to (a) diminish peripheral blood lymphocyte counts by 50% within a 4-hour period, and to (b) double the mean survival time of AKR skin grafts on $C_3H$ mice. All RAMT sera were shown to be free of anti-LCM activity as assayed by mouse neu-