Immunology 1981 **44** 489

Cellular cytotoxicity and gastrointestinal inflammation in inbred rats: induction with gut organ-specific antigens

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Accepted for publication 22 May 1981

Summary. Although the ability to induce with organ-specific antigens autoimmune inflammatory lesions in the brain and thyroid is well-established, it has not been accomplished for the gastrointestinal system. Therefore, purified rat intestinal glycoproteins (RGCG and RCG) with defined biochemical and immunological properties and known to be organ-specific, were employed to study immune responses in four highly inbred strains of rats. Animals subcutaneously immunized with RGCG/RCG (or saline in controls) and *Mycobacterium butyricum* as the primary adjuvant were followed weekly for (a) weight loss and diarrhoea; (b) development of specific antibody; (c) cell-mediated cytotoxicity by a ^{51}Cr release assay; (d) pathological changes (graded I–IV) in intestine at the time of serial killing; and (e) increase in lamina propria cell count in histological sections taken from both macroscopically normal and abnormal bowel wall.

Disease incidence was highest in LOU/Mn strain rats injected with RCG, where small bowel lesions began at the fifth week after immunization (one of three animals), their frequency progressively increasing through the seventh week (four of five animals) when weight loss was most marked. Lewis strain rats injected with RCG had similar small bowel lesions at the sixth and seventh week. Colonic lesions were found in LOU/Mn strain rats injected with RGCG. Antibody-dependent cellular cytotoxic responses to RCG were detectable at both 50:1 and 10:1 effector to target ratios, occurred principally in injected animals who progressed to disease, and were correlated in time with the onset of lesions (weeks 5–7). Pathologically, all small bowel lesions were grade III (dark red granular mucosa) or IV (granular with obvious haemorrhage). Involved segments were 4–16 cm long, confined to the ileum, and diffuse without punctate ulcers. Colonic lesions were 2 cm long and diffusely hyperemic (grade II). Histologically, sections from diseased animals showed three changes: distortion (blunting) of villus shape, a hypercellular lamina propria (grade, moderate) and disruption of columnar epithelial lining cells. Two (ACI and Wistar–Furth) of the four strains studied were non-responders with respect to disease induction. We conclude that purified organ-specific gut glycoproteins can induce bowel wall pathological changes and antibody-dependent cellular cytotoxic reactions in susceptible strains of immunized rats. This technique may be useful for studying both the origin of, and the mediators for, autoimmune disease of the intestine.

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0019-2805/81/1100-0489$02.00

INTRODUCTION

Rat goblet cell glycoprotein (RGCG) is a non-mucin macromolecule associated with gut mucosal epithelial

cells (particularly goblet cells) and present in the intestinal glycocalyx of both rat and man (Roche, Cook & Day, 1981). Its isolation is easily accomplished by a three-step procedure beginning with intestinal epithelium as the starting preparation. By passive haemagglutination inhibition and immunofluorescence, RGCG has been shown not to be present in liver, esophagus, or stomach, suggesting that it is a gut organ-specific antigen. Rat colonic glycoprotein (RCG), isolated from rat large bowel by techniques identical to that for RGCG, shares two antigenic specificities with RGCG, but probably possesses individual region-specific determinants as well (Roche *et al.*, 1981).

From previous work it is known that organ-specific antigens are quite unique among immunogens for their ability to induce autoimmune disease in the xenogeneic, allogeneic, or syngeneic host (Witebsky, Rose & Nadel, 1960). With experimental autoimmune thyroiditis, for example, humoral and cell-mediated immune responses are elicited upon immunization with a syngeneic thyroid organ-specific antigen (thyroglobulin), and adoptive transfer of serum and lymphocytes (with specificity for the organ-specific antigen) in animals has demonstrated that the antithyroglobulin immune response is etiologic for the chronic thyroiditis seen (Vladitiu & Rose, 1971; Sharp, Mullen & Kyriakos, 1974). Hence, it was of interest to determine whether immunization with recently isolated and characterized organ-specific gut antigens (RGCG or RCG) could elicit in the experimental host immune responses, both humoral and cell-mediated, directed against these same self-antigens. Such responses might lead to inflammation localized to the wall of the large/small bowel, forming, in turn, a simple method for immunologically inducing autoimmune inflammatory disease of the intestine. Therefore, it was the purpose of this study to investigate immunological and histological findings in four highly inbred strains of rats injected with these two intestinal antigens, RCG and RGCG.

MATERIALS AND METHODS

Immunogens and animals

Rat goblet cell glycoprotein and rat colonic glycoprotein were isolated by a three-step procedure (mechanical separation of lumen-lining intestinal epithelial cells, solubilization of their associated macromolecules, and ethanol fractionation) as previously described (Roche *et al.*, 1981). Inbred Lewis and Wistar–Furth strain rats were purchased from Microbiological Associates (Bethesda, Md); ACI rats, from Laboratory Supply Company (Indianapolis, Ind.). Breeding pairs of LOU/Mn strain rat were kindly provided by the Small Animal Branch, N.I.H., Bethesda, Md, from which a colony was established in this laboratory.

Immunization regimens

Seven-week-old inbred LOU/Mn rats were divided among four groups and immunized as follows. Group 1 (fourteen animals) received subcutaneous (two hind footpad) injections of 70 μg RGCG (per animal), dissolved in saline which had been emulsified in an equal volume of Freund's incomplete adjuvant (FIA, Difco Laboratories) containing 150 μg *Mycobacterium butyricum*. Group 2 (seventeen animals) underwent an immunization identical to that of Group 1 except that RCG was substituted for RGCG. Group 3 (eight animals), serving as the control for effects due to adjuvants, received 0·15 M saline emulsified in FIA containing *M. butyricum* as above. Group 4 (eight animals) were not injected but managed identically to Groups 1, 2, and 3, and served to test whether gastrointestinal lesions, which possibly could be confused with those resulting from immunization, might spontaneously develop in the LOU/Mn strain of rat; the latter is noted for its capacity for spontaneous development after one year of age of ileocecal myelomas of diverse immunoglobulin subclass (Bazin, Beckers & Querinjean, 1974; Bazin, Querinjean, Beckers, Heremans & Dessy, 1974).

A second inbred line of rats, Lewis, with known potential for developing autiommune disease upon immunization with syngeneic organ-specific antigens (myelin basic protein of brain, for example) (Paterson, 1960; Day, Roche & Varitek, 1977) was injected with RCG (or saline as control) as outlined in the regimen for Groups 2 and 3 above. A third and fourth inbred line (sixteen animals each, ACI and Wistar–Furth) were randomly assigned to receive either the Group 1 regimen with RGCG (eight animals from each strain) or the Group 2 regimen with RCG (the remaining eight animals from each strain).

Immunological studies

Subsquently, all animals were bled via the tail vein every seventh day, and RGCG- and RCG-specific antibody was assayed by a passive haemagglutination technique already described (Roche, Day & Hill, 1978; Roche, Varitek, Hill & Day, 1979). In addition, the ability of sera, serially obtained after immunization, to

participate in an antibody-dependent cellular cytotoxic reaction against RGCG- and RCG-specific targets was evaluated using a microtitre assay system developed in our laboratory (Roche, Cheung & Lang, 1981). Target to effector ratios of 1:10 and 1:50 were studied in the presence of a 1:40 and 1:160 dilution of sera, using freshly-isolated rat spleen cells as effectors. Controls for background release, ^{51}Cr labelling of cells, incubation times and calculation of specific lysis were the same as described (Roche *et al.*, 1981).

Pathological studies

Animals were weighed twice weekly and observed for symptoms, particularly listlessness, diarrhoea and blood per rectum. Beginning with the 28th day after injection and every seventh day thereafter, two to five rats from each immunized group were killed by ether inhalation, their small and large bowel excised and opened longitudinally, and pathological lesions noted and photographed. The latter were graded I–IV as follows: I, minimally abnormal mucosa (slight hyperemia); II, diffuse and definite hyperemia; III, dark red granular mucosa; IV, granular mucosa with obvious haemorrhage.

For histological analysis, a 5 × 5 mm full thickness biopsy was taken from abnormal and normal areas of each animal, oriented on plastic mesh with villus surface up, and placed in 10% Formalin/phosphate-buffered saline. After fixation, twenty consecutive 6 μ-thick sections were cut from each specimen and stained with haematoxylin and eosin. Sections were evaluated by one of us (J.K.R.) for an increase in the lamina propria cell count (indicative of inflammatory cell infiltrate) and change in villus shape (secondary to inflammation) by techniques as described, using histological gradings of mild, moderate, and severe (Rubin, Brandborg, Phelps & Taylor, 1960). Tissue sections were photographed using a Leitz LaborLux 12 microscope equipped with a Wilde MPS-51S camera and Kodak Plus-X pan film with ASA of 125.

RESULTS

Symptoms and pathology

Obvious weight loss was noted in LOU/Mn strain rats injected with RCG, where, after the fifth week, animals subsequently shown to have lesions (see below) lost an average of 10 g or more, ranging up to 16% (38 g) of total body weight. Diarrhoea and blood per rectum were not noted.

At autopsy, pathological changes were seen in the intestine of serially killed RGCG- or RCG-injected animals of two (of the four) strains studied during and following the fifth week after immunization (Table 1). At that time, segments of distal small bowel were noted to be darker in colour than others even when first inspected *in situ*. By the criteria for grading pathological changes stated in Materials and Methods, the opened intestine revealed all small bowel lesions to be grade III (dark red granular mucosa) or grade IV (granular with obvious haemorrhage)—Table 1. Involved segments were 4–18 cm long, confined to the ileum and diffuse without punctate ulcers (Fig. 1a). The transition between normal and abnormal bowel was quite abrupt, and there were obvious gross differences between segments when compared side-by-side (Fig. 1a and 1b).

Disease incidence was highest in LOU/Mn rats immunized with rat *colonic* glycoprotein. Lesions in these animals were confined entirely to small bowel, the number of affected animals progressively increasing from week five (one of three) through week seven (four of five—Table 1). Lewis strain rats injected with RCG had similar small bowel lesions at weeks 6 and 7 (Table 1).

On the other hand, if RGCG were employed as the immunogen, only colonic lesions were detected, these occurring in the fifth week after immunization in two of the three animals examined on that date. The colonic mucosa appeared deeply hyperemic (grade II) rather than granular or frankly haemorrhagic, with one or two 2-cm lesions per animal. Diarrhoea, bloody stools and weight loss were not detected. Control animals (adjuvant-injected or uninjected) were found to be uniformly free of gross pathological changes in the small bowel or colon and did not experience weight loss.

Immune responses

While RCG-specific antibody was detected in two diseased (but no normal) LOU/Mn animals 3–5 weeks after immunization (Table 1), significantly more animals were found to have made an immune response to the immunogen using the technique of antibody-dependent cellular cytotoxicity. Because of their high incidence of lesions, LOU/Mn rats injected with RCG were examined in detail. Specific lysis of RCG-labelled target cells was detected between four and seven weeks post-immunization (Fig. 2). Further, a level of 5% RCG-specific ^{51}Cr release permitted injected animals to be divided into two groups, examples of which are

Table 1. Incidence and pathological findings in Lewis and LOU/Mn strain rats injected with RGCG and RCG

Recipient (strain)	Immunization regimen		Incidence of disease		Onset		Other parameters		Location/extent of disease			
	Antigen	Dose (μg/animal)	No. injected	No. with disease	Incidence	Time after injection (weeks)	Weight loss (g)	Antibody titre*	Colon	Small Bowel	Grade†	Extent (cm)
Lewis	RCG	70·0	14§	3	0/3	4	None	None	Nl	Nl	—	—
					1/3	5	None	Neg.	Nl	Abn.	IV	12
					1/3	6	None	Neg.	Nl	Abn.	IV	14
					1/4	7	None	Neg.	Nl	Abn.	IV	16
Lewis	—	—	16‡	0	0/16	4–7	None	Neg.	Nl	Nl	—	—
LOU/Mn	RCG	70·0	17§	7	0/4	4½	None	Neg.	Nl	Nl	—	—
					1/3	5	None	+(1·8)¶	Nl	Abn.	IV	4
					2/4	6	5–12	Neg.	Nl	Abn.	III	4
					4/5	7	10–38	+(1·4)¶	Nl	Abn.	IV	5–18
LOU/Mn	RGCG	70·0	14	2	0/4	4	None	Neg.	Nl	Nl	—	—
					2/3	5	None	Neg.	Abn.	Nl	II	2
					0/4	6	None	Neg.	Nl	Nl	—	—
					0/3	6½	None	Neg.	Nl	Nl	—	—
LOU/Mn	—	—	16‡	0	0/16	4–7	Neg.	Nl	Nl	Nl	—	—

* Antibody titre determined by a passive haemagglutination assay described in detail elsewhere (Roche *et al.*, 1978).

† Pathological grades of lesions (I–IV) defined under Materials and Methods.

‡ Control animals: eight rats were injected with adjuvant (150 μg *M. butyricum* in Freund's incomplete adjuvant) without RGCG or RCG; the remaining eight rats were uninjected. All sixteen animals were followed and killed in a manner identical to the experimental groups.

§ One animal died prematurely, and was not counted in the disease onset portion of this Table.

¶ Detected in only one diseased animal.

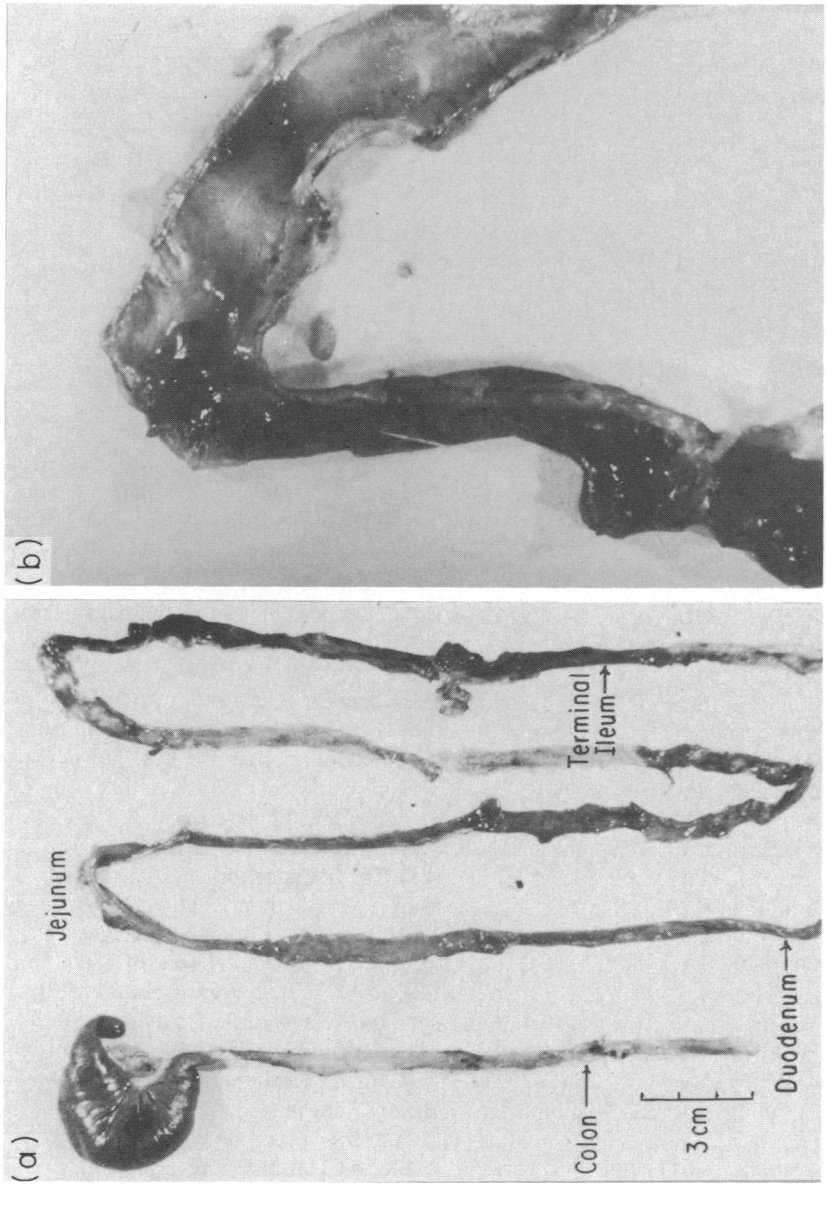

Figure 1. Pathological lesions in the RCG-injected experimental animal. (a) Small bowel and colon: At 6 weeks after immunization, lesions were evident throughout the distal one-half of the small intestine, with intervening areas of normal mucosa. (b) Ileum: this higher-power view shows a diffusely haemorrhagic segment, an area of transition toward normal mucosa (right side) and the absence of punctate ulceration (magnification × 5).

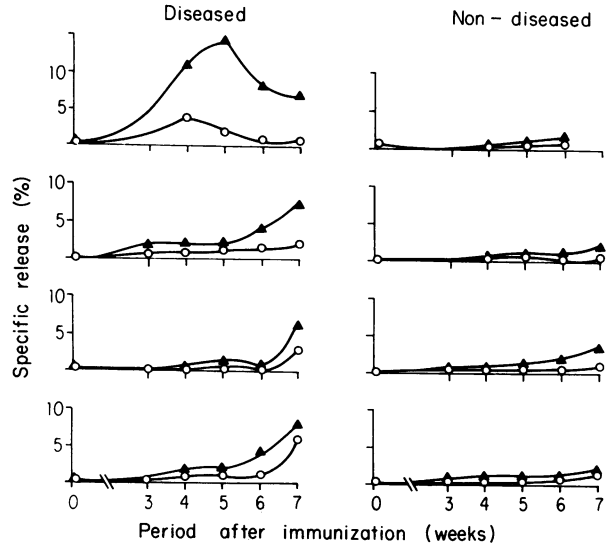

Figure 2. RCG-specific antibody-dependent cellular cytotoxicity in LOU/Mn strain animals, measured serially 3, 4, 5, 6 and 7 weeks after immunization. Release at two effector-to-target ratios [50:1 (▲) and 10:1 (○)] is shown, with patterns typical of those found in lesion-bearing animals on the left and in macroscopically normal animals on the right.

shown in Fig. 2. Five of seven LOU/Mn RCG-injected animals found to have intestinal lesions when killed (as described above) demonstrated a pattern of cytotoxicity identical or similar to those shown on the left of Fig. 2. Note that at both a 50:1 and 10:1 effector to target ratio, specific release of ^{51}Cr from RCG-coated target (chicken) red blood cells was found after non-specific release in the presence of naive sera and splenocytes used as control had been subtracted. Cytotoxicity evident at a 1:40 dilution of sera was not detectable at higher dilutions (1:160). On the other hand, six of eight animals from this same experimental group found at autopsy to be *free* of intestinal lesions demonstrated only a very low level of cytotoxicity upon serial testing of their sera, which was generally less than 2%–3% above background (examples on right side of Fig. 2); adequate sera to test were not available in one animal. While most RCG-immunized animals demonstrated a trend towards an increased cytotoxic potential with time, only animals who evolve this potential early and to a high degree were ultimately shown to develop lesions.

Histological findings and results in other strains

In adjuvant-injected (or uninjected) control animals, villi were long and feather-like in shape (Fig. 3a). Columnar cells lining the luminal surface were intact with basally-placed nuclei, and the lamina propria cell count was normal, there being considerable clear space between cells. In contrast, microscopic examination of full-thickness biopsy specimens from RCG- and RGCG-injected animals that developed lesions demonstrated changes in lamina propria cell count and in villus shape that underlay the macroscopic findings noted above. Three histological abnormalities were noted: distortion of villus shape (blunted rather than feather-like); a greatly increased lamina propria cell count per villus; and changes in epithelial cell morphology including loss of their normal (basal) nuclear polarity and vacuolization (Fig. 3b). Similar changes were seen in the colon of diseased animals after RGCG injection. By the histological grading system employed (see Materials and Methods), most lesions were judged to be 'moderate' with less than 10% in the 'severe' or 'mild' categories.

The ACI and Wistar–Furth rat strains appeared resistant to disease in terms of weight loss or obvious pathological lesions at autopsy, when immunized with regimens identical to those employed with the LOU/Mn and Lewis strains. When evaluated histologically, full-thickness biopsies revealed no abnormalities, in concert with their macroscopically normal appearance.

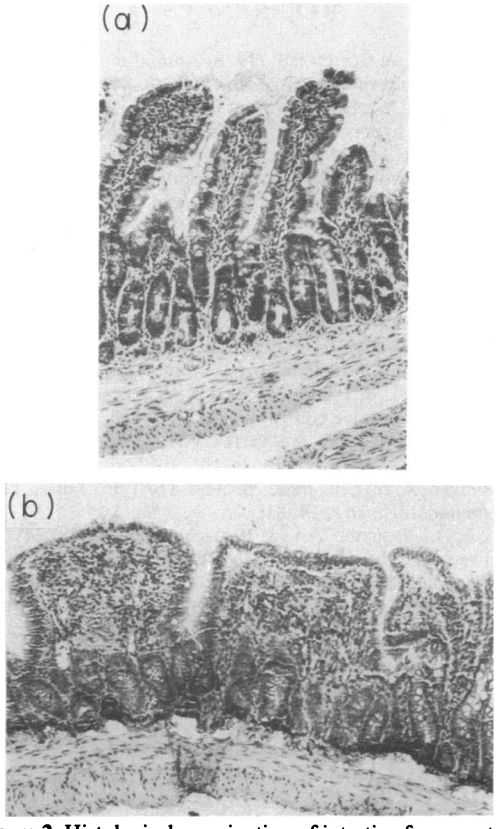

Figure 3. Histological examination of intestine from control and RCG-injected animals. Compared with villi of normal height and cell content on control animals (a), those from immune rats (b) appeared blunted and hypercellular. Loss of the basal orientation of epithelial cell nuclei indicates disturbance of the luminal lining columnar cells as well (magnification ×40).

DISCUSSION

Although a number of autoimmune reactions, both humoral (anti-colon antibodies) and cell-mediated (lymphocyte-mediated cytotoxicity for colon cells) have been detected and confirmed to occur in the chronic inflammatory bowel diseases of man (Broberger & Perlmann, 1959; Watson, Quigley & Bolt, 1966; Stobo, Tomasi, Huizenga & Shorter, 1976), their etiological significance is unclear. Further, because experiments using human subjects which might rigorously test the pathogenetic importance of these immune reactions (adoptive transfer of sera and/or lymphocytes into patients, for example) are not ethically possible to carry out, efforts have turned to experimentally-induced animal models of gut inflammation as the best possible arena for testing hypotheses bearing on etiology. A number of methods for experimentally-inducing gut inflammation have been proposed for this purpose. Gastrointestinal lesions elicited in these efforts include the predominantly caecal inflammation occurring after repeated ingestion of carrageenan in animals (Anver & Cohen, 1976); local rectal inflammation after systemic and then rectal exposure to DNCB (Askernase, Boone & Binder, 1978; Bicks, Azar, Rosenberg, Dunham & Luther, 1967); and diffuse intestinal haemorrhagic lesions after transfer to dogs of hyperimmune rabbit antisera to homogenized canine colon (Shean, Barker & Fonkalsrud, 1964). Unfortunately, none of these models has been accepted as providing an optimal avenue for investigation, generally because their pathogenetic basis has seemed unlikely to be truly autoimmune in nature and because the identity and nature of the relevant self-antigen(s) has not been elucidated.

We report here the successful induction of inflammatory intestinal disease in two of four inbred rat strains, with an associated immune response to the immunogen (measured by antibody-dependent cellular cytotoxicity) which correlated with disease onset and incidence over time. A crucial difference between this and previously proposed models is the employment of a gastrointestinal organ-specific antigen, present in both rat and man, which has been purified and partially characterized (Roche *et al.*, 1981). Further, the model described here uses a mode of induction (subcutaneous injection of a purified antigen at a site distant from the target organ) which closely parallels that of the well-established autoimmune models of experimental allergic encephalomyelitis and autoimmune thyroiditis (Paterson, 1960; Day *et al.*, 1977; Sharp, Mullen & Kyriakos, 1974). Further, additional manoeuvres, such as local application of irritants or the injection of preformed antigen–antibody complexes (often necessary in other models) are avoided. The autoimmune nature of lesions in the current report is suggested by their occurrence within one inbred strain, i.e. by the finding that the *Lewis* strain rat (in addition to the LOU/Mn strain rat) demonstrated intestinal lesions when injected with syngeneic (Lewis) RCG.

Intriguing was the occurrence of small bowel lesions in animals sensitized to colonic glycoprotein, as well as vice versa. Using heterologous sera in immunodiffusion, we have previously shown that RGCG (small bowel origin) has multiple specificities, two of which

cross-react with the colonic glycoprotein (RCG, Roche *et al.*, 1981), while others appear to be region- (i.e. small bowel) specific. Thus, the presence of antigenic determinants held in common by RGCG and RCG could begin to explain the presence of colonic or small bowel lesions when RGCG or RCG, respectively, is used as the immunogen. The mechanism by which remaining region-specific and other determinants are responsible for eliciting an immune response and lesions only in adjoining portions of bowel (the small bowel after RCG injection, for example) remains an important goal for future study.

The differential susceptibility to disease among the four rat strains studied is of interest and parallels findings in the experimental allergic encephalitis model, where the Lewis strain is highly susceptible to disease induction while the BN strain is considerably less so (Williams & Moore, 1973). Potential mechanisms for increased resistance to autoimmune disease found with the ACI and Wistar–Furth animals studied in the current report include: absence of B- and T-cell clones with specificity for the organ-specific (self-) antigen (Ortiz-Ortiz & Weigle, 1976); higher levels of circulating syngeneic antigens which serve continuously to tolerize specific B and T cells which might otherwise respond to self-antigens (Fujinami, Paterson, Day & Varitek, 1978; Day, Varitek & Paterson, 1978); and the presence of immune regulatory T cells which tightly control B and other cells which have the potential for an autoimmune response (Talal, Dauphinee, Pillarisetty & Goldblum, 1975).

The ability consistently and reproducibly to elicit gut inflammation by immunological means is a worthwhile and important goal. Although not expected to duplicate precisely the histology or pattern of distribution of lesions found in the gut of patients with chronic idiopathic inflammatory bowel disease, such a model should be of immense help in defining initial events leading to, and the immune mediators responsible for, immunologically-induced gut inflammation.

AKNOWLEDGEMENTS

This study was supported by grants AM-20201 and NS-10237 from the National Institutes of Health as well as by a Career Development Award to J. K. R. from the National Foundation for Ileitis and Colitis, Inc.

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