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The effects of splenectomy, mesenteric lymphadenectomy and portacaval shunt on antibody responses to antigens within the small intestine

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Summary. The primary and secondary systemic antibody responses to protein antigens introduced into the gut (diphtheria toxoid, lauroylated human serum albumin) were not diminished or enhanced by previous splenectomy, mesenteric lymphadenectomy or portacaval shunt. Immunization with diphtheria toxoid before such operations did not produce significant alterations in the responses.

The cells forming antibody were found predominantly in the lamina propria of the small bowel and to a lesser extent in the subcapsular sinuses of the mesenteric lymph nodes when tissues were examined by the fluorescent antibody technique, ten days after the secondary stimulus of intestinal diphtheria toxoid.

The experiments show that the responses, stimulated locally by gut absorbed protein are little affected by an altered portal circulation.

INTRODUCTION

Since Ehrlich in 1891 first demonstrated prompt systemic antibody responses to orally administered proteins (the vegetable poisons ricin and abrin), the mechanisms of antigen absorption and antibody production have been greatly elucidated. The gastrointestinal mucosa has been found to be permeable to many antigens present in the intestinal lumen (Alpers & Isselbacher, 1967; Bernstein & Ovary, 1968; Cornell, Walker & Isselbacher, 1971; Walker, Cornell, Davenport & Isselbacher, 1972; Worthington, Boatman & Kenny, 1974). The subsequent transport of molecules penetrating the mucosa has been studied with labelled molecules (Warshaw, Walker, Cornell & Isselbacher, 1971). These have been shown to enter either the mesenteric lymphatics or tributaries of the portal venous system. The amount of antigen finally entering the systemic circulation is reduced by filtration of the lymph in the mesenteric lymph nodes and of the portal blood in the liver. If exposure to antigen continues, synthesis of antibody in the spleen (Rowley, 1950a, b) and in the mesenteric nodes will, with the resulting immune complex formation, further reduce the amount of antigen entering or remaining in the systemic circulation (Thomas & Vaez-Zadeh, 1974).

In this study of systemic antibody responses to antigens instilled into the lumen of the small intestine, the relative contributions of the different antigen-handling and antibody-forming tissues have been assessed. We have investigated the separate effects of

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removal of the local mesenteric nodes, of splenectomy and of by-passing the portal venous return to the liver by a portacaval venous shunt and have also examined the local synthesis of antibody in the intestinal wall by the fluorescent antibody technique. Lauroylated albumin was included as an antigen in this study because it was known to be a good immunogen when given into the gut (Heatley & Stark, 1975).

MATERIALS AND METHODS

Animals

Female Wistar rats were used of 200–250 g weight.

Antigens

Lipid conjugated (Lauroylated) human serum albumin (LCHSA) and diphtheria toxoid (Wellcome Batch BJGL 736) were used. Human serum albumin (HSA) lot no. 22C8170, Sigma (London) Chemical Company Ltd, Kingston-on-Thames, London, was heavily acylated with lauroyl chloride and the degree of acylation measured as previously described (Heatley & Stark, 1975). Approximately 85 per cent of the available amino groups were acylated.

Operative procedures

The following procedures were performed at appropriate times in the immunization schedules on separate groups of animals after laparotomy under intraperitoneal sodium pentobarbitone and ether anaesthesia: (a) *splenectomy*: the spleen was removed by dissection from the stomach and ligation and transection of the vessels. (b) *Lymphadenectomy*: the mesenteric lymph nodes were removed by dissection of the covering peritoneum, followed by careful excision of the nodes and preservation of the underlying lymphatic trunk. At operation after completion of the immunization experiments the animals were examined for evidence of lymphatic obstruction by infusion of methylene blue dye with a 30-gauge needle into the Peyer's patches of the small intestinal wall. The stained lymphatic vessels were examined under the dissecting microscope to confirm that there was no obstruction to lymph flow. (c) *Portacaval shunt*: portacaval shunt was performed by ligation and transection of the portal vein in the porta hepatis and end-to-side anastomosis of the proximal portion of the vein to the inferior vena cava (Lee & Fisher, 1961). Patency of the shunts was confirmed at the termination of the experiments by infusion at laparotomy of 30 per cent Hypaque into the spleen through a 30-gauge needle. Careful inspection under image intensification of the portacaval shunt confirmed passage of the radio-opaque dye directly from the portal vein into the inferior vena cava. (d) *Sham operation*: at laparotomy the liver, spleen and mesenteric lymph nodes were mobilized and the abdomen closed.

Immunization of rats

Each antigen preparation was given to groups of six rats. All antigens were injected into the small bowel lumen with a 30-gauge needle at laparotomy under ether anaesthesia. Two ml of solution was injected 3 cm above the ileo–caecal junction. In a group of animals not otherwise included in the experiments, a similar volume of methylene blue dye was injected. Observation confirmed that there was no leakage of inoculum into the peritoneal cavity.

In the operative study with LCHSA, the primary antigenic stimulus (10 mg) was given 21 days after the operation and the secondary stimulus (1 mg) was given 21 days later to each rat in the splenectomy, lymphadenectomy, portacaval shunt, and sham-operated groups. Blood was sampled by cut-down on the jugular vein under ether anaesthesia before immunization and on the 24th, 26th, 28th, 30th and 33rd days after primary injection in all groups except the portacaval shunt group which were bled on the 24th, 28th and 33rd days. With diphtheria toxoid as antigen there were two series of experiments. The first was as the LCHSA experiment where both antigenic stimuli (primary, 400 μg and secondary, 100 μg toxoid) were post-operative. Blood samples were taken before immunization and on the 21st, 25th and 31st days after injection of primary stimulus. In the second series the primary dose (400 μg toxoid) was given 21 days pre-operatively. On the 21st post-operative day the secondary dose (100 μg toxoid) was given. The animals were bled on the 42nd, 46th and 52nd post-operative days.

Antibody measurement

The total antibody responses were estimated by a modified Farr ammonium sulphate precipitation technique (Mitchison, 1971) at a concentration of 1 μg of antigen per ml in the reacting mixture. Radio-labelled antigen bound by antibody was precipitated by an equal volume of saturated ammonium sulphate solution in the estimation of antibody to

HSA whereas 80 per cent saturated ammonium sulphate was used for antibody estimation to diphtheria toxoid (Mitchison, 1968). Logarithmically converted antigen-binding capacities (ABC) in micrograms per millilitre of serum were used in the statistical comparisons (by Student's *t*-test) and in the graphs.

Estimation of specific anti-HSA IgG and IgA. Duplicate dilutions of immune rat sera (and of an unimmunized rat as control) were made as in the Farr test in 1/30 normal rat serum and 0·1-ml volumes allowed to react at 4° with an equal volume of radio-labelled HSA solution (2 μg/ml). After 3 days 0·1 ml of a 1/12 dilution of rabbit anti-rat (IgG1 + IgG2) (Mercia Diagnostics Ltd, Watford, Hertfordshire) or of rabbit anti-rat IgA (a gift from Dr J. P. Vaerman) was added to each of the paired dilutions which were allowed to stand for 16 h. They were then spun in an MSE Mistral centrifuge for 30 min. at 2500 r.p.m. at 4°. The supernates were carefully removed by pipette and the tubes inverted and allowed to drain. The radioactivity of the immune complexes precipitated was determined in a Packard gamma spectrometer Model 5110 and the Antigen Binding Capacities of (IgG1 + IgG2) and of IgA calculated for each individual and group as in the Farr test. Statistical comparisons were made by the Student's *t*-test.

Localization of antibody by the sandwich fluorescent antibody technique

Preparation of fluorescein isothiocyanate-conjugated diphtheria antitoxin. Peptic refined diphtheria antitoxin (Wellcome DP 2542/64) was diluted in pH 9 carbonate buffer containing 2 mg/ml fluorescein isothiocyanate (FITC) isomer I (Sigma London) to give a final concentration of 10 mg protein: 1 mg FITC. After being stirred at 4° for 24 h the conjugate was passed through a G-25 Sephadex column and the fractions containing the FITC conjugate reconcentrated to the original volume. The fluorescein-to-protein ratio of the conjugate was 0·8. After absorption with cryostat sections of unfixed rat liver, the preparation was diluted in saline containing 10% horse serum to a final concentration of 1/10 for use.

Localization of cells bearing antibody to diphtheria toxoid

The control group of animals which received diphtheria toxoid were killed on the 10th day after the secondary antigen injection. Frozen 10 μ sections of the terminal small bowel, ascending colon, mesenteric lymph nodes, spleen and liver were incubated for 30 min at room temperature with diphtheria toxoid (10 μg/ml) diluted in 10 per cent horse serum. They were then washed three times in phosphate-buffered saline, partially dried and incubated with fluorescein-conjugated diphtheria antitoxin. The prepared sections were mounted in 50 per cent buffered glycerol (pH 8·1) and examined using a Reichert fluorescence microscope fitted with a wide-angle darkfield condenser. Examination was made with transmitted UV light and a ×40 objective. Appropriate control sections were examined including adjacent sections developed by the fluorescein-conjugated antitoxin of toxoid given in life by the intestinal route. Adjacent sections stained with haematoxylin and eosin were also examined to determine the localization of the fluorescence.

RESULTS

Surgical procedures

Examination after the final bleeding for antibody estimation confirmed that the portacaval shunts had remained patent and that the continuity of the main mesenteric lymphatic channel had not been interrupted by the removal of the mesenteric nodes.

Antibody responses to LCHSA

Strong antibody responses were obtained, the log ABC in the control group at 10 days post-secondary stimulus being 2·02 ± 0·25. With the lauroylated HSA antigen, systemic antibody rose to a maximum 7 days after the second dose of antigen (Fig. 1). The several operative procedures had not influenced the responses as there were no significant differences between the groups (Fig. 1).

The content of specific IgG and IgA anti-HSA detectable in the sera taken on the 27th and 33rd days was not significantly different in the test groups from that of the controls (Table 1). In individual animals the rise in the content of IgG and IgA paralleled each other in this period.

Antibody responses to diphtheria toxoid

Significant antibody responses were detectable in all groups 21 days after the first administration of

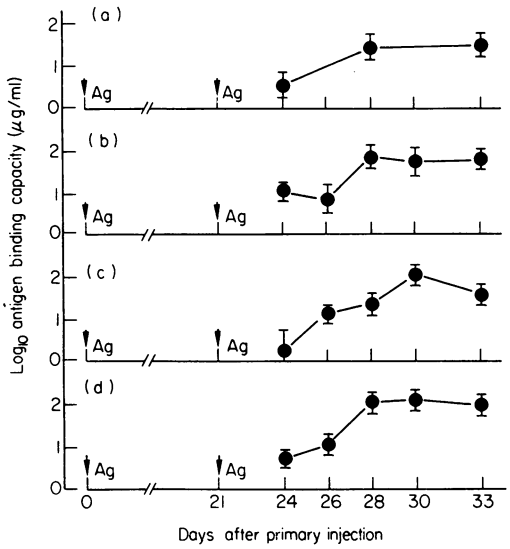

Figure 1. Post-operative rat antibody responses to lauroylated human serum albumin (LCHSA) in the gut. The effect of various operative procedures (control = sham operated) on the systemic antibody response to primary (10 mg LCHSA) and secondary (1 mg LCHSA) stimuli in the gut. Each point represents mean $\log_{10}$ antigen-binding capacity ± s.e. for five animals when tested at 1 μg/ml. (a) Portacaval shunt; (b) splenectomy; (c) lymphadenectomy; (d) control.

antigen by the gut, the average log ABC before antigen administration being $\bar{2}\cdot72 \pm 0\cdot16$, and $\bar{1}\cdot59 \pm 0\cdot08$ after antigen injection in, for example, the post-operative control group ($P < 0\cdot02$). In those animals where the first immunization was pre-operative there was no significant difference in the circulating antibody at 21, 25 and 31 days after operation (Fig. 2a) from that of the control group. In the post-operative groups the time course of the response was similar, the only significantly different response lying in the splenectomized group which on the 10th day after secondary stimulation (Fig. 2b) showed a significantly greater response ($\bar{1}\cdot81 \pm 0\cdot02$, $P < 0\cdot05$) than the control group ($\bar{1}\cdot38 \pm 0\cdot15$).

Cellular localization of antibody to diphtheria toxoid in intestinally immunized animals

Immunofluorescent examination by the sandwich technique to detect specific antibody revealed large numbers of cells with a brightly fluorescent cytoplasm in the lamina propria of the ileum (Fig. 3a). Very few, scattered, poorly fluorescent cells were to be seen in sections from the spleen, colon, and Peyer's patches of the small intestine and none in those from the liver. The mesenteric lymph nodes

Table 1. Serum immunoglobulin responses to lauroylated human serum albumin given into rat bowel

	Day*	ABC† (log μg/ml) ± s.e.	ABC‡ $IgG_1 + IgG_2$ (log μg/ml) ± s.e.	ABC§ IgA (log μg/ml) ± s.e.
Control	24	$0\cdot66 \pm 0\cdot23$	$\bar{1}\cdot03 \pm 0\cdot46$	$\bar{1}\cdot57 \pm 0\cdot64$
	33	$1\cdot96 \pm 0\cdot24$	$0\cdot70 \pm 0\cdot45$	$0\cdot38 \pm 0\cdot73$
Portacaval shunt	24	$0\cdot51 \pm 0\cdot29$	$\bar{2}\cdot74 \pm 0\cdot75$	$\bar{1}\cdot28 \pm 0\cdot79$
	33	$1\cdot49 \pm 0\cdot27$	$1\cdot75 \pm 0\cdot93$	$0\cdot74 \pm 0\cdot94$
Lymphadenectomy	24	$0\cdot29 \pm 0\cdot53$	$\bar{1}\cdot40 \pm 0\cdot45$	$\bar{1}\cdot69 \pm 0\cdot40$
	33	$1\cdot56 \pm 0\cdot23$	$0\cdot21 \pm 0\cdot71$	$0\cdot24 \pm 0\cdot71$
Splenectomy	24	$0\cdot98 \pm 0\cdot22$	$\bar{1}\cdot04 \pm 0\cdot52$	$\bar{1}\cdot11 \pm 0\cdot56$
	33	$1\cdot76 \pm 0\cdot24$	$\bar{1}\cdot30 \pm 0\cdot64$	$\bar{1}\cdot31 \pm 0\cdot64$

* Day after primary stimulus (10 mg lauroylated HSA) into bowel lumen. Secondary stimulus of 1 mg lauroylated HSA on day 21.

† Mean log antigen binding capacity for human serum albumin (HSA) by the Farr ammonium-sulphate precipitation method.

‡ Mean log antigen binding capacity for HSA by precipitation with rabbit anti-rat (IgG1 + IgG2).

§ Mean log antigen binding capacity for HSA by precipitation with rabbit anti-rat IgA.

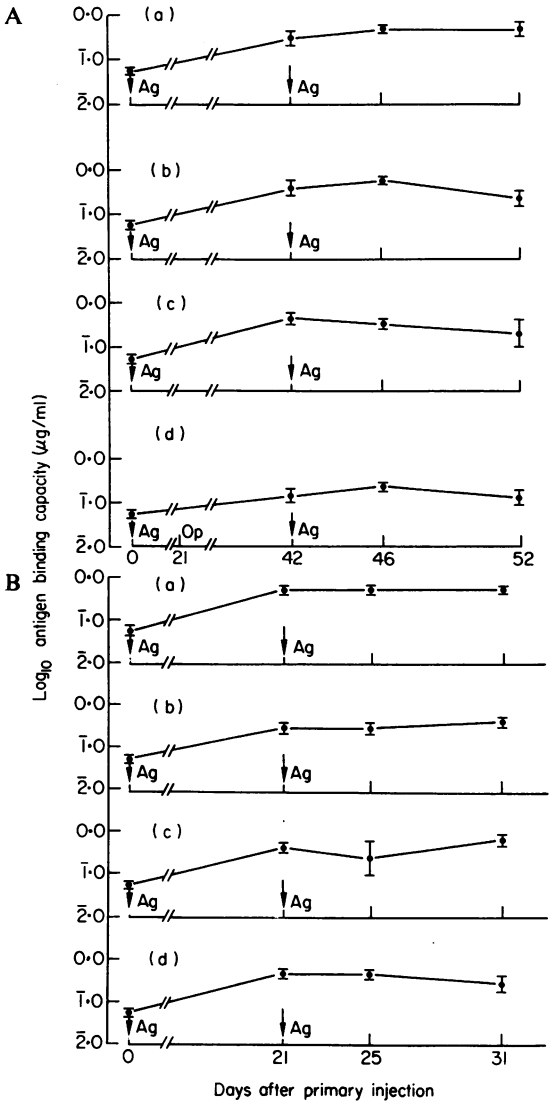

Figure 2. Rat antibody responses to diphtheria toxoid in the gut. (A) Pre-operative immunization. The effect of operative procedures 21 days after first antigenic stimulus (400 μg toxoid). Secondary stimulus (100 μg toxoid) 21 days post-operatively. ABC tested at 1 μg/ml. (B) Post-operative immunization. The effect of operative procedures 21 days before first stimulus (400 μg toxoid). Secondary stimulus 21 days later. (a) splenectomy; (b) lymphadenectomy; (c) portacaval shunt; (d) control.

contained scattered fluorescent cells in the medullary region. However, many brightly fluorescing specific staining spherical cells were observed in a linear

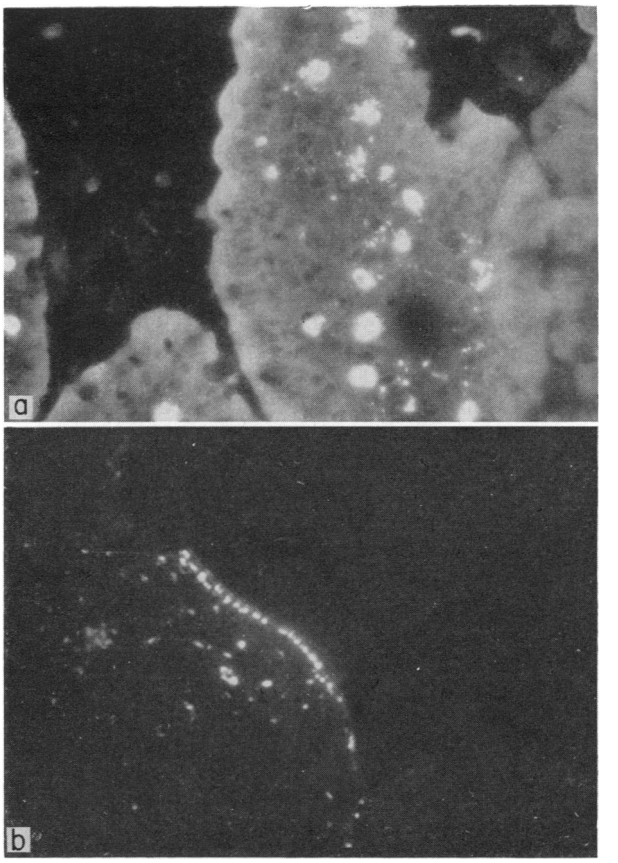

Figure 3. Fluorescent micrographs of rat tissues by the sandwich technique with fluorescein labelled diphtheria antitoxin to detect specific antibody. Rats had been given a secondary antigenic stimulus (100 μg diphtheria toxoid) 10 days previously. (a) Ileum with cytoplasmically staining, antibody-containing cells in the lamina propria. (Magnification ×280.) (b) Mesenteric lymph node showing, in the peripheral sinus, cells with antibody. (Magnification ×70.)

pattern in the sub-capsular sinuses of the nodes (Fig. 3b).

DISCUSSION

If gut-associated immune tissue is essential to the systemic antibody response following antigen challenge in the intestine, removal of part of that tissue would be expected to diminish antibody formation. In this series of experiments removal of either the mesenteric lymph nodes or spleen, although themselves the sites of some specific antibody-forming

cells, did not significantly influence the concentration of systemic antibody against the antigens used. Furthermore, the lack of enhancement by venous bypass of the liver seems, at first sight, to question the importance of the liver in modifying antibody formation by preventing antigenic material from the gut reaching the systemic circulation.

An antibody response implies the coincidence of stimulating antigen molecules and responding cells. Antigen entering from the gut will meet cells of the recirculating pool of lymphocytes which travel from blood to the lymphoid tissues of the gut and return via the lymph to the blood (Gowans & Knight, 1964; Craig & Cebra, 1971). The large lymphocytes of this population, for reasons not yet elucidated, home predominantly to the lamina propria of the small gut where they differentiate to primitive plasma cells (Gowans & Knight, 1964). There they respond if the relevant antigen molecules persist in the internal milieu or are again absorbed. In the experiments with diphtheria toxoid ten days after the secondary stimulus most of the cells forming antibody did indeed lie in the gut wall (Fig. 3a). In the mesenteric nodes at that time there were few antibody-forming cells in the medulla. A striking feature of some nodes was the presence of antibody-containing cells in the peripheral sinuses (Fig. 3b). The siting and morphology of these cells suggests that they are antibody-forming cells in transit from distal sites, possibly the Peyer's patches.

The difference in the contribution of various tissues to the response will depend, in part, on the intimate fate of crucial immunogenic molecules *in vivo*. The antigen molecules in the LCHSA experiment would be expected because of their hydrophobocity and consequent increased adhesiveness (Heatley & Stark, 1975) to become, more readily than many antigens, associated with cell surfaces in the locality of their absorption. Less lauroylated antigen, in fact, was found in the draining intestinal lymph in rats than with unmodified HSA (Heatley & Stark, 1975), presumably because a higher proportion of the dose had become adherent to cells peripherally. This adhesiveness could enhance immunogenic performance in the Peyer's patches. The likelihood of LCHSA molecules encountering primed lymphocytes in the lamina propria would also be increased as many molecules would remain attached to cells locally rather than be carried off quickly in draining lymph.

The lack of effect of the portacaval shunt implies that with the antigens tested important immunogenic events are unaffected by the liver. This contrasts with the findings in cirrhosis where the liver fibrosis constricts the portal vessels and reduces the venous flow from gut to liver. Systemic antibody to bacterial antigens is increased in cirrhotic patients and this has been attributed to either an altered balance of antigen trapping between liver and spleen or a reduced liver uptake of endotoxin, itself a non-specific immunogenic stimulus (Thomas, McSween and White, 1973). The liver also plays a part in the development of tolerance inducible by oral antigen (the Sulzberger–Chase phenomenon). After a portacaval shunt in dogs, instillation of antigen in the intestinal lumen or in the portal circulation no longer suppressed the response to antigens administered by other routes (Cantor & Dumont, 1967). This can be interpreted as showing that the shunt abolishes the liver's screening of the more immunogenic, denatured molecules which allows only tolerogenic molecules into the systemic circulation.

The implications of these previous reports would not be contradicted by the present findings if the antibody-forming cells at the times observed were mainly confined to the gut wall. The later handling of gut-absorbed antigen by the liver would then influence immunogenicity much less. Such an explanation could also be given for the lack of effect of surgical removal of the spleen or mesenteric nodes. The fluorescent antibody technique provided direct evidence of this localization of antibody-forming cells in the gut wall with diphtheria toxoid. As has been discussed above, a similar localization of cells forming antibody to LCHSA is likely because of the molecular nature of LCHSA. Other workers using protein antigens have found the antibody-forming cells to be confined to the lamina propria (Farr & Dickinson, 1961; Goldberg, Kraft, Petersen & Rothberg, 1971) or to be predominant in that site (Crabbe, Nash, Bazin, Eyssen & Heremans, 1969). Particulate antigens by the same route set up responses more successfully in other sites such as mesenteric nodes and spleen (Cooper, Halliday & Thonard, 1967; Felsenfeld & Greer, 1968; Bazin, Levi & Doria, 1970; Werner, Lefèvre & Raettig, 1971). It may be that only when the protein antigen is in a particulate form, such as when complexed with antibody (Thomas, McSween & White, 1973), is its immunogenicity likely to be affected by filtration in the liver. Certainly the present report confirms that gut-absorbed soluble protein antigens tend to stimu-

late immune responses locally and shows that these responses are little affected by an altered portal circulation.

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