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No impairment of local intestinal immune response to keyhole limpet haemocyanin in the absence of Peyer's patches

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Summary. The role of Peyer's patches in the local intestinal and serum antibody responses to keyhole limpet haemocyanin (KLH) was studied in rabbits with chronically isolated ileal loops. Four weekly doses of 400 μg KLH were administered into loops prepared with and without Peyer's patches. Isotype-specific IgA and IgG anti-KLH in loop secretions collected twice each week and in sera collected weekly were assessed by enzyme-linked immunosorbent assay. Fluid IgA anti-KLH in loops without Peyer's patches first showed a statistically significant increase on day 25, 1 week later than control loops with Peyer's patches. However, some animals in the group without Peyer's patches showed a rise as early as day 7, and the differences from controls were not statistically significant at any time. No statistically significant rise in fluid or serum IgG anti-KLH occurred in either group. Thus, Peyer's patches were not essential for local intestinal antibody response to KLH, a soluble macromolecular antigen. The findings suggest that the innumerable small lymphoid nodules in the gastrointestinal tract, or other mechanisms of antigen processing, play an important role in local intestinal immune responses.

Abbreviations: KLH, Keyhole limpet haemocyanin; DNP-KLH, Dinitrophenol-keyhole limpet haemocyanin; PBS, Phosphate buffered saline; ELISA, Enzyme-linked immunosorbent assay; SEM, Standard error of the mean.

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INTRODUCTION

Study *in vivo* of the kinetics of local intestinal immune responses and of the factors required for optimum intestinal immunization is difficult due to the relative inaccessibility of the intestine. We and others have used intact loops of ileum which have been chronically isolated from the faecal stream in rabbits as a model to study the local intestinal immune responses to intraluminally and systemically administered antigens (Keren, Elliott, Brown & Yardley, 1975; Robertson & Cebra, 1976; Keren, Holt, Collins, Gemski & Formal, 1978; Yardley, Keren, Hamilton & Brown, 1978; Hamilton, Yardley & Brown, 1979).

Peyer's patches, the visible aggregates of lymphoid tissue in the intestine, have been proposed to be important in the processing of luminal antigens for stimulation of immunocompetent cells and in influencing the intestinal distribution of the resulting IgA antibody-

producing plasma cells (Muller-Schoop & Good, 1975; Wansbrough-Jones, Pepys & Doe, 1976; Robertson & Cebra, 1976; Husband & Gowans, 1978). Since the isolated loops in our model can be prepared with and without Peyer's patches, we and others have used the model to investigate *in vivo* the role of the patches in local intestinal IgA responses. Absent or weak local IgA response to dinitrophenol-keyhole limpet haemocyanin (DNP-KLH) was found in loops without Peyer's patches in one study (Robertson & Cebra, 1976), suggesting the patches were essential for optimal response (Cebra, Gearhart, Kamat, Robertson & Tseng, 1977a; Cebra, Kamat, Gearhart, Robertson & Tseng, 1977b). On the other hand, in another study a delay but no impairment in the intestinal IgA response to locally invasive *Shigella* was found in loops without Peyer's patches (Keren *et al.*, 1978). Because the *Shigella* were invasive, however, they could have bypassed the usual mechanisms of antigen processing in the intestine. As a result of these conflicting results, we have studied in a larger number of animals than in previous investigations the role of Peyer's patches in immune responses to a soluble macromolecular antigen, keyhole limpet haemocyanin (KLH).

MATERIALS AND METHODS

Preparation and care of loops

An isolated 20 cm loop of distal ileum was prepared by our previously described technique (Keren *et al.*, 1975) in female outbred New Zealand white rabbits weighing 3–4 kg. A segment of ileum lacking a Peyer's patch was identified and isolated from the faecal stream in the experimental group. A Peyer's patch was specifically included in the loop of the control animals. Silastic tubing (Dow Chemical Corp., Midland, Mich., U.S.A.) was sewn into each end of the selected segment of bowel. Intestinal continuity was then restored by end-to-end anastomosis. The two tubes from the isolated loop were passed through the muscle wall via the abdominal incision and tunnelled subcutaneously to the nape of the neck. The loop was flushed with saline daily to prevent excess mucus accumulation.

Immunization of animals and specimen preparation

Ten animals were included in the experimental group without Peyer's patches and nine in the control group. Based upon the results of preliminary studies, all animals were immunized intraloop with 400 μg KLH (Sigma Chemical Co., St. Louis, Miss., U.S.A.) in 4 ml 0·01 M phosphate buffered saline with pH 7·2 containing 0·02% sodium azide. Four weekly intraloop immunizations were given, starting on day zero (3 to 4 days after operation). The distal (efferent) tube from the loop was clamped for 24 h after instillation of the antigen solution.

Loop fluids were collected on the day of first intraloop immunization (day zero) and every 3 to 4 days thereafter. Specimens were frozen at −20°. After thawing, mucus and any cellular debris were removed by low-speed centrifugation, yielding clear supernatants that were used for antibody studies. Blood for serum was collected before the first immunization and at weekly intervals thereafter.

ELISA for isotype-specific anti-KLH antibody

The enzyme-linked immunosorbent assays for fluid IgA and IgG anti-KLH and for serum IgG anti-KLH were carried out as described previously (Yardley *et al.*, 1978; Hamilton *et al.*, 1979). Briefly, polystyrene tubes were coated with KLH by incubation of 1 μg of antigen in 0·5 ml phosphate buffered saline (PBS) at 37° for 3.25 h. After the tubes were rinsed, diluted loop fluids or sera were incubated in the tubes. Following another rinse, alkaline phosphatase-conjugated goat anti-rabbit alpha chain or anti-gamma chain was incubated with the tubes. After a final rinse, nitrophenyl phosphate substrate was incubated with the tubes for 60 min. The reaction was stopped with sodium hydroxide, and the reaction mixture was read at 400 nm in a spectrophotometer.

Specimens from each immunization group were tested in each run. 'Standard' and control fluids and sera from immunized animals were included in each assay. The absorbance values of the standards were adjusted to an arbitrary value of 2·000 by a correction factor which was then applied to the specimen values to compensate for day-to-day variations in the test system. Relative quantities of IgA or IgG anti-KLH were then expressed as absorbance at 400 nm of the chromagen resulting from the alkaline phosphatase activity on the colourless substrate. As described previously (Yardley *et al.*, 1978; Hamilton *et al.*, 1979), in preliminary studies relative absorbances of specimens at any given dilution generally showed the same relationships as the titres of the specimens, i.e. the specimens with the highest titres usually had the highest absorbances at any given dilution. On the basis of this relationship, the lowest specimen dilution in which the enzyme-linked immunosorbent assay (ELISA) system

was generally not exhausted of substrate for each type of specimen and each isotype was chosen as the test dilution, i.e. 1:40 for IgA and IgG antibody in fluids, and 1:1600 for IgG antibody in sera. Specimens approaching substrate exhaustion were retested with incubation for 30 min, and the absorbance was then corrected to a 60-min value by doubling the result. Although the absorbance values of the IgA test system could not be compared directly with those for IgG, the time courses of the IgA anti-KLH response in loop fluids and of the IgG anti-KLH responses in sera and fluids could be followed.

The anti-alpha and anti-gamma enzyme conjugates were prepared as described previously (Keren, 1979) and were highly specific for their respective isotypes. The ELISA gave a linear response with tubes coated by solutions containing from 20 μg/ml to about 400 μg/ml of purified IgA or IgG. The day-to-day coefficients of variation for the assay systems were 9·0% for fluid IgA anti-KLH at mean value of 2·2 absorbance units and 3·2% for serum IgG anti-KLH at mean value of 3·1 absorbance units.

Statistical analysis

Mean and standard error (SEM) were calculated for ELISA values of each group of animals. The Kolmogorov–Smirnov test (Wu, Twomey & Thiers, 1975) was used to determine that data under consideration did not have a non-Gaussian distribution. Statistical significance of differences in means was determined by two-tailed t test based upon paired data for within-group comparisons and based upon unpaired data for comparisons between groups.

RESULTS

IgA anti-KLH in loop fluids (Fig. 1 and Table 1)

Although two of the nine animals in the control group showed a rise in fluid IgA anti-KLH as early as 10 days after first intraloop immunization, a statistically significant rise first occurred on day 18 ($P < 0{\cdot}02$), after the third dose of KLH. A further rise to a peak on day 25 ($P < 0{\cdot}01$) occurred after the fourth intraloop dose of KLH, followed by a decline on day 28.

Loop fluid IgA anti-KLH in the experimental group without loop Peyer's patches showed a suggestion of a rise on days 18 and 21 ($P < 0{\cdot}10$), and two of the ten animals showed a statistically significant rise as early as day 7. However, the first significant rise for the group occurred on day 25 ($P < 0{\cdot}01$), after the fourth dose of intraloop KLH. Although this increase in fluid IgA anti-KLH occurred 7 days later than in the control group, the experimental group was not statistically significantly different at any time.

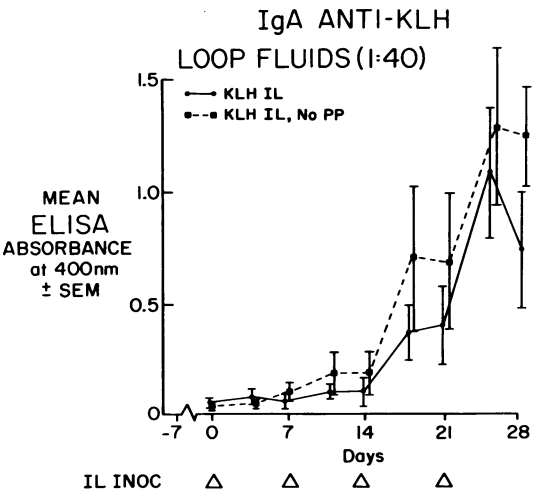

Figure 1. IgA anti-KLH by ELISA in fluids from chronically isolated Thiry-Vella ileal loops in rabbits given 4 weekly intraloop (IL) doses of 400 μg KLH. The experimental group without loop Peyer's patches (No-PP) first showed a statistically significant rise on day 25, 1 week later than the control group, but the differences were not significant at any time.

IgG anti-KLH in loop fluids (Table 1)

Fluid IgG anti-KLH showed a strikingly different pattern of response to antigen administration than IgA anti-KLH as no significant rise occurred in either the experimental or control group.

IgG anti-KLH in sera (Table 1)

Although increases in serum IgG anti-KLH occurred in some animals in each group, no statistically significant rises were found.

DISCUSSION

Peyer's patches, the visible aggregates of lymphoid tissue in the intestine, contain lymphoid cells which are precursors to IgA-producing cells and which are cap-

Table 1. Summary of anti-KLH ELISA results

Day of experiment	Anti-KLH ELISA absorbance (Mean ± SEM)								
	0	4	7	11	14	18	21	25	28
Specimen									
Fluid IgA									
Control	0·03 ± 0·02	0·07 ± 0·03	0·05 ± 0·03	0·09 ± 0·04	0·10 ± 0·06	0·38 ± 0·12	0·41 ± 0·18	1·11 ± 0·29	0·76 ± 0·26
No Patch	0·02 ± 0·01	0·03 ± 0·01	0·09 ± 0·04	0·18 ± 0·10	0·18 ± 0·10	0·71 ± 0·33	0·68 ± 0·31	1·30 ± 0·36	1·27 ± 0·23
Fluid IgG									
Control	0·03 ± 0·02	0·04 ± 0·02	0·03 ± 0·02	0·03 ± 0·02	0·00 ± 0·00	0·02 ± 0·01	0·01 ± 0·01	0·07 ± 0·03	0·03 ± 0·01
No Patch	0·00 ± 0·00	0·02 ± 0·01	0·03 ± 0·02	0·03 ± 0·01	0·02 ± 0·01	0·02 ± 0·01	0·03 ± 0·01	0·06 ± 0·03	0·06 ± 0·02
Serum IgG									
Control	0·01 ± 0·01	—	0·01 ± 0·00	—	0·04 ± 0·02	—	0·05 ± 0·06	—	0·25 ± 0·14
No Patch	0·02 ± 0·01	—	0·05 ± 0·04	—	0·16 ± 0·14	—	0·19 ± 0·13	—	0·31 ± 0·15

able of responding to a variety of antigens (Coppola, Jones & Wilkes, 1976). Specialized follicle-associated epithelium containing phagocytic M cells which can take up antigen from the intestinal lumen overlies the lymphoid tissue (Owen, 1977). Therefore, Peyer's patches have been proposed to be important in processing luminal antigen for stimulation of antigen-specific immunocompetent cells which then proliferate and migrate to populate the lamina propria with plasma cells producing antibody to the antigen (Rudzik, Perey & Bienenstock, 1975; Cebra *et al.*, 1977b). Few *in vivo* studies of the role of Peyer's patches in intestinal immune response are available, however, and have produced conflicting results.

Robertson & Cebra (1976) found absent or weak local IgA response to DNP-KLH, a soluble macromolecular antigen, in chronically isolated ileal loops without Peyer's patches. Although the number of animals in the study was small (five), the authors suggested on the basis of their results that Peyer's patches were essential for optimal intestinal immune responses to luminal antigens. On the other hand, Keren *et al.* (1978) found a delay but no lessening of the magnitude of the intestinal IgA response to locally invasive *Shigella* X16 bacteria in loops without Peyer's patches. However, intra-epithelial trapping of the protozoan *Giardia* by macrophages in the dome epithelium of Peyer's patches has recently been demonstrated (Owen, Allen & Stevens, 1980). This finding of a mechanism of antigen processing not involving the M cells raises the possibility that the invasive *Shigella* could produce an immune response in the absence of Peyer's patches by direct invasion of the mucosa. Nonetheless, our findings in loops with and without Peyer's patches of no significant difference in local response to KLH, an inert macromolecule which would be processed by the M cells of the follicle-associated epithelium, provides strong evidence that Peyer's patches are not essential to optimal local intestinal immune response to such antigens. The differences between our study and the previous one using a similar macromolecular antigen (Robertson & Cebra, 1976) may be attributable to the variability of outbred animals and the lessening of statistical sampling error by the use of a larger number of animals (ten) in our study.

The explanation for optimal intestinal immune response in the absence of Peyer's patches for processing antigen remains conjectural. Alternative routes of antigen processing, such as phagocytosis by macrophages unassociated with the Peyer's patches, are possibilities. However, the microscopic lymphoid aggregates which are scattered throughout the intestine have the same follicle-associated epithelium with M cells as the visible Peyer's patches (Keren *et al.*, 1978). Thus, these microscopic lymphoid aggregates may have the functional capabilities of the larger Peyer's patches and be present in sufficient numbers to ensure adequate immune response to luminal antigens in the absence of the patches. In fact, when the tiny total mucosal surface area overlying the Peyer's patches is considered relative to the total mucosal surface area of the gastrointestinal tract, the importance in antigen processing of the innumerable small lymphoid aggregates may equal or even exceed that of the visible Peyer's patches.

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