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Intestinal uptake of macromolecules

Differences in distribution and degradation of protein antigen in control and immunised rats*

K Y PANG,† W A WALKER,‡ AND K J BLOCH

From the Pediatric Gastrointestinal and Nutrition Unit and the Clinical Immunology and Allergy Units of the Medical Services, Massachusetts General Hospital, and the Departments of Pediatrics and Medicine, Harvard Medical School, Boston, Massachusetts, USA

SUMMARY The present study examined intraluminal events in the *in vivo* processing of a protein antigen by the intestine of normal and orally immunised rats. One hour after the administration of ^{125}I-bovine serum albumin (^{125}I-BSA) and unlabelled BSA by gavage, the majority of the radioactivity was found in the distal small intestine of control and immunised rats but there was a difference in the distribution of radioactivity. In contrast with controls, immunised rats retained a lesser percentage of radioactivity in the proximal small intestine and a greater percentage of radioactivity in the distal small intestine. Radioactive substances present in intestinal rinse fluids and mucosal extracts were characterised by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), density gradient ultracentrifugation, and by immunochemical methods. Rinse fluids and mucosal extracts from immunised rats fed ^{125}I-BSA by gavage contained high molecular weight components with characteristics of antigen-antibody complexes. Rinse fluids and extracts of normal rats contained more intact BSA and less fragments of BSA than did rinse fluids and extracts from immunised animals. These findings suggest that oral immunisation alters the distribution of antigen administered into the gut and that immunisation enhances the intraluminal degradation of antigen.

In studies performed with everted gut sacs *in vitro*[1 2] and mesenteric lymph fistulas *in vivo*,[3] it was found that exogenous macromolecules (horseradish peroxidase) were taken up by pinocytosis into enterocytes and subsequently transported to the extracellular space of the lamina propria and from there into the lymph. After oral[4] or parenteral immunisation,[5] there was less uptake by gut sacs from immunised than from control animals. In the present study, we examined intraluminal events in the *in vivo* processing of antigen in normal and immunised rats. After gavage with labelled BSA, there was a difference in distribution of antigen in the gut of immunised and control animals.

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‡ Address for correspondence: Professor W A Walker, Pediatric Gastrointestinal and Nutrition Unit, Massachusetts General Hospital, Boston, Massachusetts 02114, USA.

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Methods

ANIMALS AND IMMUNISATION

Adult female Sprague-Dawley rats (Charles River Labs., Wilmington, MA) ranging in weight from 200 to 250 g were used in these studies. Groups of four to six rats, which had been on milk protein-free rat chow diets (Ralston Purina, Inc., St. Louis, MO) for at least six weeks, were immunised according to the following schedule: 100 mg crystalline BSA (Sigma Chemicals, St. Louis, MO) in 1 ml 1·3% $NaHCO_3$ was administered by gavage under light ether anaesthesia each day for five days and once each week for five weeks. The total amount of BSA received by a rat during the entire immunisation process was 1 g.[6] Control rats were given 1 ml 1·3% $NaHCO_3$ by gavage according to the same schedule.

PREPARATION OF RADIOLABELLED ANTIGEN

BSA was labelled with Bolton-Hunter reagent (New England Nuclear, Boston, MA) according to the

method of Bolton and Hunter.[7] Fifty ug BSA in 100 ul phosphate-buffered saline (PBS) at pH 7·4 were mixed with 1 mCi (2000 Ci/mmol) of Bolton-Hunter reagent at 0°C. The reaction mixture was stirred for two to three hours before the addition of 0·2 ml 0·2 M glycine solution. The labelled BSA was separated from other reagents by gel filtration on a Sephadex G-25 (Pharmacia, Inc., Piscataway, NJ) column. The typical specific activity obtained by this method was 2×10^6 cpm/ug BSA.

ANTIGEN DISTRIBUTION STUDIES

One week after the completion of the immunisation schedule, rats received 2 drops of saturated potassium iodide solution in 2 ml water and were fasted overnight. On the following day, animals were challenged by gavage under light ether anaesthesia with either 1 mg BSA plus 100 mg casein (Miles Chemicals, Elkhart, IN) and trace amounts of ^{125}I-BSA in 1 ml PBS or 100 mg BSA and trace amount of ^{125}I-BSA in 1 ml PBS. Each rat received between 4 to 6×10^6 cpm of ^{125}I-BSA per gavage feeding. One hour later, rats were anaesthetised with ether; blood was obtained by cardiac puncture, and the stomach, small intestine, large intestine, and liver were removed. The small intestine was divided into two equal segments, one proximal and one distal. Five millilitres of cold PBS was infused into each segment and the intraluminal fluid was advanced through the segment by gently massaging the small intestine wall; the fluid was recovered. Thereafter, the gut segments were slit longitudinally and the mucosal layer was recovered by scraping with a glass slide. The rinse fluids and mucosal scrapings were incubated at 56°C for 30 minutes to inactivate intestinal enzymes,[8] and then were homogenised in a 30 ml Teflon pestle homogeniser (Arthur H Thomas & Co., Philadelphia, PA) at 4°C to disperse mucus and particulate matter. The rinse fluids were cleared of insoluble material by centrifugation at 4000*g* for 30 minutes, and the supernatants were retained for further analysis. The supernatants contained more than 90% of the radioactivity initially present in the rinse fluids. Homogenates of scrapings were mixed with 5 ml PBS and centrifuged under the same conditions. The supernatants were recovered, designated mucosal extracts, and saved for further analysis. The supernatants from each of these extracts contained more than 80% of the radioactivity initially present in the homogenate. Unless otherwise specified (see below), characterisation of the labelled proteins in rinse fluids or mucosal extracts was performed with the supernatants of such processed fluids or extracts.

Radioactivity in serum, stomach, large intestine, and liver, as well as in the unprocessed rinse fluids and mucosal extracts of the small intestine, was measured in a Beckman Gamma 7000 crystal scintillation spectrometer (Beckman, Fullerton, CA). The total radioactivity in proximal and distal small intestine was represented by the sum of the radioactivity in the unprocessed rinse fluids and mucosal scrapings of each segment. The blood volume of the rat was calculated from its body weight according to a previously described method[9] and the radioactivity in the circulation was determined.

In separate experiments, 1,2-^{14}C-polyethylene glycol (^{14}C-PEG, New England Nuclear, Boston, MA) was used as a marker of intestinal motility.[10 11] Rats which had been orally immunised with BSA and control rats were fasted for 24 hours before administration of 1 ml PBS containing 100 mg 5× crystallised egg albumn (ICN Pharmaceuticals, Cleveland, OH) and 2×10^6 cpm ^{14}C-PEG (7mCi/g, Lot No. 1291–124). One hour later, the animals were bled by cardiac puncture and subjected to a laparotomy as previously described. The stomach was opened, its contents recovered, and the volume of gastric fluid adjusted to 5 ml with PBS. The small intestine was divided into two segments and rinse fluids and mucosal scrapings were prepared. After homogenising the rinse fluids and mucosal scrapings, the total volume of the homogenates was adjusted to 10 ml with PBS. The large intestine was slit longitudinally, the mucosal layer was removed, homogenised, and the total volume was adjusted to 5 ml with PBS. Fifty or 100 μl of stomach contents, rinse fluids, and mucosal scrapings from proximal and distal small intestine and the mucosal scrapings from the large intestine were mixed with 10 ml Aquasol II (New England Nuclear, Boston, MA) before being counted in a Searle liquid scintillation spectrometer (Searle Instruments, Chicago, IL). The total radioactivity in the stomach, small and large intestine was calculated.

CHARACTERISATION OF ANTIGEN- FRAGMENTS OF ANTIGEN, AND ANTIGEN-ANTIBODY COMPLEXES IN RINSE FLUIDS AND MUCOSAL EXTRACTS FROM SMALL INTESTINE

SDS-PAGE

Sodium dodecylsulphate-polyacrylamide gel electrophoresis was used to detect the presence of BSA and its breakdown products in small intestinal rinse fluids and mucosal extracts from both control and immunised rats challenged with 100 mg BSA plus trace amounts of ^{125}I-BSA. Ten per cent polyacrylamide gel slabs were prepared in a Bio-Rad gel cell (Bio-Rad, Richmond, CA) according to the method of Laemmli.[12] After electrophoresis of rinse fluids and mucosal extracts (under non-reducing conditions), the gel slabs were either stained with 0·5% Coomassie blue or were cut into 1 mm slices in an electric slicer

(Bio-Rad) and the radioactivity of the slices was determined. ^{125}I-BSA and SDS-PAGE low molecular weight markers (Bio-Rad) were included on each gel slab.

Sucrose density gradient ultracentrifugation and immunoprecipitation

Rinse fluids and mucosal extracts from immunised and control rats given 100 mg BSA and trace amounts of ^{125}I-BSA were further characterised by sucrose density gradient ultracentrifugation as previously described.[13] These gradients were used to examine the distribution of BSA and its digestion products in rinse fluids and mucosal scrapings from immunised and control rats. Samples (0·1 ml to 0·2 ml) of each test solution were applied to a 6% to 16% sucrose density gradient. After ultracentrifugation, 10 drop fractions were collected and the total radioactivity in each fraction was determined.

Additional density gradient ultracentrifugation experiments were performed with gradients varying from 15% to 45% sucrose further to characterise the radioactive substances heavier than BSA. One hundred μl of rinse fluid or mucosal extract were applied to the gradient. After ultracentrifugation, 10 drop fractions were collected from the bottom of the gradient into tubes containing 1·5 ml normal saline; the total radioactivity in each tube was determined.

In three separate experiments, immunoprecipitation was performed on fractions obtained from the gradients. A sample of each fraction (0·5 ml) was added to 0·1 ml normal rat serum used as a source of rat IgGl, plus 0·05 ml of rabbit anti-rat IgGl (Miles Lab., Elkhart, IN). The mixture was held at 37°C for 48 hours and was then centrifuged. The radioactivity remaining in the supernatant was determined.

STATISTICAL METHOD

Student's *t* test was used to assess the significance of the difference in results obtained with immunised and control animals.

Results

INTESTINAL DISTRIBUTION OF RADIOACTIVITY AFTER ADMINISTRATION OF ^{125}I-BSA TO NORMAL AND IMMUNISED RATS

Control and BSA-immunised rats were gavaged with either 1 mg or 100 mg of BSA mixed with trace amounts of ^{125}I-BSA. One hour later, the stomach liver, and large intestine were removed and their content of radioactivity was determined. The amount of radioactivity in the unprocessed rinse fluid and mucosal scraping of the proximal and distal segments of the small intestine was determined separately.

Between 15% to 25% of the administered radioactivity remained in the stomach of most of the animals. In two of 10 normal rats and one of 11 orally immunised rats fed 100 mg BSA plus trace amounts of ^{125} I-BSA, the retention of radioactivity in the stomach approached 40% of the dose administered. None of the rats fed 1 mg plus trace amounts of ^{125} I-BSA showed abnormally high retention of radioactivity in the stomach. In both groups, the major portion of the radioactivity administered (50%–70%) was found in the small intestine and only trace amounts (less than 1%) appeared in the large intestine. Two to 4% of the radioactivity was detected in the liver and about 2% in the circulation. As differences in the rate of emptying of the stomach led to the presence of variable amounts of radioactivity in the small intestine, we chose to report the distribution of radioactivity in the proximal and distal segments as a percentage of the total radioactivity in the entire small intestine.

In the Table, the distribution of radioactivity in the unprocessed rinse fluid and mucosal extract pre-

Table *Percentage of radioactivity in unprocessed rinse fluids and mucosal extracts in proximal and distal small intestine of control and immunised rats one hour after administration of trace amounts of ^{125}I-BSA and either 1 or 100 mg unlabelled BSA.*

	Dose of BSA administered by gavage					
	1 mg			*100 mg*		
	*Control (4)**	*Immunised (5)*	*P*	*Control (10)*	*Immunised (11)*	*P*
Proximal small intestine						
Rinse fluid	36±3†	21±2	<0·001	23±1	17±1	<0·02
Mucosal extract	7±2	6±1	NS	11±1	7±2	NS
Total	42±4	27±3	<1·05	34±3	24±2	<0·05
Distal small intestine						
Rinse fluid	51±4	66±2	<0·01	53±5	58±4	NS
Mucosal extract	7±1	8±1	NS	11±2	18±4	NS
Total	58±4	73±3	<0·05	65±4	76±2	<0·05

*: Number of animals tested.
†: Mean and SE.
NS: not significant at level of $P < 0·05$.

pared from the proximal and distal segments of the small intestine of control and immunised rats is compared. In rats given 1·0 mg BSA and trace amounts of ^{125}I-BSA, there was a greater percentage of radioactivity in rinse fluid from the proximal segment of control compared with immunised rats ($P < 0.001$) and a greater percentage of radioactivity in rinse fluid from the distal segment of immunised compared with control animals ($P < 0.01$). There was no difference in the percentage of radioactivity present in mucosal extracts from proximal and distal segments of control and immunised rats. In rats given 100 mg BSA plus trace amounts of ^{125}I-BSA, there was a greater percentage of radioactivity in rinse fluids from the proximal segments of control compared with those from immunised rats ($P < 0.02$) and a greater percentage of radioactivity in rinse fluid from the distal segment of immunised compared with control rats (but the latter differences were not significant). The distribution of radioactivity in proximal and distal mucosal extracts showed the same pattern as the rinse fluids but the differences between control and immunised rats were not significant.

Nevertheless, upon comparing the combined percentage of radioactivity in rinse fluids and mucosal extracts from proximal and distal segment (Table), there was a significantly greater percentage of radioactivity in the proximal segment of control compared with immunised rats fed 1·0 mg BSA ($P < 0.05$) and in the distal segment of immunised compared with control rats ($P < 0.05$). The same pattern of distribution of radioactivity was seen in control versus immunised rats fed 100 mg BSA plus trace ^{125}I-BSA ($P < 0.05$; Table).

^{14}C-polyethylene glycol was used as a marker to study the motility of the small intestine of immunised and control rats in the presence of an unrelated protein. One hour after feeding 100 mg egg albumen with trace amounts of ^{14}C-PEG, the distribution of ^{14}C-PEG in the proximal and distal segments of the small intestine of rats orally immunised with BSA and control rats was similar (Fig. 1).

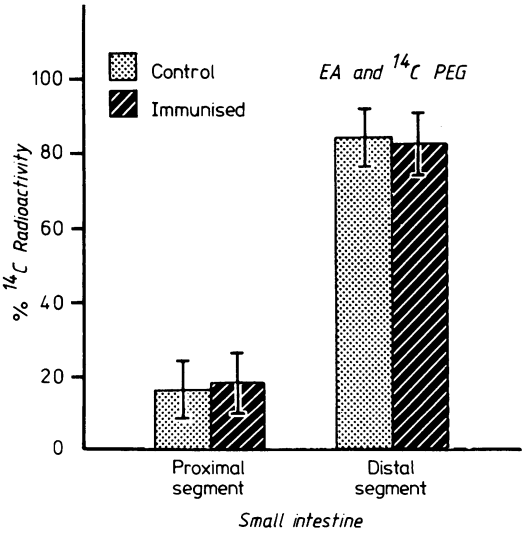

Fig. 1 *Distribution of ^{14}C-polyetheylene glycol in the small intestine of BSA-immunised and control rats one hour after feeding 100 mg egg albumin and trace amounts of ^{14}C-PEG in 1 ml PBS.*

CHARACTERISATION OF ANTIGEN, FRAGMENTS OF ANTIGEN, AND ANTIGEN-ANTIBODY COMPLEXES IN RINSE FLUIDS AND MUCOSAL EXTRACTS

Characterisation by SDS-PAGE

SDS-polyacrylamide gel electrophoresis was performed on rinse fluids and mucosal extracts from immunised and control rats challenged with 100 mg BSA and trace amounts of ^{125}I-BSA to examine the distribution of radiolabelled substances in these solutions. The radioactivity profiles of the gels could be divided into three sections based on the size of the radiolabelled substance—that is, those substances larger than 68000 m.w., substances ranging from 10000 to 68000 m.w., and substances smaller than 10000 m.w. but larger than small peptides. The distribution of these radiolabelled substances in the proximal and distal small intestine of immunised and control rats was determined in six experiments; the combined results of all experiments are shown in Fig. 2. The radioactivity in three sections of the gel profile of rinse fluid and mucosal extract was expressed as the percentage of the total radioactivity recovered in either the proximal or distal segment of the small intestine. With rinse fluids and extracts from either control or immunised rats fed labelled BSA, there was more radioactivity present in the gel section containing substances in the range of 10 000 to 68 000 m.w. than in the gel sections containing substances larger than 68 000 m.w. or substances smaller than 10 000 m.w. In both the proximal and distal small intestine of immunised rats, there was a greater percentage of radioactivity in the sections containing substances larger than 68 000 m.w. and in the sections containing substances smaller than 10 000 m.w. The higher percentage in the section containing substances larger than 68 000 was contributed by both rinse fluids and mucosal extracts from immunised rats, whereas there was virtually none present in rinse fluids and a very small percentage in mucosal extracts from control rats. In contrast, the control rats had a greater percentage of labelled substances with a m.w. of 10 000 to 68 000.

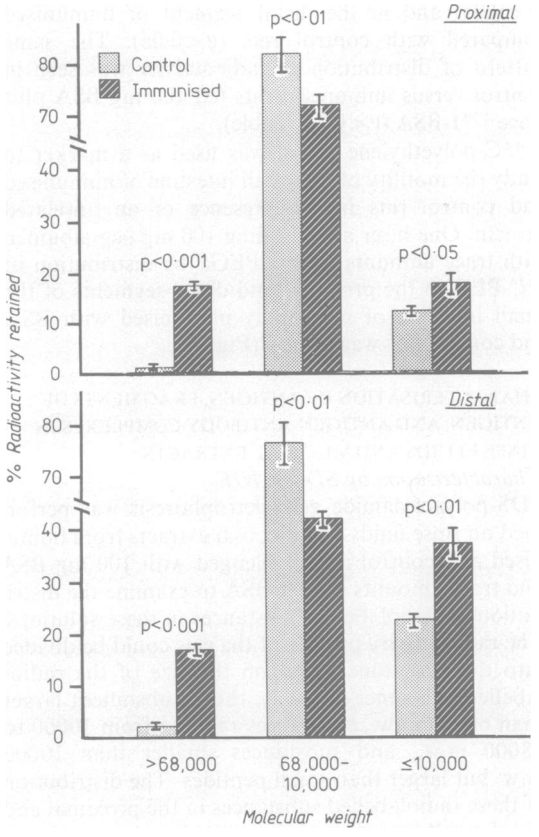

Fig. 2 *Distribution of radioactivity associated with BSA or BSA fragments in regions of an SDS-PAGE gel. The rinse fluid and mucosal extract obtained from the proximal (upper panel) and distal segment (lower panel) of the small intestine were separately applied to a 10% SDS-PAGE gel; the combined results of these analyses on rinse fluid and extracts from six immunised and six control rats are shown.*

CHARACTERISATION BY SUCROSE DENSITY GRADIENT ULTRACENTRIFUGATION AND IMMUNOPRECIPITATION

Further to characterise the labelled substance in the range of 10 000 to 68 000 m.w., experiments were performed with density gradients varying from 6% to 16% sucrose. Rinse fluids and mucosal extracts from the proximal and distal segments of the small intestine of immunised and control rats gavaged with 100 mg BSA plus trace amounts of ^{125}I-BSA were applied to these gradients. After ultracentrifugation of rinse fluids and mucosal extracts from control animals, a peak of radioactivity was detected in fractions corresponding to the location of an ^{125}I-BSA marker; larger amounts of radioactivity were found in the upper zone of the gradient. In contrast, after ultracentrifugation of rinse fluids and mucosal extracts from immunised rats, no peak was observed

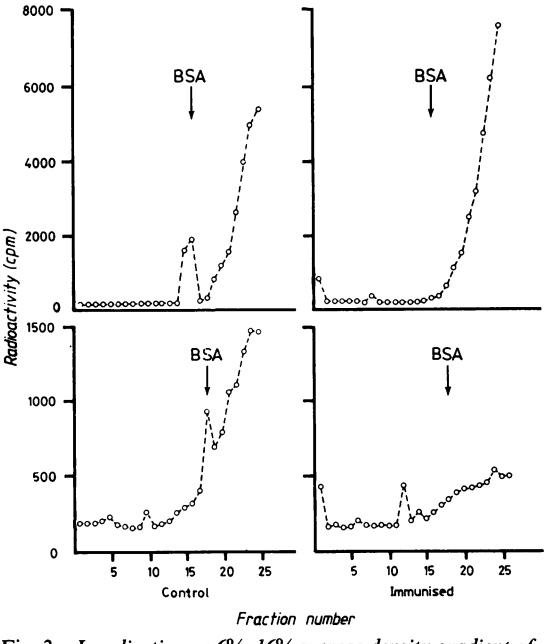

Fig. 3 *Localisation on 6%–16% sucrose density gradient of radioactivity in rinse fluids (upper panel) and mucosal extracts (lower panel) from the distal small intestine of a control and a BSA-immunised rat fed one hour earlier with 100 mg BSA and trace amounts of ^{125}I-BSA.*

in fractions corresponding to the ^{125}I-BSA marker. Larger amounts of radioactivity were again found in the upper zone of the gradient and a small amount of radioactivity was found near the bottom of the gradient. Representative sucrose density gradient profiles obtained with rinse fluids and mucosal extracts from the distal segments of the small intestine of control and immunised animals are shown in Fig. 3.

Further to characterise the radiolabelled substances with a m.w. greater than 68 000 m.w., rinse fluids and mucosal extracts from proximal and distal segments of small intestine of immunised and control rats gavaged with 100 mg BSA plus trace amounts of ^{125}I-BSA were applied to density gradients varying from 15% to 45% sucrose. Representative gradient profiles obtained with rinse fluids and mucosal extracts are shown in Fig. 4. Radioactivity was detected in fractions collected near the bottom of the gradient, from fractions near the middle of the gradient, and from fractions near the top of the gradient to which rinse fluid or mucosal extract from an immunised rat had been applied. In contrast, the profile obtained with rinse fluid and mucosal extract from a control rat showed radioactivity only at the top of the gradient.

To investigate the possibility that some of the

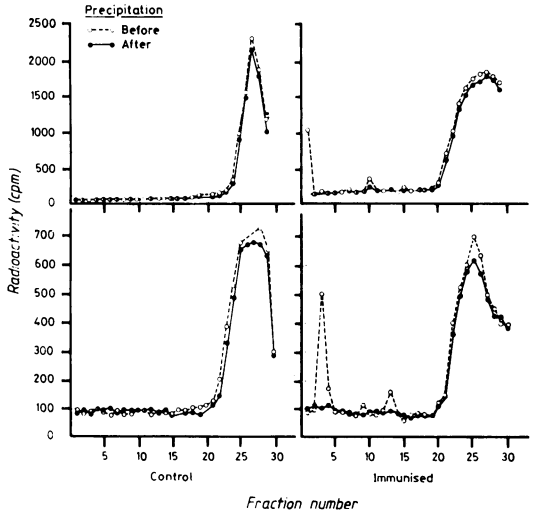

Fig. 4 *Localisation on 15% to 45% sucrose density gradient of radio-activity in rinse fluids (upper panel) and mucosal extracts (lower panel) from the distal small intestine of a control and a BSA-immunised rat before (O- - -O) and after (●——●) coprecipitation of each fraction with a rabbit anti-rat IgGI antiserum-rat IgG1 antigen system.*

radioactive components might represent antigen-antibody complexes, fractions from these gradients were mixed with normal rat serum to serve as a carrier and were incubated with rabbit anti-rat IgGl antiserum. After incubation, the mixtures were centrifuged and the radioactivity remaining in the supernatant was determined. Fig. 4 shows the results of one of these experiments. The radioactivity near the bottom of the gradient to which an extract from an immunised rat had been applied, was reduced by more than 90% after immunoprecipitation. The radioactivity at the top of the gradients, to which rinse fluid or mucosal extract from either immunised or control rats had been applied, was not significantly affected by immunoprecipitation.

Discussion

In this study, we have attempted to extend previous *in vitro* observations regarding the effect of oral immunisation on intestinal antigen processing by using an *in vivo* animal model. In this model system, both small and large amounts of BSA antigen plus trace amounts of labelled BSA were administered by gavage and the distribution and breakdown of antigen within the intestine of control and immunised rats were compared. One hour after gavage feeding of antigen, most of the radioactivity administered was present in the small intestine; less than 1% was detected in the large intestine of either control or immunised rats. There was, however, a difference in the distribution of radioactivity in the small intestine. Control animals retained a significantly greater percentage of radioactivity in the proximal segment, while immunised rats retained a greater percentage of radioactivity in the distal small intestine (Table). Our finding of 34% retention of ^{125}I-BSA in the proximal small intestine of control animals is similar to that reported in an earlier study by Parkins *et al.*[14] in which 42% of a dose of ^{125}I-human serum albumin was retained in the proximal small intestine of normal rats one hour after administration. As, in our experiments, immunised rats did not have an increase in radioactivity in the large intestine, it may be concluded that the difference in distribution of radioactivity between immunised and control rats did not reflect a general increase in distal propulsion of gut contents.

This impression is confirmed by the studies conducted with labelled polyethylene glycol. ^{14}C-PEG is used as an indicator of intestinal motility because it is not absorbed by the gut.[10 11] After the administration of ^{14}C-PEG and egg albumen to either normal or BSA-immunised rats, there was no difference in the distribution of ^{14}C-PEG in the small intestine of either animal group. In both, approximately 85% of the radioactivity was found in the distal small intestine. This percentage is similar to the percentage of radioactivity found in the distal small intestine of immunised rats given ^{125}I-BSA (78%, $P > 0.05$) and is considerably greater than the percentage of radioactivity found in the distal small intestine of control rats given ^{125}I-BSA (66%, $P < 0.01$). These findings suggest that, in immunised animals, radiolabelled BSA or its fragments 'move' like ^{14}C-PEG, whereas, in control rats, BSA and its fragments are restricted in their movement by events occurring in the proximal intestine.

In previous experiments performed with everted small intestinal sacs from immunised and normal rats,[13] we observed that there was adsorption of antigen to gut sacs from both types of animals, but that initial adsorption was greater with gut sacs from immunised rats. Furthermore, gut sacs from immunised rats showed enhanced breakdown of labelled antigen on prolonged incubation. After incubation, gut sacs were rinsed and the amount of radioactivity eluted was determined to be greater in the first rinse fluid of gut sacs from immunised rather than from control rats. We suggested that the latter phenomenon was related to the presence of antigen-antibody complexes loosely associated with the gut sac surface and therefore readily displaced by rinsing. We assume that in the present *in vivo* experiments, a portion of the administered BSA was also adsorbed to the surface of the gut and that, in the immunised rats, some of the antigen combined with

surface-associated antibodies. Our previous studies[15] have shown that such complexes are capable of stimulating goblet cell mucus release and, presumably, of enhancing the movement of the surface-associated mucus coat. In the present experiments, the formation of antigen-antibody complexes and the subsequent stimulation of mucus release may account for the failure of antigen to be retained in the proximal intestine of immunised rats and for its enhanced transport to the distal small intestine.

Evidence for the formation of immune complexes in the intestine of immunised rats is provided by the results of the SDS-PAGE and ultracentrifugation studies performed with rinse fluids and mucosal extracts. On SDS-PAGE analysis, approximately 20% of the radioactivity in rinse fluids and extracts from immunised, but not from control, rats was associated with molecules larger than 68 000 molecular weight (Fig. 2). In addition, after density gradient ultracentrifugation of rinse fluid and mucosal extract from immunised rats, radioactive components recovered from the bottom of the gradients could be coprecipitated by anti-IgGl complexes (Fig. 4), suggesting that the 'heavy' components consisted of radiolabelled BSA (or BSA fragments) and IgGl anti-BSA antibody complexes.

The present study also provided evidence for enhanced breakdown of antigen by the gut of immunised rats. On SDS-PAGE analysis of rinse fluids and extracts from the small intestine of immunised rats, there was a greater percentage of radioactivity associated with substances smaller than 10 000 m.w. and less radioactivity associated with substances ranging from 10 000 to 68 000 m.w. than was found in the controls. On density gradient ultracentrifugation of rinse fluids and extracts from immunised rats, there was also less radioactivity in fractions of the gradient that correspond to the position of an intact BSA marker. As previous *in vitro* studies demonstrated that gut sacs from immunised rats showed a diminished uptake of antigen compared with controls, it is unlikely that failure to detect BSA molecules in rinse fluids and extracts from immunised rats *in vivo* was due to enhanced uptake of BSA by these animals. It seems more likely that the present *in vivo* observations support previous *in vitro* findings which suggested that immunisation was associated with enhanced intestinal breakdown of protein antigen.[13]

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