AUTOANTIBODIES TO COLON IN GERMFREE RATS MONOCONTAMINATED WITH CLOSTRIDIUM DIFFICILE*

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Sera from patients with ulcerative colitis contain autoantibodies against colon antigen of both human and animal origin. Sera from patients with unrelated diseases, including disorders of the gastrointestinal tract, contain no, or only low titered, antibodies of this kind (1–5). The antigen is a polysaccharide, present in the mucus-producing cells of the colon mucosa and in the mucus (2, 3, 6). It is organ-specific but not exclusively confined to the lower gastrointestinal tract (7).

Formation of autoantibodies against colon can also be induced in rabbits and rats by immunization with antigens from certain bacteria such as *Escherichia coli*, *Salmonella*, and *Proteus*, or with heterologous colon tissue (8–10). Closer analysis of the antibodies to germfree rat colon of rats immunized with fetal rabbit colon showed a cross-reactivity between these two colons and antigen from *E. coli* O14. This cross-reactivity was very similar to that typical for the autoantibodies in certain sera of patients with ulcerative colitis. Autoantibodies of the same specificity were also found in some of the animals injected with allogenic rat colon. Other animals in this group, as well as a few in the group injected intraperitoneally with Freund's complete adjuvant, formed an anti–rat colon antibody reacting with determinants found only in the rat colon polysaccharide. Finally, a few animals in the latter group had antibodies reacting with determinants common for rat and rabbit colon but absent from *E. coli* O14 polysaccharide. Thus, depending on the immunizing antigen, antibodies were formed which reacted with different carbohydrate determinants of the germfree rat colon extract (10).

When used for monocontamination of germfree mice (11) or rats,[1] anaerobic bacteria of the species *Clostridium difficile* appreciably reduce the size of the cecum. During the first days of monocontamination with *Cl. difficile*, the exgermfree rats also exhibited transient diarrhea.[1]

This study was undertaken in order to establish (a) whether monocontamination of germfree rats with *Cl. difficile* or other bacteria gives rise to autoantibody production against colon, and if so (b) how these antibodies are related to the anti-colon antibodies produced in rats by immunization with colon antigen.

Material and Methods

Rats.—The germfree rats were descendants of the 16th–18th generation of the inbred Swedish germfree strain reared according to the technique of Gustafsson (12, 13). Conventional rats originating from the germfree strain, or outbred Sprague-Dawley rats served as controls. The germfree animals and their conventional counterparts were fed with the autoclaved semisynthetic diet D 7 (13) and the Sprague-Dawley rats with a pelleted commercial diet which was not sterilized.

Bacteria.—*Clostridium difficile* was originally from the American Type Culture Collection, Rockville, Md. (ATCC 9689) and used in our laboratory (B.E.G.) for studies of its effect on enlarged ceca in germfree animals. Another *Clostridium* species (our strain G 62) was isolated from fecal cultures of conventional rats and shown to metabolize bilirubin to stereobilin (14). The sera from animals monocontaminated with other bacteria were obtained from rats monoinfected for other purposes. These bacteria and their origin are listed in Table I. Before inocculation, the bacteria were grown for 24–48 hr at 37°C in a fluid thioglycolate medium (Difco Laboratories, Detroit, Mich.), or in dextrose broth. *E. coli* O14, used for antigen preparation, were from the International *E. coli* Center, Copenhagen, Denmark (*E. coli* O14: K7(6):H-, obtained through the courtesy of Dr. F. Örskov). They were grown on a synthetic medium as earlier described (10).

Monocontaminations.—These were performed in standard germfree units or in individual germfree isolators by feeding the germfree animals with cultures of the different bacteria (14, 15).

Antigen.—Colon antigen from germfree rats and bacterial antigen were prepared by phenol-water extraction according to Westphal et al. (16) as earlier described (7). The microorganisms were collected by centrifugation, washed in physiological saline, and either heat-inactivated (2 hr, 100°C) or killed by incubation with 0.5% formaldehyde for 72 hr at 37°C.

Sera.—Sera were from germfree rats, monocontaminated (i.e., exgermfree) rats, conventional rats, and from conventional rats immunized intraperitoneally with fetal rabbit colon plus Freund's complete adjuvant (10). Blood was collected aseptically by heart puncture. The sera were stored in small phials at −20°C. Before use, all sera were inactivated at 56°C for 30 min and absorbed with equal volumes of (a) packed and washed sheep erythrocytes, and subsequently (b) human erythrocytes, type A₁.

Hemagglutination.—The indirect method as previously described was used throughout (7, 10). The concentrations of antigen applied for sensitization of 0.025 ml packed and washed sheep red cells were: for germfree rat colon antigen, 2.0 mg/ml; for *Cl. difficile*, 0.50 mg/ml; and for *E. coli* O14, 0.050 mg/ml. For the bacterial antigens, these concentrations gave optimal sensitization of the erythrocytes. The concentration of rat colon antigen was slightly suboptimal (4, 17). Hemagglutination was performed in Perspex trays and read macroscopically after incubation for 18 hr at room temperature (7).

Hemagglutination Inhibition.—0.1 ml aliquots of serum dilutions containing 4–8 hemagglutinating antibody units (HU) were 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 2 to 1000 μg/ml. After 30 min, 0.1 ml of sensitized sheep cells were added and hemagglutination was recorded as usual. A detailed description of the procedure and the controls which were included has previously been given (7).

Fluorescent Antibody Staining.— The indirect method was used (18). Pieces of freshly dissected proximal colon of germfree rats were snap-frozen in liquid nitrogen, cut in a cryostat into 5 μ thin sections, and fixed in ethanol. The dried sections were then incubated with rat

TABLE I

Number of Sera from Monocontaminated Germfree Rats and Their Controls with Hemagglutinating Titers $>$ *$\frac{1}{4}$ against Polysaccharide from Germfree Rat Colon, Cl. difficile and E. coli O14, Respectively*

Rats	Age	Treatment*	Time of monocontamination	No. of animals	No. of sera with titers $>\frac{1}{4}$ against sheep cells sensitized with		
					Rat colon (germfree)	Cl. difficile	E. coli O14
	days		*days*				
Swedish inbred, exgermfree	180–540	Cl. difficile	0–60	12	2	11	0
	"	" "	60–360	4	3	4	0
	"	Clostridium, sp. (G62)	0–60	2	0	0	0
	"	" "	60–360	2	2	0	0
	"	G 62 + E. coli	302 + 32	1	1	0	1
	"	Various organisms§	0–60	6	0	1	1‡
	"	" "	60–360	3	0	2	0
Swedish inbred, germfree	180–540	—	—	3	0	0	0
	655–1097	—	—	5	0	0	0
Swedish inbred, conventional, of germfree origin	180–540	—	—	10	0	5	3
	586–702	—	—	5	1	5	3
Sprague-Dawley, conventional	180–540	—	—	12	0	3	6
	"	Immunized with fetal rabbit colon‖	—	4	4	0	4

* Monocontaminated with the bacteria indicated.

‡ In serum of rat infected with E. coli for 33 days.

§ Monocontamination of rats with: E. coli, 2 (6 and 33 days); Bacillus subtilis, 1 (48 days); Streptobacillus sp., 1 (21 days); Streptococcus sp., 1 (90 days); anaerobic species from rat intestine, 4 (7, 24, 89, and 213 days).

‖ For immunization procedure see reference (10).

serum, washed with physiological saline, and incubated with rabbit anti-rat γ-globulin, conjugated with fluorescein. This antiserum contained precipitating antibodies to rat IgG and IgM. Conjugation of the γ-globulin fraction of the rabbit antiserum was performed with fluorescein isothiocyanate on Celite (Microbiological Associates, Bethesda, Md.) according to Rinderknecht (19). The fluorescein protein ratio was 3.5 $\times$ 10^{-3}. Before use, the conjugate was absorbed twice with an equal volume of an acetone powder of calf liver. Details have previously been given (5).

RESULTS

Table I summarizes the results of the hemagglutination experiments. As can be seen, rats monocontaminated with *Cl. difficile* or *Clostridium* strain G 62 produced antibodies to germfree rat colon. Their titers were in the range of 1/16 to 1/128. Such antibodies were not found in (*a*) rats monocontaminated with other bacteria, (*b*) germfree rats, (*c*) conventional rats of the same strain (for one exception see below), or (*d*) conventional Sprague-Dawley rats. All Sprague-Dawley rats immunized with fetal rabbit colon in Freund's complete adjuvant also produced antibodies to rat colon (titers 1/32–1/512).

The occurrence of circulating antibodies to colon in monocontaminated rats is obviously dependent on the time of infection. Thus, animals kept mono-contaminated for more than 60 days show a higher incidence of anti-colon antibodies than those monocontaminated for shorter time periods. In this series, anti-colon antibodies never occurred earlier than 35 days after mono-contamination. Old age of the animals per se does not explain the presence of anti-colon antibodies, since germfree animals as old as 3 yr were all negative. However, low titered antibodies to rat colon (titer 1/8) were found in one old (2 yr) of the 27 conventional controls.

Table I also demonstrates that all except one of the rats monoinfected with *Cl. difficile* responded by producing antibodies to phenol water extracted antigen from this organism (titers 1/16–1/2048). The nonresponding animal had been monocontaminated for 14 days, with positive subcultures from the feces, but this period was probably too short for the induction of antibodies at measurable titers. The antibody response to the homologous organism in rats monoinfected with *Clostridium* G 62 was not investigated.

Sera of germ free rats, or of rats inocculated with the *Clostridia* or with various other bacteria did not contain antibodies to *E. coli* O14. This was in contrast to a large fraction of those from conventional animals, and those from all animals immunized with fetal rabbit colon. In the latter group, the titers were 1/16–1/256. Anti-*E. coli* O14 titers were also demonstrated in sera from two of three exgermfree animals infected with *E. coli* of unknown serotype (Table I).

Sera with hemagglutination titers to rat colon were also investigated by indirect immunofluorescence. When applied to tissue sections of germfree rat colon, the goblet cells of the crypts were weakly but specifically stained. The staining was similar to that obtained with sera of ulcerative colitis patients (5). Three out of three sera (hemagglutination titers 1/32, 1/64, and 1/128) from *Cl. difficile* infected animals gave positive staining. The same type of staining was also obtained with two out of two sera from Sprague-Dawley rats, immunized with fetal rabbit colon (hemagglutination titers 1/256, 1/512). However, the intensity of the staning obtained with these two sera was stronger, probably due to their higher antibody titers (5, 6). Sera from four conventional

and from one germfree rat were also tested. None of these gave positive staining.

Table II shows the results of a typical experiment in which the specificity of the anti-colon antibodies in sera from rats monocontaminated with *Cl. difficile* was studied by means of hemagglutination inhibition. For comparison, the results of a similar test with the serum from a rat immunized with fetal rabbit colon in Freund's complete adjuvants have also been included. As can be seen, colon antigen used as inhibitor completely inhibits the reaction at low concentrations. In contrast, antigen from *Cl. difficile* gave no inhibition, even

TABLE II

Hemagglutination Inhibition Assay of Cross-Reactivity of Anti-Rat Colon Antibodies with Antigen from Cl. difficile and E. coli O14—Sheep Cells Coated with Germfree Rat Colon Antigen

Inhibitor	Sera from three rats monocontaminated with *Cl. difficile*			Serum from rabbit colon immunized rat
	No. 1	No. 2	No. 3	
Titer HU	256 8	64 8	64 4	256 4
Germfree rat colon	4*	64	2	16
E. coli O14	>1000	>1000	N.D.‡	125
Cl. difficile	>1000	>1000	>1000	>1000

* The numbers are μg/ml of antigen giving complete inhibition of the number of hemagglutinating units (HU) indicated. >1000, no inhibition with highest doses tested.
‡ N.D., not done.

at the highest concentration tested. As earlier demonstrated (10), rats immunized with fetal rabbit colon form anti-rat colon antibodies, which cross-react with *E. coli* O14. However, the anti-colon antibodies in the monocontaminated animals did not cross-react with this antigen (no inhibition with 1000 μg/ml of *E. coli* O14 antigen). It should also be mentioned that neither colon antigen nor *E. coli* O14 inhibited the reaction of *Cl. difficile* sensitized erythrocytes with antibodies from the *Clostridium* infected rats (unpublished material).

DISCUSSION

In this study, antibodies to rat colon were demonstrated in rats monocontaminated with two different species of *Clostridium*. Since the test antigen was obtained from germfree rats of the same inbred strain, the antibodies were probably autoantibodies in a true sense. No such antibodies were seen in the controls, comprising both exgermfree rats monocontaminated with other

bacteria, conventional rats, and germfree rats, some of very old age. Fluorescent antibody staining showed that the antigen was localized to mucosal sites similar to those stained with sera from rats immunized with rabbit colon, or with sera from patients with ulcerative colitis (5). In spite of the fact that the test antigen was identical with that used earlier, hemagglutination inhibition conclusively proved that the reactive structures of the colon antigen in the present case were not related to E. coli O14 antigen. Thus, different determinants (or antigens) present in the same extract, are involved in the two different immune responses. However, a few of the rats previously injected intraperitoneally either with rat colon or with Freund's adjuvant alone also developed anticolon antibodies which did not cross-react with E. coli O14 (10). It is not known whether these antibodies reacted with the same colon determinants as the antibodies of the Clostridium-infected rats, or whether different determinants were involved. In any case, the antigens were immunologically distinct from those having blood group A- or H-specificity, also present in rat colon (7, 20), since no reaction was obtained when the rat sera were tested with human A_1- or O-erythrocytes (unpublished experiments).

Obviously, the formation of anti-colon antibodies is causally related to infection with the Clostridia used here. The question arises how autoantibody formation was set in motion. A number of possibilities will be discussed.

1. Anti-colon antibodies may arise secondarily to intestinal lesions caused by the bacteria. These lesions may lead to an unwarranted confrontation of the immune apparatus with the colon antigen. Although the germfree animals infected with Cl. difficile have diarrhea; this is transient and only very seldom leads to the death of these animals. Monoinfection also results in transient reduction of the cecum contents and a partial reduction of the weight of the cecum wall.[1] Most likely, the anti-colon antibodies are not due to lesions underlaying these conditions, which appear very early after infection. In contrast, antibody titers become measurable late, often later than 2 months after inocculation. Moreover, the other Clostridium species tested, strain G 62, also gives rise to formation of anti-colon antibodies, in spite of the fact that neither cecum reductions nor diarrhea are observed in these rats. However, this does not entirely exclude that anti-colon antibodies are secondary sequelae of intestinal lesions of an unknown nature.

2. Antibody formation to colon may represent a case of lost tolerance, after stimulation with bacterial antigens, immunologically related to rat antigen. Autoimmunity through breakage of tolerance by microbial antigens which cross-react with host antigens can be induced experimentally. Similar mechanisms have also been implicated in human disease (21, 22). In fact, anti-colon antibodies can be induced in rabbits by immunization with bacterial antigen, related to colon antigen (9). It has also been shown that colon polysaccharides from germfree rats share determinants with the "common an-

tigen" of *Enterobacteriaceae* (10, 17). This antigen, described by Kunin et al. (23) and by Whang and Neter (24) is more abundant (or more immunogenic) in *E. coli* O14 than in any other known *E. coli* species. The anti-colon antibodies of patients with ulcerative colitis also cross-react with *E. coli* O14 (17). We have previously suggested that breakage of tolerance, induced by this ubiquitous bacterial antigen, may give rise to autoimmunity in ulcerative colitis. (4, 17). No such cross-reactions were observed in the rats monoinfected with *Clostridium*. All *Cl. difficile*-infected rats developed a high titer against homologous bacterial polysaccharides. However, hemagglutination inhibition experiments provided good evidence that the clostridial polysaccharides were immunologically unrelated to rat colon (and to *E. coli* O14 as well). These findings speak against breakage of tolerance by bacterial antigen as the cause of autoimmunity to colon in this model.

3. Chemical alteration of autologous material may also give rise to auto-antibody formation (22, 25). The *Clostridia* may make host material immunogenic by altering it chemically. Enzymes from anaerobic inhabitants of the gastrointestinal tract of both men and rats have been shown to degrade mucins of germfree rats. This leads to a loss of blood group A and H activity (26, 27). Since the colon antigen is closely related chemically to the blood group active mucins (7, 26)[2] it is possible that enzymatic degradation in the intestine of the monoinfected rats may lead to the production of partially altered mucin structures. These may give rise to the production of autoantibodies, reacting with unaltered structures as well. Lindstedt et al. (28) have shown that *Cl. difficile*, although capable of reducing the cecal size in vivo, did not degrade the mucus from germfree rats in vitro when tested under conditions where a full intestinal flora was highly active. However, these authors did not look for possible changes in the immunological fine structures of the mucins. Their observation that the full flora leads to a much more pronounced degradation may actually explain the lack of immunogenicity of the mucins in conventional animals, whose flora also contains *Cl. difficile*.

4. Finally, autoimmunity may also arise because of an adjuvant effect of the bacteria or their products, leading to an altered reactivity of the immune apparatus, with no implication of antigenic changes (21). Such a hyperreactivity alone, or perhaps in combination with some of the mechanisms mentioned above, could be responsible for the autoimmune state. However, direct evidence for this assumption is lacking.

While the mechanisms for autoantibody production in these rats remains to be explored, the data unequivocally show that infection with certain bacteria under strictly controlled conditions may give rise to autoimmunity. This autoimmune state does not seem to be connected with disease and the auto-

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[2] Hammarström, S. Manuscript in preparation.

antibodies have no obvious pathogenic significance. Whether they exert a protective function, play a role as scavengers, or have no physiological significance at all remains to be established.

SUMMARY

Germfree rats monocontaminated with the anaerobic microorganisms *Clostridium difficile* or another *Clostridium* species (strain G 62) produce auto-antibodies to colon antigen. The antigen can be extracted with phenol water from the feces of germfree rats. Antibodies, demonstrable by means of passive hemagglutination of antigen sensitized sheep erythrocytes appear after mono-contamination for 35 days or longer. The indirect immunofluorescence techniques, applied to sections of germfree rat colon, gave positive mucosal staining. The staining was similar to that obtained with sera from patients with ulcerative colitis or from rats immunized with rabbit colon. No antibodies were found in the sera of germfree rats, germfree rats monocontaminated with various other bacteria, conventional rats of germfree origin, or conventional Sprague-Dawley rats.

Although the anti-colon antibodies of the *Clostridium* infected rats reacted with the same feces extract as the antibodies of ulcerative colitis patients or of rabbit colon immunized rats, their specificity was different. While the latter cross-react with polysaccharide from *E. coli* O14, those from the *Clostridium*-infected exgermfree rats did not. Rats monocontaminated with *Cl. difficile* also developed antibodies to this organism, but no cross-reaction between *Cl. difficile* antigen and colon antigen could be demonstrated. This speaks against breakage of tolerance by cross-reacting bacterial antigen as the cause of autoimmunity in these rats. Other possible mechanisms for autoantibody production in this model are immunogenic alteration of gastrointestinal mucins by bacterial degradation, adjuvant effects of bacterial products, or both.

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