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Goblet cell glycoprotein: an organ-specific antigen for gut. Isolation, tissue localization and immune response

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Summary. We report, for the first time, immune responses within two lines of inbred rats to a purified Lewis rat glycoprotein antigen which is organ-specific for intestine. The antigen was prepared by solubilization of gut epithelial cell-associated macromolecules, fractionation in ethanol, and molecular sieve chromatography over Sepharose 2B. Homogeneity of the end product (RGCG-PK1) was supported by results of both double diffusion in agar and SDS polyacrylamide gel electrophoresis. Amino acid analysis and specific sugar determination proved that RGCG-PK1 was not a classical mucin because of its comparatively high tyrosine and low galactosamine + glucosamine content, and the absence of glycosidic linkages to serine and threonine. Organ-specificity was shown by the ability of RGCG (but not liver homogenate) to inhibit precipitation and haemagglutination by heterologous specific sera. Organ-specificity was confirmed by the demonstration of RGCG-PK1-specific immunofluorescence staining of rat small and large intestine, but not esophagus, stomach or liver. RGCG-PK1 determinants within rat and human small bowel were found to be confined to goblet cells and intestinal glycocalyx. Anti-RGCG-PK1 serum showed no reactivity with highly purified xenogeneic mucins nor with syngeneic small bowel mucin. Specific antibody (as well as antibody-dependent cellular cytotoxicity) to RGCG was elicited and detected for up to 10 days in two lines of inbred rats, including the one (Lewis) from which the antigen was isolated. The duration and peak of the humoral immune response were abbreviated compared with that of a xenogeneic control glycoprotein studied in parallel, probably due to immunoregulatory mechanisms operative for self antigens.

Abbreviations: RGCG, rat goblet cell glycoprotein; RGCG-PK1, peak 1 of Sepharose 2B-purified RGCG; OSM, ovine submaxillary mucin; BSM, bovine submaxillary mucin; PSM, porcine submaxillary mucin; RGCM, rat goblet cell mucin; RGCM-FF-1, peak 1 of Sepharose 4B-purified RGCM; RGCM-F-2, peak 2 of Sepharose 4B-purified RGCM; RCG, rat colonic glycoprotein; FCA, Freund's complete adjuvant; FIA, Freund's incomplete adjuvant; FCS, foetal calf serum; PHA, passive haemagglutination; PAS, Periodic Acid Shiff method; MC, mononuclear cells.

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INTRODUCTION

Organ-specific antigens are generally involved in eliciting autoimmune diseases, e.g. those of the thyroid (Witebsky & Rose, 1959), brain (Patterson, 1960), adrenal (Milgrom & Witebsky, 1962), and testicular (Freund, Lipton & Thompson, 1953) tissues. With regard to the intestinal tract, recent studies by Rabin (1976) would suggest that gut mucosa contains organ-, species- and region-specific antigens. However, highly-purified candidate organ-specific antigens of this system have not been rigorously characterized nor investigated for their ability to produce autoimmune responses and disease. If an immune response were detected, it could serve as a tool to study the origin and

regulation of similar events (anti-colon antibody and cytotoxic lymphocytes) described in certain chronic intestinal diseases (Langercrantz, Hammarstrom, Perlmann & Gustafsson, 1966; Watson, Quigley & Bolt, 1966).

Gastrointestinal glycoproteins such as mucin are likely candidates for organ-specific antigens for several reasons. Firstly, their distribution (goblet cells and glycocalyx) matches that described by Rabin for organ-specific antigen in gut (Rabin, 1976; Forstner, Taichman, Kalnins & Forstner, 1973). Secondly, they are closely associated with the target cells (lumenal epithelium) to which peripheral lymphocytes are sensitized, and for which circulating antibody is specific, in chronic inflammatory bowel diseases of man which are thought to be autoimmune in nature (Shorter & Shephard, 1975). Mucins, for example, are synthesized by one type of epithelial cell (the goblet cell; Goodman, 1975) and coat other epithelial cells as a component of the glycocalyx.

It was the purpose of this series of investigations to (i) isolate and characterize an organ-specific antigen (subsequently designated rat goblet cell glycoprotein or RGCG) from rat small bowel; (ii) describe aspects of the immunochemistry of RGCG, particularly its specificity and cross reactivity with glycoproteins from other portions of intestine and with the highly purified mucins; (iii) test whether humoral and antibody-dependent cell-mediated immune responses could be elicited by this antigen when injected into each of two inbred strains of rats.

MATERIALS AND METHODS

Analytical procedures

Carbohydrate and protein determinations. Total carbohydrate content was measured by the phenol-resorcinol procedure (Dubois, Gilles, Hamilton, Rebers & Smith, 1956), with galactose (Cal Biochem, La Jolla, Calif.) as standard and colorimetric readings at 490 nm. Protein determinations were by the method of Lowry, Rosenbrough, Farr & Randall, (1951), with twice recrystallized bovine serum albumin (Sigma Chemical Company, St. Louis, Mo.) as a standard.

Polyacrylamide gel electrophoresis. Samples were prepared under reducing conditions (β-mercaptoethanol) with heating for 1 hr at 100° in pH 8·0 phosphate buffer which was 1·0% in SDS. Electrophoresis was carried out by a standard procedure (Weber & Osburn, 1969), testing in duplicate up to 100 μg of sample per 5% gel. Standards of known molecular weight (ovalbumin, 43,000 mol. wt; light chain of rabbit IgG, 22,000 mol. wt; bovine myelin basic protein, 18,400 mol. wt; cytochrome C, 11,700 mol. wt) were run concomitantly, as was a highly-purified reference glycoprotein (ovine submaxillary mucin or OSM) having a molecular weight and carbohydrate/-protein content similar to that of RGCG. Proteins were stained with Coomassie Blue.

Amino acid analysis before and after β-elimination of carbohydrate side chains. The β-elimination reaction in 1 M $NaBH_4$ was accomplished using a standard procedure (Marshall & Neuberger, 1972). Subsequently, 500 μg of unmodified RGCG-PK1 as well as a similar quantity of RGCG-PK1 which had undergone β-elimination were studied with a single column amino acid analyser (Beckman Model 120C) to quantify specific sugars and changes in amino acid residues (Ser→Ala; Thr→α-aminobutyrate) characteristic of glycosidic linkages to serine and threonine, as in mucins (Marshall & Neuberger, 1972). A standard procedure (Spackman, Stein & Moore, 1958) was employed with hydrolysis carried out in the presence of 6 M HCl at 100° for 20 hr *in vacuo*.

Isolation of rat goblet cell glycoprotein

Epithelial cell isolation. The entire small bowel of ether-anaesthetized 8-week-old male Lewis rats was resected into cold isotonic saline, and faecal contents were removed by gentle flushing with cold saline. Epithelial cells and adherent mucus were then stripped from the mucosa by the method of Culling, Reid, Trueman & Dunn (1973). Cells (>95% morphologically epithelial in type), freed from the gut mucosa, were pelleted by centrifugation (2000 r.p.m., 10 min) and, upon homogenization in 0·1 M NaCl, were designated as extract C. Total carbohydrate present in this cell-derived fraction was less than 20% of that in the EDTA extraction fluid (extract A) spun free of cells.

Ethanol fractionation. To determine optimal conditions for precipitation of carbohydrate-rich macromolecules, aliquots of material prepared above were fractionated with ethanol (35–80% v/v), Fig. 1. Preferential precipitation of carbohydrate relative to protein occurred at ethanol concentrations between 55% and 75%, particularly with extract A, where 48% of the carbohydrate and 12% of the protein precipitated at

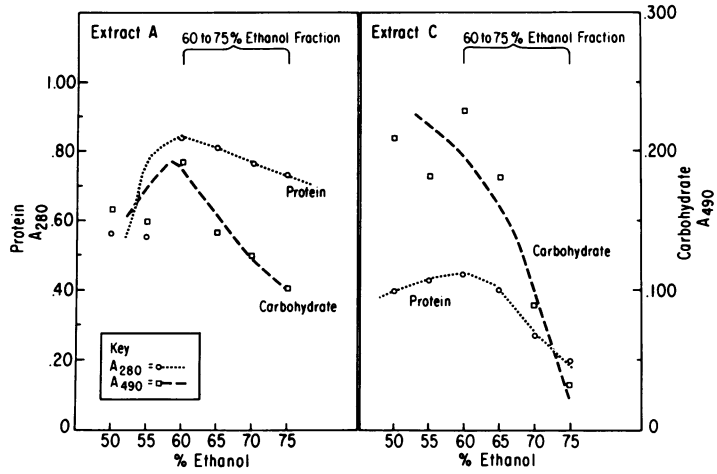

Figure 1. Step fractionation in ethanol of small bowel extracts. Extract A consists of substances soluble in the PBS/0·01% EDTA solution used for mechanical separation of intestinal epithelial cells described in Materials and Methods. Extract C was prepared by homogenization in 0·01 M NaCl of rat small bowel epithelial cells. One millilitre aliquots of each extract were brought to a preselected concentration of ethanol at 4°, stirred for 60 min, centrifuged at 27,000 *g* for 30 min, and analysed. The carbohydrate content of supernatants, determined by the phenol–resorcinol procedure, is shown by absorbance at 490 nm (□- - -□), while the protein content (particularly aromatic amino acids) was followed by absorbance at 280 nm (O- - -O).

the highest ethanol concentration. Hence, our procedure for preparative work with extract A involved precipitation in 35, 55, and 75% ethanol followed by centrifugation at 27,000 *g*. Material precipitating between 55% and 75% ethanol, after being redissolved and lyophilized, was designated rat goblet cell glycoprotein.

Sepharose 2B chromatography. Lyophilized RGCG dissolved in 0·15 M ammonium acetate buffer (pH 7·0),

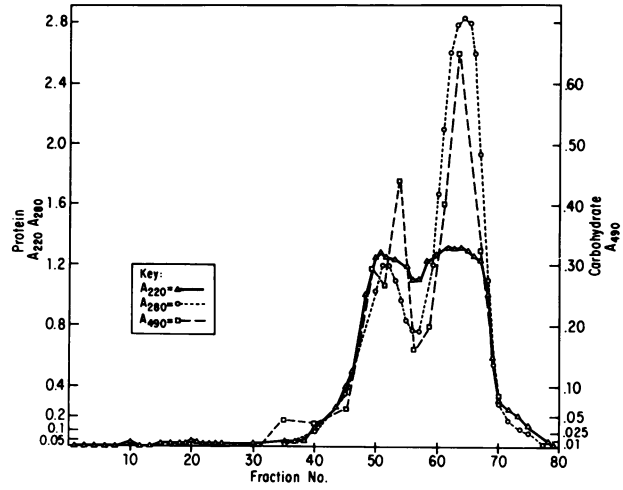

Figure 2. Preparation of purified components of rat goblet cell glycoprotein (RGCG) by molecular sieve chromatography. Twenty milligrams of lyophilized RGCG, prepared from Extract A by precipitation between 55% and 75% ethanol, was applied to a 73 × 2 cm column of Sepharose 2B (Pharmacia, Uppsala, Sweden) equilibrated with 0·15 M NH_4OAc at pH 7·0. One millilitre fractions were collected, and then analysed as described in the legend for Fig. 1. In addition, absorbance at 220 nm (Δ- - -Δ) was monitored to follow proteins relatively poor in aromatic amino acids, as in the case of mucin macromolecules (Hill, Reynolds & Hill, 1977; Bella & Kim, 1972).

was applied to a 73 × 2 cm column of Sepharose 2B (Pharmacia, Uppsala, Sweden), eluted with the same buffer, and analysed for protein and carbohydrate. Two separate peaks could be distinguished by collecting fractions which were 1% of bed volume (Fig. 2). Eluate with the highest carbohydrate to protein ratio was found nearest the void volume (peak 1), while the bulk of protein was associated with peak 2. Fractions within each carbohydrate-rich peak were combined, dialysed exhaustively against water, lyophilized, and stored in a desiccator. While a similar chromatographic pattern developed with the homogenized epithelial cell preparation (extract C), the protein present in peaks 1 and 2 was five-fold greater, yielding material with inferior (four- to five-fold lower) carbohydrate to protein ratios.

Other isolation procedures investigated. Major purification based upon pH was not possible as over 60% of the carbohydrate-containing substances in extracts A and C were denatured and precipitated at pH 4·5 while most of the protein (77%) remained in solution. In addition, chromatography of RGCG on hydroxylapatite and DEAE-cellulose, employing step gradients from 0·01 M to 0·5 M phosphate buffer, did not provide further clear-cut separation.

Immunological techniques for characterization of RGCG

Elicitation of heterologous antisera to gut glycoproteins. New Zealand White rabbits were immunized subcutaneously (footpad) with either 1·5 mg of RGCG (glycoprotein precipitating between 55% and 75% ethanol; rabbits SB-1, -2), or 0·7 mg of RGCG-PK1 (peak 1 of Sepharose 2B-purified RGCG; rabbits SB-3, -4). Each immunogen was dissolved in saline and emulsified in an equal volume of Freund's complete adjuvant (FCA), and the resulting antisera at peak titre [1:4000 by passive haemagglutination (PHA)] were used as standard reagents for antibody testing and immunofluorescence (below).

Detection of anti-mucin antibody. Modifications of methods previously described (Roche, Day & Hill, 1978; Roche, Varitek, Hill & Day, 1979) were used to detect specific antibody to RGCG and to highly-purified xenogeneic mucins. For PHA, 100 μl of RGCG (7 mg/ml) was used as the optimal amount of antigen for sensitizing cells. Immunodiffusion was carried out in 1% agarose made up in pH 8·2 tris buffer, using 5 μl wells 10 mm apart. Reactions were read at 72 hr and photographed. The sources of antigen used in immunodiffusion and PHA are indicated below.

The specificity of rat and rabbit sera for subfractions of RGCG (PK1 and PK2) was determined by their ability to inhibit haemagglutination or precipitation in agar gel diffusion. Seventy-five microlitres of immune serum were pretreated with 1–8 μl of each potential inhibitor (7 mg/ml) for 1 hr at room temperature, centrifuged (10 min, 2000 r.p.m.), and tested for residual specific antibody activity.

Immunofluorescence. Tissues (liver, esophagus, stomach, small and large intestine) from germ-free and conventional Lewis strain rats were embedded in 10% gelatin, snap frozen in isobutane cooled by liquid nitrogen and fixed in acetone (20 min) as 4 μ-thick cryostat sections. Histologically normal human small intestine was processed in an identical manner. Optimal conditions for indirect immunofluorescence (1:80, RGCG-specific serum; 1:40, fluorescence-conjugated immunoglobulin) were determined by evaluating dilutions of our antibody reagents in a checkerboard fashion. To identify the cellular localization of RGCG-PK1 determinants within gut, cryostat sections examined by the immunofluorescence technique were subsequently stained by the Periodic Acid Shiff (PAS) method to delineate goblet cells, counterstained with haematoxylin and rephotographed.

Sensitization of inbred murine lines with gut glycoprotein

Regimens. Seven-week-old inbred LOU/Mn rats (Small Animals Branch, NIH, Bethesda, Md) and Lewis strain rats (Microbiological Associates, Walkersville, Md) underwent subcutaneous (footpad) and intravenous (tail vein) immunization with RGCG employing several regimens previously shown by us to successfully elicit humoral responses to the more thoroughly purified and studied glycoproteins, the ovine and porcine submaxillary mucins (Roche *et al.*, 1979). RGCG and mucins were bound to red blood cells for injection as described above. To verify that conditions were optimal in our experiments for both elicitation and detection of anti-glycoprotein antibodies, parallel immunizations were carried out with highly purified OSM and PSM. Other control animals received saline with adjuvant or chicken red blood cells alone. Subsequently, animals were weighed and bled at 3 day intervals, and humoral responses measured by the passive haemagglutination assay.

Detection of antibody-dependent cell-mediated responses. Antigen-labelled chicken red blood cells were incubated with 100 μCi ^{51}Cr over 2 hr, washed three times, and dispensed in triplicate into wells of a microtitre plate (Falcon Labware, Oxnard, Calif.) to achieve a final density of 5×10^4 cells per well. Mononuclear cells (MC) were isolated from freshly defibrinated blood of healthy adults (Boyum, 1976) and from rat spleen. After three washes and viability testing, 10^5 to 10^8 MC in medium M199 (Gibco, Grand Island, N.Y.) with 10% foetal calf serum were pipetted into wells. Heat-inactivated test serum was diluted as required in medium and pipetted into wells. The resultant final volume was 200 μl, and effector:target (E:T) ratios varied from 1:1 to 50:1. Tested concomitantly were known positive and negative rabbit sera, having a PHA titre to RGCG of 1 to 4000 and less than 4, respectively. Cultures were incubated at 37° for 4 hr in 95% air–5% CO_2. The percentage of ^{51}Cr released into the medium was used as a measure of cell injury. For actual assays, background release (leakage) from labelled and unlabelled red blood cells averaged 6% or less over 4 hr. Cytotoxicity (percentage specific release) was determined by the formula:

$$\frac{2\,(\text{c.p.m.}_{\text{experimental}} - \text{c.p.m.}_{\text{control}})}{\text{c.p.m.}_{\text{total}} - \text{c.p.m.}_{\text{control}}} \times 100$$

where c.p.m.$_{\text{experimental}}$ are supernatant counts per minute (wells contain test sera, MC and targets); and c.p.m.$_{\text{control}}$ are the supernatant counts per minute in wells containing non-immune sera, MC and targets. c.p.m.$_{\text{total}}$ are the total counts released when the standard number of target cells (5×10^4) were lysed in the presence of 5% Triton X-100. Targets exposed to antibody alone were also routinely run as controls. However, since their ^{51}Cr release did not exceed background, these control data have not been included here.

RESULTS

Biochemical characterization of RGCG

Polyacrylamide gel electrophoresis. When RGCG-PK1 (1 mg/ml) was loaded onto 5% gels in volumes up to 100 μl, no components entered. In this regard it behaved identically to ovine submaxillary mucin, a very high molecular weight glycoprotein, run concomitantly. For the less purified rat small bowel extract, RGCG, a single component did enter the gel and had an apparent molecular weight of 11,700 by comparison with known mol. wt standards.

Carbohydrate and protein content. RGCG-PK1 had a high carbohydrate content, as did classical mucins analysed for comparison: PSM and RGCM-FF-1 (Table 1). RGCG-PK2, on the other hand, was relatively carbohydrate-poor, as was its less purified precursor, RGCG, and rat colonic glycoprotein (RCG).

Amino acid analysis. When RGCG-PK1 both before and after undergoing a β-elimination procedure, was studied in an amino acid analyser, characteristics distinct from classical mucins (Marshall & Neuberger, 1972) were noted (Tables 2 and 3). Specifically, (i) tyrosine content (26·94 nM/100 nM of amino acids) was two and a half times that encountered with mucin isolated from rat small bowel (RGCM-FF-1); (ii) total galactosamine was considerably less than the sum of threonine and serine; (iii) glucosamine was not abundant; and (iv) chemical conversions typical of a substance with glycosidic linkages to Ser and Thr, i.e.

Table 1. Relative carbohydrate and protein content of RGCG, its Sepharose 2B-purified components, RCG, and the mucins RGCM and PSM

	Content†	
Substance analysed*	Carbohydrate	Protein
RGCG		
Unfractionated	0·15	0·85
Peak 1	0·58	0·42
Peak 2	0·16	0·84
RCG	0·08	0·92
RGCM (Forstner)		
Unfractionated	0·09	0·91
FF-1 (peak 1)	0·63	0·37
F-2 (peak 2)	0·12	0·88
PSM	0·54	0·46

* Rat colonic glycoprotein (RCG) was prepared in a manner identical to the procedure for RGCG (mechanical isolation, ethanol fractionation). Rat goblet cell mucin (RGCM) was prepared from the small bowel of Lewis rats according to the procedure of Forstner *et al.* (1973); FF-1, as originally designated, refers to peak 1 material after Sepharose 4B chromatography. Highly purified xenogeneic mucin (PSM), analysed for comparison, was prepared as previously described (Roche *et al.*, 1978).

† Expressed as a percentage of total carbohydrate plus protein. The procedure and standards used in measuring carbohydrate and protein content are described under Materials and Methods.

Table 2. Comparison of the amino acid and specific sugar composition of a purified component of RGCG and a well-characterized intestinal mucin, RGCM-FF-1*

Amino acid	Content in nmol per 1000 nmol of amino acid	
	RGCG-PK1	RGCM-FF-1
Tyrosine	26·94	10·84
Galactosamine	68·31	170·93
Threonine and Serine	143·75	180·42
Glucosamine	85·71	320·0

* One milligram of substrate (RGCG-PK1 or RGCM-FF-1) was hydrolysed in 6 M HCl at 110° for 20 hr *in vacuo*. After calibration with known quantities of amino acids and specific sugars, a Beckman Model 120C single column amino acid analyser was used to study 500 μg samples of each substrate by the procedure described by Spackman *et al.* (1958).

Ser→Ala and Thr→α-aminobutyrate (Marshall & Neuberger, 1972) were not detected. RGCM-FF-1, on the other hand, was confirmed to contain abundant glycosamine and galactosamine while having little tyrosine (Table 2).

Immunological characterization and localization

Specificity and cross-reactivity of RGCG. Rabbit antiserum elicited by RGCG exhibited two specificities by Ouchterlony analysis, while antiserum raised to peak 1 of Sepharose 2B-purified RGCG showed a single precipitin band which cross-reacted with rat colonic glycoprotein but not xenogeneic mucins (Fig. 3). That the principal immunogen eliciting the xenogeneic humoral response was present primarily in peak 1 and not peak 2 RGCG was shown by using the purified peak 1 and peak 2 components themselves as (i) reactants in agar gel (Fig. 4) and (ii) inhibitors of precipitation by immune sera. In the latter case, serum was readily inhibited by peak 1 but not peak 2 RGCG. There was no cross-reactivity with either peak 1 (FF-1) or peak 2 (F-2) of mucin (RGCM) isolated from rat small bowel (Fig. 4) over a range of concentrations (1–7 mg/ml) consistent with the differences detected by amino acid analysis described above (Table 2).

Evidence that RGCG determinants were unique to bowel was obtained by both immunodiffusion and by inhibition of agglutination. Pretreatment with ≥5 μl of Lewis or LOU/Mn-strain RGCG (but *not* rat liver homogenate) blocked subsequent precipitin reactions of anti-RGCG sera. Similar results were obtained using as little as 1 μl of inhibitor in blocking haemagglutination (Fig. 5).

Localization and organ-specificity. An indirect immunofluorescence technique was used to confirm the organ-specificity, as well as to assess the cellular localization within tissues, of RGCG-PK1. Of all rat tissues tested, only the single cell epithelium lining the lumen of the intestine distal to the stomach possessed

Table 3. Effect of β-elimination conditions upon amino acid content of RGCG-PK1*

Amino acid	Content in nmol per 1000 nmol of amino acid	
	Before	After
Serine	55·36	45·11
Alanine	75·40	66·23
Threonine	64·88	76·36
α-aminobutyrate	0	0

* Five hundred micrograms of substrate (RGCG-PK1) was incubated at 55° for 12 hr in an O_2-free 1 M $NaBH_4$ solution which was 0·01 M in NaOH. After acidification and dialysis, the sample was analysed in a single column amino acid analyser as described in the legend for Table 2.

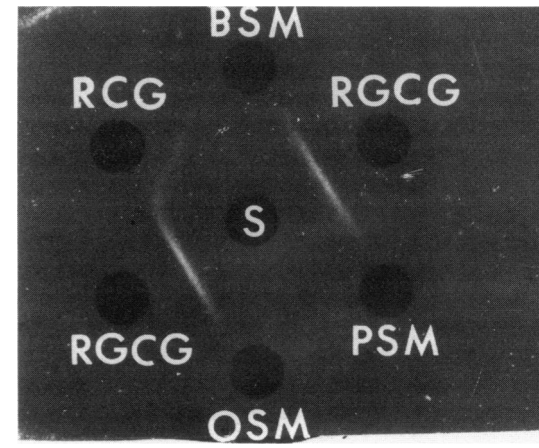

Figure 3. Double diffusion in agar of rabbit anti-RGCG serum against the indicated antigens. Highly purified xenogeneic mucins [bovine submaxillary mucin (BSM), ovine submaxillary mucin (OSM), and porcine submaxillary mucin (PSM)] analysed for comparison, were prepared as previously described (Roche *et al.*, 1978). Antigen concentrations were 7 mg/ml. Centre well (S) contains rabbit antiserum to Sepharose 2B-purified peak 1 RGCG.

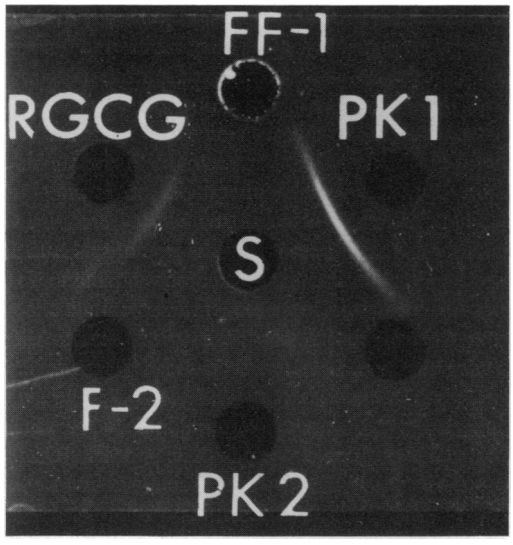

Figure 4. Ouchterlony double diffusion analysis of rabbit anti-RGCG-PK1 serum against purified fractions of Lewis rat small bowel. FF-1 (rat goblet cell mucin) and F-2 are Sepharose 4B-purified components of a small bowel homogenate prepared by the method of Forstner *et al.* (1973). PK1 and PK2 are components of rat goblet cell glycoprotein (RGCG) isolated by Sepharose 2B chromatography. Serum in the centre well (S) is rabbit anti-RGCG-PK1.

RGCG determinants (Fig. 6). Fluorescence was not seen within the lamina propria, muscle layers, serosa, or in collagen fibres of the intestine at any level (esophagus to colon); nor in the epithelial (including goblet) cells of stomach or esophagus, nor in the liver (same figure). Germ-free and conventional tissues did not differ in this respect.

Identification of the cell type having RGCG-PK1 determinants was ascertained by accomplishing both the fluorescence and PAS-staining procedures on the same cryostat section (Fig. 7). Only the goblet cells (positive by PAS) were fluorescence-positive, whether in the intestinal crypts or in villi tips. In addition, fluorescence and PAS-positive amorphous material was present in the intervillus spaces and likely represented RGCG-PK1 determinants secreted or extruded into the lumen by goblet cells. Of interest, RGCG-PK1 determinants were also identified by fluorescence in human small bowel (not shown), and exhibited a tissue distribution and cellular localization identical to that of rat small bowel and colon, and, in this sense, behaved as an organ-specific antigen.

Immune responses to rat gut glycoproteins within inbred murine lines

Humoral response. In the LOU/Mn rat, RGCG-specific antibody was elicited by all but one of the immunization regimens tested (Table 4). Kinetics were not unusual (onset, day 9; duration, ≤ 14 days) and mean reciprocal PHA titres were 20 to 72 at the peak. Inhibition of agglutination with RGCG documented the specificity of the reactions (data not shown). Titres in animals immunized with a glycoprotein of comparable mol. wt and carbohydrate/ protein ratio (OSM) were 10–100 fold higher.

By contrast, in the Lewis strain rat, only very low titre antibody (generally 1:4–1:8) was elicited, occurring within the first 2 weeks after immunization, but lasting usually 3 days or less in an individual animal (Table 4). These reactions, too, were antigen-specific, for they could be inhibited by preincubation of sera with RGCG before exposure to antigen-labelled

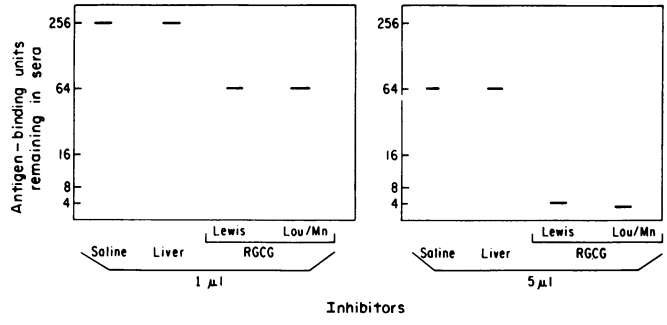

Figure 5. Passive haemagglutination of RGCG-labelled goat red blood cells by anti-RGCG-PK1 serum pretreated with potential blocking substances. To 150 µl of serum was added 1 or 5 µl of the indicated inhibitor. After 1 hr at room temperature, sera were centrifuged at 3000 *g* for 15 min and supernatants tested for residual reactivity to RGCG. The 20% liver homogenate was prepared in a Waring blender using freshly-resected Lewis strain rat liver in 4 vol of saline, followed by centrifugation (20 min, 5000 r.p.m.) to yield a clear supernatant.

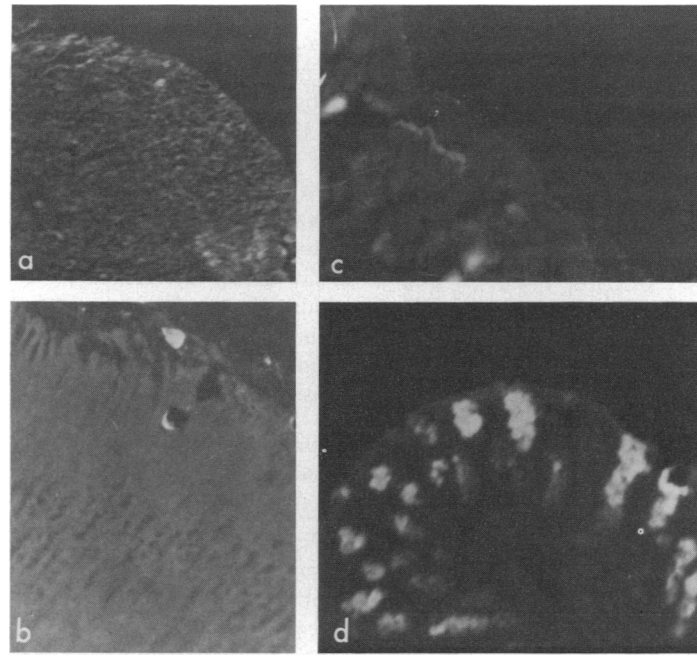

Figure 6. Immunofluorescent staining of selected rat organs treated with anti-RGCG-PK1 serum. Tissues were taken from Lewis strain rats and were: (a) liver; (b) stomach; (c) esophagus; and (d) colon. Control sections were treated with (a) fluorescinated second antibody alone; (b) rabbit anti-RGCG serum after complete absorption with homologous antigen (verified by PHA); (c) normal sera in place of immune sera. The fluorescence-positive colonic epithelium lines the surface of intestinal crypts, which are sectioned perpendicularly at the top and right, but tangentially at the left of photograph labelled (d). Results were recorded through a Zeiss fluorescence microscope equipped with fluorescence-specific filters and a Nikon camera (magnification × 40).

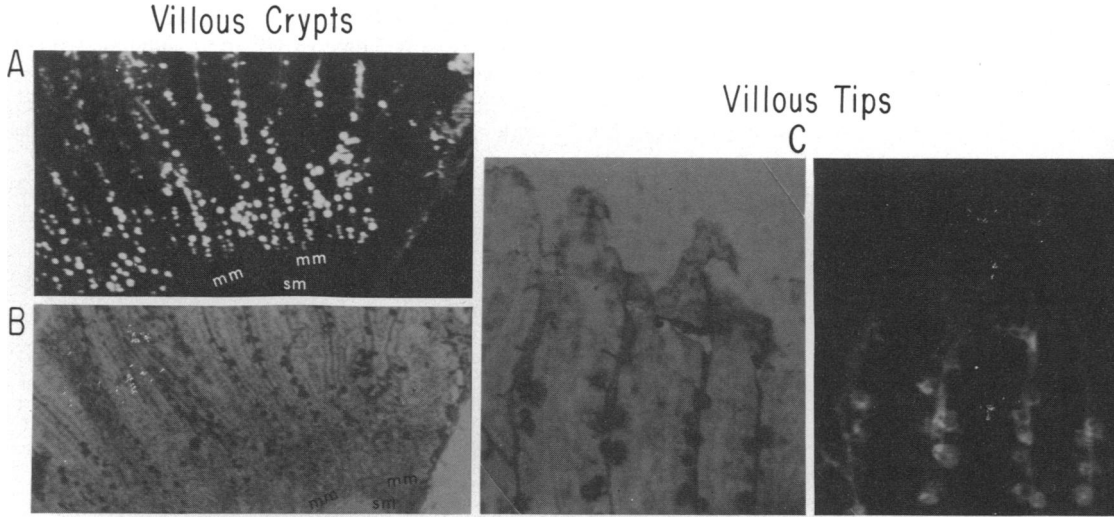

Figure 7. Dual immunofluorescent/PAS staining of rat small intestine treated with anti-RGCG-PK1 serum. Sections were taken both deep within the glands of the intestine (A, B), as well as at their interface with the bowel lumen (C, D). Sections stained for RGCG-PK1 determinants by immunofluorescence are A, D; for carbohydrate, B, C. A one-to-one correspondence is seen between PAS and fluorescence-positive areas. mm, muscularis mucosa; sm, submucosa of intestinal wall (magnification × 100, A and B; magnification × 250, C and D).

Table 4. Response of LOU/Mn and Lewis rats to immunization with RGCG and control xenogeneic glycoprotein

Immunization regimen			Response to RGCG		Response to OSM		
Dose/ animal (μg)	Route	Number of responders/ total	Reciprocal titre at peak response (mean†/range)	Duration (days)	Number of responders/ total	Reciprocal titre at peak response (mean/range)	Duration (days)
LOU/Mn strain rats							
700	Subcutaneous (s.c.)*	4/4	20/16–32	10	—	—	—
70	s.c.*	4/4	22/8–32	14	4/4	≥4096/2048 to ≥4096	28
<0·5	IV‡	2/4	72/16–128	4	—	—	—
<5	IV‡	0/4	—	—	3/3	≥2048/1024 to ≥4096	14
Lewis strain rats							
700	s.c.*	4/4	4–8	4	2/4	256/256§	—
70	s.c.*	3/4	4–8	<3	3/4	32/32§	—
0	s.c.*	0/4	—		—	—	—
<0·5	IV‡	3/4	4–8	4	3/4	128/64–256	—
<5	IV‡	3/4	4–8	<3	—	—	—
<5	s.c.*	2/4	4	<3	4/4	172†/ 64–356	—
0	IV	0/4	—	—	0/4	—	—

* In Freund's incomplete adjuvant containing 2·5 mg/ml *Mycobacterium butyricum*.
† Calculated as the antilog of the mean of the log titres.
‡ RGCG bound to chicken red blood cells; 0·25 ml of a 1% or 10% suspension injected.
§ Peak titre was the same in all animals.

indicator cells (data not shown). Animals injected with a xenogeneic (control) glycoprotein (OSM or PSM) responded with each regimen tested, with peak titre of specific antibody ranging from 1:32 to 1:256.

Cell-mediated response. Sera elicited in the LOU/Mn strain rat by subcutaneous injection of 700 μg of RGCG/animal were effective in ADCC assays and were able to trigger cell-mediated cytotoxic reactions against RGCG-labelled targets, with purified human peripheral lymphocytes or rat spleen cells as effectors (Fig. 8). Specific release of ^{51}Cr was

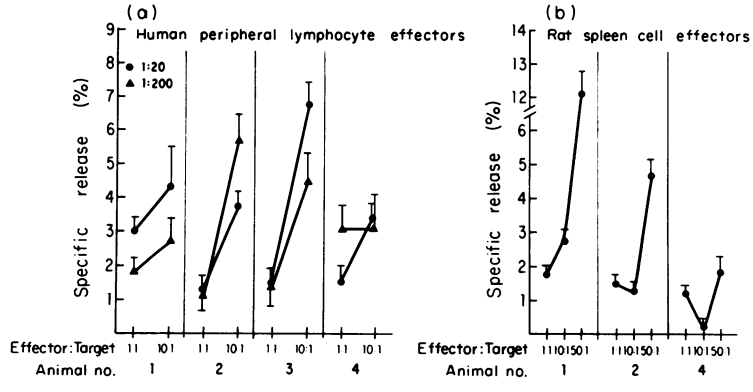

Figure 8. Antibody-dependent release of ^{51}Cr from RGCG-coated chick red blood cells by immune rat sera. LOU/Mn strain rats were immunized subcutaneously with RGCG 0·07 mg/animal, emulsified in an equal volume of Freund's incomplete adjuvant containing 2·5 mg/ml of *M. butyricum*. Serum taken at day 19 post-immunization was tested at a 1:20 (○——○) or 1:200 dilution (△——△), with effectors consisting of purified human peripheral lymphocytes (a) or rat spleen cells (b). Serum from animal 3 was not available for testing in (b). Details of the assay are described under Materials and Methods.

generally greater at the higher effector/target ratios employed, and at the lower (1:20) serum dilution tested.

DISCUSSION

While circulating antibody specific for antigens on (or adjacent to) colon epithelial cells has been reported to be present in up to 60% of patients with certain chronic inflammatory conditions of the gastrointestinal tract, particularly ulcerative colitis (Langercrantz *et al.*, 1966), similar autoimmune phenomena involving gut antigens have only rarely been elicited and studied in the experimental animal. Further, an autoimmune humoral or cell-mediated response to a *purified* gut component has not been reported within inbred animal lines to our knowledge.

Although the existence of organ- and region-specific antigens for gut has been proposed by others (Rabin, 1976), extensive attempts to isolate such materials have been infrequent (Nairn, Fothergill & McEntergart, 1963; Kopp, Trier, MacKenzie & Donaldson, 1968). The first goal of the current study was to purify and characterize antigens that might elicit autoimmune responses to the single cell epithelial (lumenal) lining of the intestine. Techniques for antigen purification were selected, therefore, that could yield glycoproteins localized exclusively to this portion of bowel. A mechanical separation technique followed by centrifugation resulted in an initial preparation essentially free of non-epithelial gut cell types as well as of intracellular antigens that would have been liberated had tissue homogenization been necessary. Precipitation with ethanol, the second step in purification, accomplished a rapid concentration of antigen, demonstrated a respectable solubility differential between intestinal glycoprotein and protein macromolecules, and exerted antibacterial activity to protect antigen from degradation by contaminating intestinal organisms. Our final purification step, Sepharose 2B chromatography, permitted fractionation of RGCG into two components which differed markedly in their properties. Evidence for homogeneity of first peak material (RGCG-PK1) included (i) absence of bands on a 5% polyacrylamide gel, indicating less than 1% contamination with individual small mol. wt substances; and (ii) presence of a single precipitin band in agar gel when RGCG-PK1 was reacted with polyspecific sera to our initial preparation, RGCG. Initially, RGCG-PK1 was thought to be an intestinal mucin, perhaps the same one isolated by Forstner *et al.* (1973) because of certain characteristics in common: high molecular weight, inability to enter a 5% polyacrylamide gel, high carbohydrate content, and localization to intestinal goblet cells and glycocalyx. However, neither the composition of specific amino acids and sugars, nor changes upon β-elimination of carbohydrate moieties from RGCG-PK1 in the presence of 1 M $NaBH_4$, were characteristic of classical mucins; in particular, the content of tyrosine, galactosamine and glucosamine in RGCG-PK1 differed considerably from that of rat small intestinal mucin (RGCM-FF-1), analysed in parallel in this study.

Several immunochemical techniques were employed to test whether RGCG-PK1 was truly organ-specific for the gastrointestinal tract. Findings were confirmatory in that (i) RGCG-PK1 was shown by indirect immunofluorescence to be confined to a single cell type in the colon and small bowel; neither the liver, esophagus nor stomach contained these RGCG-PK1 determinants; (ii) rat liver homogenate, when employed in amounts far greater than that required to achieve inhibition with homologous antigen, was incapable of blocking anti-RGCG-PK1 serum reactions in agar or in inhibiting haemagglutination of RGCG-labelled cells; (iii) RGCG-PK1 determinants in gut were detected across species lines (murine and human intestine showed an identical fluorescence-positive pattern and localization of these determinants) and across strain lines [LOU/Mn and Lewis intestinal extracts (but not liver) blocked to an equal extent haemagglutination by anti-RGCG-PK1 serum].

A second goal was to test the hypothesis that purified components isolated from non-inflamed gut could elicit immune responses in inbred lines of the animal species from which the components were originally isolated. Organ-specific antigens in particular have this capacity to induce autoimmune responses and disease (Witebsky & Rose, 1959) as demonstrated in several current experimental animal models (Paterson, 1960; Nakamura & Weigle, 1969; Day & Wexler, 1974). Yet most previous studies of immune responses to gut antigens have used crude intestinal homogenates for immunization of outbred animals, circumstances where the autoimmune nature of the response may be difficult to assess and document. Ford & Kirshner (1967) reported that the sera of two of six rabbits multiply-injected with formalin-inflamed gut tissues and FCA contained antibody to a soluble

fraction of normal rabbit colon. However, their antibody reacted with kidney/liver extracts in agar gel, and the presence of specificities for strain determinants or extraneous antigens in their homogenate was not ruled out by absorption studies. Holborow, Asherson & Wigley (1963) overcame some of these objections by using, as the substrate for immunofluorescence, tissue from the immunized animal itself. While their rabbit sera had specificity for antigens in rabbit colon, complement fixation showed concomitant specificities for stomach and liver, which may have accounted for positive fluorescence findings but on a non-autoimmune basis.

In the current study, both humoral and antibody-dependent cellular immune responses could be demonstrated with sera from rats of two inbred lines immunized with RGCG. Compared with control glycoproteins of similar molecular weight and carbohydrate/protein content, peak titre and duration of specific antibody to RGCG were less, although the regimens employed for successful sensitization were identical. Included among possible mechanisms to account for this finding are host regulatory processes that would suppress reactions to self-antigens while not affecting responses to the xenogeneic mucins (OSM and PSM) used as control glycoproteins. It is of interest that recent studies in our laboratory have shown that small bowel hemorrhagic lesions develop in each of the two murine strains (LOU/Mn, Lewis) reported here as capable of autoimmune responses, and not in two strains in which circulating RGCG-specific antibody could not be demonstrated (Roche, Cook & Day, 1981) Further work is needed to define the mechanism(s) which regulates autoimmune responses to gut self-antigens, and how these are circumvented in the LOU/Mn and Lewis rat strains.

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