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Lack of site of origin effects on distribution of IgA antibody-containing cells

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Summary. Previous experiments (Husband, 1982) indicated that IgA-specific antibody-containing cells (ACC), appearing among thoracic duct lymphocytes (TDL) following challenge of intestinal segments of i.p. primed rats, display antigen-dependent distribution in the small intestine by 12 hr after their i.v. injection into autologous recipients. Data are presented here which demonstrate that ACC among TDL collected from rats bearing double Thiry-Vella loops challenged with two different antigens still appear almost exclusively in loops immunized with the antigen corresponding to their antibody specificity, even when injected into recipients in which immunized loops were prepared from different levels of the small intestine to that of the donors. These experiments indicate that, in animals given whole gut priming (by i.p. immunization) and segmental challenge (by lumenal challenge of isolated loops), the site of origin of IgA-ACC precursors does not influence antigen-induced distribution patterns.

To determine whether segmental priming followed by whole gut challenge results in site of origin effects, rats were primed by subserosal immunization of a single Peyer's patch, either in the proximal or distal intestine, and then challenged intraduodenally. The ultimate IgA-ACC distribution was similar, regardless of the site of priming.

These results indicate that, in a model employing priming with antigen in Freund's complete adjuvant, there is no site of origin effect on ultimate IgA plasma cell location within the small intestine, whether whole gut priming and segmental challenge or segmental priming and whole gut challenge, are used.

Abbreviations: ACC, antibody-containing cells; AOCC, anti-ovalbumin-containing cells; ATCC, anti-tetanus toxoid-containing cells; FCA, Freund's complete adjuvant; FITC, fluorescein isothiocyanate; i.d., intraduodenal; OVA, ovalbumin; PP, Peyer's patches; TDL, thoracic duct lymphocytes; TRITC, tetramethylrhodamine isothiocyanate; TT, tetanus toxoid.

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INTRODUCTION

Intraperitoneal (i.p.) administration of antigen in Freund's complete adjuvant (FCA) has been demonstrated to prime the gut-associated lymphoid tissue of rats, such that a substantial IgA-specific antibody-containing cell (ACC) response occurs in the gut following subsequent intraduodenal (i.d.) administration of soluble antigen; this response is much greater than the response to i.d. antigen alone (Pierce & Gowans, 1975). This has provided a useful model for studying the induction and dissemination of IgA-ACC (Husband & Gowans, 1978; Husband, 1982), and has been shown to be an effective immunization strategy for the control of infectious enteritis in large animals (Husband, 1978; Husband & Seaman, 1979; Husband, 1980; Bennell & Husband, 1981).

Previous studies using this immunization protocol have established that the precursors of IgA-ACC arise in Peyer's patches (PP) in response to gut lumenal

antigens, and migrate to the intestinal lamina propria via intestinal and thoracic duct lymph and the blood circulation (Husband, Monié & Gowans, 1977; Husband & Gowans, 1978). These studies also demonstrated that extravasation of IgA precursors in the gut lamina propria occurs at random, but that the site of antigen administration influences their ultimate location by retention and proliferation of extravasated ACC in specifically immunized tissue.

These studies were confirmed and extended by further experiments (Husband, 1982) in which double Thiry-Vella loops were prepared in rats which had been previously primed by i.p. injection of ovalbumin (OVA) and tetanus toxoid (TT) in FCA. Each loop was later challenged with one of the two antigens and the thoracic duct cannulated. When autologous thoracic duct lymphocytes (TDL), collected at the peak of the ACC response, were infused i.v., at 12 hr after infusion only ACC specific for the antigen used to immunize each loop were detectable in the loops, indicating the antigen-dependent nature of the ultimate distribution of ACC.

However, data presented recently by Pierce & Cray (1982) raise an issue which requires re-assessment of the above findings. These authors showed, using enteric rather than i.p. priming, that the distribution of IgA-ACC specific for cholera toxin was also influenced by their site of origin within the gastrointestinal tract following challenge (i.e. precursors arising from colonic challenge homed selectively to the colon in preference to the jejunum, and vice versa). If this principle applies to segments within the small intestine, the implication to the above studies (Husband, 1982) is that precursors arising from an immunized small intestinal loop may tend to return to that loop through a site of origin effect rather than, or in addition to, an antigen-specific effect.

To determine whether this phenomenon exists within the small intestine, and whether this in any way influenced previous results, those experiments using an i.p. priming model have been repeated but, instead of injecting autologous TDL,TDL from donors bearing loops intralumenally-challenged with antigen in the reverse order to the recipients were injected, such that the antigen for which the ACC were specific was present in a different segment of the intestine to that from which they arose in response to segmental challenge of donors. In addition, further experiments have been conducted to determine the effect of segmental priming followed by whole gut challenge, by priming a single PP, either in the proximal or distal small intestine with antigen in FCA, followed by i.d. challenge of intact animals, with or without TDL drainage.

These experiments demonstrate that, when priming is achieved by administration of antigen in FCA, ultimate ACC distribution after extravasation within the small intestine is entirely antigen-dependent and not influenced by site of origin following either segmental challenge or segmental priming.

MATERIALS AND METHODS

Rats

Male or female rats of the hooded (PVG) inbred strain were maintained in a disease-free colony and used at 90–100 days of age.

Thiry-Vella loops

Double Thiry-Vella loops were prepared as previously described (Husband & Gowans, 1978).

Antigens and immunization

Crude tetanus toxoid (TT) was prepared from a culture of *Clostridium tetani* by Arthur Webster Pty. Ltd., Northmead, Australia. The activity of this preparation was such that it produced 10 IU of antitoxin in guinea-pigs. Purified TT (1000 l.f.u./ml) and tetanus toxin (1 mg/ml) were obtained from Wellcome Reagents Ltd., London, U.K. Crystalline OVA (Grade VI) was obtained from Sigma Chemical Co., St Louis, MO.

Priming was achieved, either by i.p. injection of 50 μl crude TT, 50 μl OVA (10 mg/ml), and 150 μl phosphate-buffered (pH 7·4) saline (PBS) emulsified in 200 μl FCA, or by injection of approximately 2 μl aliquots of a mixture containing 50 μl OVA (10 mg/ml), 200 μl PBS and 250 μl FCA under the serosa overlying a single PP, after exposure of the small intestine through a small laparotomy. In an attempt to achieve segmental priming, either the first PP in the duodenum distal to the colonic attachment were injected, or the last PP occurring before the ileocaecal junction.

Lumenal challenge in rats bearing Thiry-Vella loops was performed 14 days after whole gut priming by injection of 0·5 ml crude TT or 0·5 ml OVA (10 mg/ml) directly into the lumen of a loop exposed by laparotomy. To ensure antigen retention, each loop was sealed with adhesive plastic tape for 24 hr after injection, after which time loops were washed through

daily with sterile PBS. Lumenal challenge in intact rats receiving segmental priming was performed 14 days later by injection of 0·5 ml OVA (10 mg/ml) directly into the lumen of the duodenum exposed by laparotomy.

Fluorescent reagents

Rabbit anti-TT and rabbit anti-OVA antibodies were purified and conjugated with fluorescein isothiocyanate (FITC) as previously described (Husband, 1982). Goat anti-rat Iga (heavy chain-specific) was obtained from Miles Laboratories, Springvale, Australia, and an IgG fraction prepared by ammonium sulphate precipitation and elution on DEAE-cellulose. This reagent was absorbed with rat spleen cells before use. Tetramethylrhodamine isothiocyanate (TRITC)-conjugated rabbit anti-goat IgG was obtained from Atlantic Antibodies (Scarborough, ME) and absorbed with rat spleen cells before use.

ACC in thoracic duct lymph

The thoracic duct of rats was cannulated as previously described (Gowans & Knight, 1964). Total and IgA-specific ACC were simultaneously stained by sequential incubation with either purified TT (1 mg/ml), FITC-anti-TT, goat anti-rat IgA and TRITC-anti-goat IgG for detection of ACC specific for TT (ATCC) or OVA (1 mg/ml), FITC-anti-OVA, goat anti-rat IgA and TRITC-anti-goat IgG for detection of ACC specific for OVA (AOCC). No ATCC or AOCC were seen in smears from immunized rats if TT or OVA, respectively, were omitted from the staining sequence. No IgA-containing cells were detected if goat anti-rat IgA was omitted from the staining sequence or if smears were stained with unconjugated species-specific antisera before staining with conjugated antisera.

ACC in gut lamina propria

Segments of rat intestine were processed for fluorescent histology as previously described (Husband & Gowans, 1978). Total and IgA-specific ACC were simultaneously detected in sections of intestine using the staining sequence outlined above. Counts of ACC were made at magnification ×250 (field diameter = 0·57 mm). By scanning from the base of the mucosa to the tips of the villi in 30–50 fields, it was possible to calculate the mean number of ACC/cm of intestine in the plane of the section. A mean of one fluorescing cell per vertical scan was equivalent to 17·5 cells/cm. Counts were only performed in areas of intestine that did not contain PP. Controls for non-specific fluorescence were as previously described (Husband, 1982).

Experimental procedure

In experiments to determine the effect of whole gut priming with segmental challenge, rats were primed i.p. with TT and OVA, and double Thiry-Vella loops prepared 10 days later. At 14 days after priming, the thoracic duct was cannulated and either TT or OVA were injected into one or other of the two loops. A 24 hr collection of lymph was obtained from each rat commencing on day 17, the TDL washed and, after removal of an aliquot for counting and preparation of smears, the remaining cells were resuspended to 2 ml in sterile PBS and injected i.v. as a single dose into a recipient animal prepared in a similar manner, but with intralumenal challenge antigens given into opposite loops to that of the donor. The thoracic duct cannulae of recipient rats were allowed to drain throughout the experiments to ensure that injected cells were the only cells of thoracic duct origin that could populate the intestine. Rats were killed at 12 hr after TDL injection, and ATCC and AOCC were counted in sections of proximal and distal loops.

In experiments to determine the effect of segmental priming, rats were primed with OVA via one PP (either proximal or distal small intestine), given whole gut challenge by i.d. injection of OVA 14 days later, killed on day 19, and sections prepared from duodenum (5 cm distal to the colonic attachment) and ileum (5 cm proximal to the ileocaecal junction). In a second group of rats immunized in this way, the thoracic duct was cannulated at the time of i.d. challenge and AOCC enumerated among TDL collected daily over 5 days to determine the lymph-borne nature of precursors arising from this immunization regime and the kinetics of the response among TDL. In a third group, TDL were collected for 24 hr from day 3 to day 4 after i.d. challenge (the peak of the AOCC response among TDL), each rat injected i.v. with washed autologous TDL, killed 12 hr later and sections prepared from duodenum and ileum.

RESULTS

The cell numbers and ACC content of thoracic duct lymph collected from i.p. primed and segmentally challenged donor rats, and the distribution of ATCC and AOCC between distal loops, proximal loops and the intact (non-immunized) jejunum after TDL injec-

Table 1. Dose and proportion of IgA-specific ATCC and AOCC injected into segmentally challenged recipients after collection from reciprocally challenged donors

Expt no.	Donor challenge protocol: Proximal loop	Donor challenge protocol: Distal loop	No. rats	TDL/dose	ATCC: ATCC/dose	ATCC: %IgA	AOCC: ACC/dose	AOCC: %IgA
Expt 1	TT	OVA	4	$1{\cdot}29 \times 10^8$ ($0{\cdot}74$–$2{\cdot}21 \times 10^8$)	$1{\cdot}04 \times 10^6$ ($0{\cdot}72$–$1{\cdot}42 \times 10^6$)	81·29 (72·34–84·14)	$0{\cdot}98 \times 10^6$ ($0{\cdot}61$–$1{\cdot}37 \times 10^6$)	80·98 (74·32–82·91)
Expt 2	OVA	TT	4	$1{\cdot}03 \times 10^8$ ($0{\cdot}69$–$2{\cdot}10 \times 10^8$)	$1{\cdot}11 \times 10^6$ ($0{\cdot}33$–$1{\cdot}68 \times 10^6$)	79·62 (68·29–81·11)	$1{\cdot}29 \times 10^6$ ($0{\cdot}71$–$1{\cdot}55 \times 10^6$)	84·69 (71·47–86·69)

Donor rats were primed i.p. with OVA and TT in FCA, loops prepared 10 days later, and the thoracic duct cannulated and loops challenged with the antigens indicated on day 14. TDL were collected over days 17–18. Values represent means with range in parentheses.

Table 2. Distribution of ATCC and AOCC after i.v. injection into segmentally challenged recipients of TDL from reciprocally challenged donors

Expt no.	Recipient challenge protocol: Proximal loop	Recipient challenge protocol: Distal loop	No. rats	Proximal loop: ATCC/cm	Proximal loop: AOCC/cm	Distal loop: ATCC/cm	Distal loop: AOCC/cm	Jejunum: ATCC/cm	Jejunum: AOCC/cm
Expt 1	OVA	TT	4	12·14 ±5·94	112·24 ±5·94	125·83 ±12·43	0·44 ±0·29	0·89 ±0·70	0·44 ±0·29
Expt 2	TT	OVA	4	117·80 ±6·23	5·78 ±4·15	3·78 ±0·63	120·24 ±10·32	0·00 ±0·00	1·52 ±1·52

Recipient rats were primed i.p. with OVA and TT in FCA, loops prepared 10 days later, and the thoracic duct cannulated and loops challenged with the antigens indicated on day 14. On day 18, rats were injected i.v. with TDL from identically prepared but reciprocally challenged donors (see Table 1). Recipients were killed 12 hr after TDL injection and sections prepared from loops and intact jejunum. Values represent means ± SE.

tion into reciprocally challenged recipients, is shown in Tables 1 and 2.

The results in Table 1 indicate that, in accord with previous findings (Husband, 1982), whole gut priming by i.p. injection of antigen in FCA, followed by intralumenal challenge, results in the appearance of large numbers of IgA-specific ACC among TDL collected 3–4 days after challenge. Previous data (Husband & Gowans, 1978; Husband, 1982) have demonstrated that PP are primed, probably via the serosal route (Beh & Lascelles, 1981), after i.p. administration of antigen in FCA, and that subsequent lumenal challenge stimulates the release of ACC precursors from the PP into thoracic duct lymph, and that these subsequently extravasate from blood into the intestinal lamina propria. Thus, for example, in Experiment 1 (Table 1) ATCC would have risen from PP in the donor proximal loop and AOCC from PP in the donor distal loop.

The question arises, particularly in view of the findings of Pierce & Cray (1982) with respect to the influence of site of origin on cells homing to large or small bowel, of whether the ultimate distribution of cells within the lamina propria of the small intestine is influenced by the level of small intestine from which they originated. To examine this possibility, TDL were injected into segmentally challenged recipients after collection from reciprocally challenged donors. Thus,

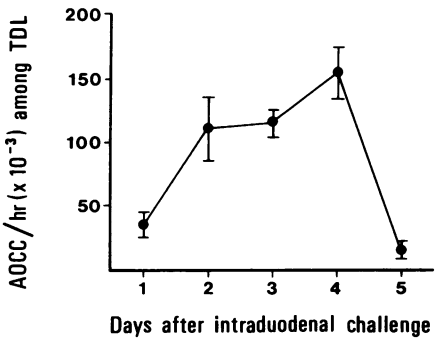

Figure 1. Output of AOCC in thoracic duct lymph after i.d. challenge of rats segmentally primed 14 days previously by injection of OVA in FCA under the serosa overlying a single PP, either in the proximal (three rats) or distal (three rats) small intestine. The results for all six rats were pooled as there was no significant difference between the two groups. Plotted points represent means, and vertical bars are standard errors.

in Experiment 1, for example, TDL doses containing ATCC arising from proximal loops and AOCC from distal loops were injected into recipients whose proximal loops were challenged with OVA and distal loop with TT. The data in Table 2 demonstrate that, by 12 hr after injection, the overwhelming majority of cells were located in the loop challenged with the antigen for which they were specific, and negligible counts were detected in the loop corresponding to their site of origin. In all cases, a high percentage of these ACC were IgA-specific (mean 77·3%, range 65·31–83·29%).

While the above experiments indicate that in animals in which all PP are primed, the site of origin of cells arising from subsequent segmental challenge does not influence antigen-induced distribution patterns, it was of interest to determine whether segmental priming followed by whole gut challenge results in site of origin effects. Rats were segmentally primed by injection of OVA and FCA under the serosa overlying a single PP, either in the proximal or distal small intestine, and the entire small gut challenged 14 days later by i.d. injection of antigen. To ensure that this procedure gave rise to lymph-borne ACC precursors, as did i.p. priming and intralumenal challenge, the thoracic ducts of six rats primed in this way were cannulated at the time of i.d. challenge and AOCC numbers among TDL enumerated (Fig. 1). The output of AOCC following this procedure was similar in

Tab'e 3. Distribution of AOCC in segmentally primed animals following challenge of the entire small intestine

Expt no.	No. rats	Site of primary	TDL drainage	AOCC/cm Proximal intestine	AOCC/cm Distal intestine
Expt 3	6	Proximal	–	158·08 ± 22·86	175·18 ± 26·31
Expt 4	6	Distal	–	126·89 ± 30·28	168·01 ± 18·09
Expt 5	4	Proximal	+	41·37 ± 7·90	34·79 ± 4·82
Expt 6	5	Distal	+	36·07 ± 2·83	30·55 ± 2·81

Rats were primed by injection of OVA in FCA under the serosa covering either a proximal or distal PP then the entire small intestine challenged 14 days later by i.d. injection of OVA in PBS. Rats in Experiments 3 and 4 were killed 5 days after challenge, and sections prepared from proximal and distal segments of small intestine. In Experiments 5 and 6, the thoracic duct was cannulated in each rat at the time of challenge and allowed to drain continuously. TDL were collected for 24 hr at the peak of the AOCC response (days 3–4 after challenge), washed and reinfused i.v. For Experiment 5, mean number of TDL per dose = $2·02 \times 10^8$ (range $1·23–2·93 \times 10^8$); mean number of AOCC per dose = $1·07 \times 10^6$ (range $0·79–1·19 \times 10^6$). For Experiment 6, mean number of TDL per dose = $2·40 \times 10^8$ (range $0·91–3·91 \times 10^8$); mean number of AOCC per dose = $1·23 \times 10^6$ (range $0·68–1·29 \times 10^6$). These rats were killed 12 hr after infusion and sections prepared from proximal and distal small intestine. Values in Table represent means ± SE of observations from the number of animals indicated.

kinetics, although slightly reduced in magnitude, to that observed after i.d. challenge of i.p. immunized rats (Husband & Gowans, 1978). Sections were taken from the mid-jejunum in these rats, and these revealed the virtual absence of ACC. The abrogation of a gut response by thoracic duct drainage indicates that in animals immunized in this way, the thoracic duct is the only source of ACC precursors, as reported previously for i.p. primed and i.d. challenged rats (Pierce & Gowans, 1975).

The ultimate location of these precursors was then established by enumerating ACC distribution between proximal and distal small intestine in another 21 rats immunized in this way, but which were either not cannulated and killed 5 days after i.d. challenge, or cannulated, TDL collected on days 3–4 after i.d. challenge, reinfused i.v. and killed 12 hr later. The results in Table 3 demonstrate that, regardless of the site of priming, there was an even distribution of AOCC at both levels of the intestine, a high percentage of which were IgA-specific (mean 81·06%, range 63·02–92·00%).

In cannulated rats given AOCC i.v. as a single dose (Experiments 5 and 6) by 12 hr after infusion, at which time AOCC distribution is not influenced by recently extravasated cells (Husband, 1982), there was still no significant difference between proximal and distal intestine. Overall AOCC numbers were lower in these rats than in intact rats because of the smaller supply of AOCC precursors available in a single 24 hr TDL collection.

To ensure that subserosal injection of PP does, in fact, achieve segmental priming via the underlying PP, a further six rats were given subserosal priming at a point in the intestine where there was no PP (at a proximal site in three rats and at a distal site in three rats). These rats were given i.d. challenge at day 14 and killed at day 19. Only occasional AOCC were detected in sections of either proximal or distal intestine.

DISCUSSION

In previous experiments (Husband, 1982), i.p. primed rats bearing double Thiry-Vella loops, each immunized with a different antigen, were injected with autologous TDL and ACC enumerated in the loops at various times after injection. These studies demonstrated that initial extravasation is antigen-independent, and ACC appear in specifically and non-specifically immunized loops in equal numbers up to 6 hr after TDL injection, but by 12 hr only ACC with specificity corresponding to the challenge antigen in each loop persist (as a result of retention and proliferation) and non-specific ACC virtually disappear by this time. The data in Tables 1 and 2, in which rats, prepared and immunized in an identical fashion, were injected with TDL from reciprocally immunized donors, are essentially the same as those obtained previously at 12 hr after injection. Thus, antigen-induced retention and proliferation effects operate, whether or not IgA-ACC are migrating to segments from which they originated.

These experiments indicate that in animals given whole gut priming (by i.p. immunization) and segmental challenge (by lumenal challenge of isolated loops), the site of origin of IgA-ACC precursors does not influence antigen-induced distribution patterns. It was therefore important to determine whether segmental priming (by subserosal immunization of a single PP) followed by whole gut lumenal challenge (by the i.d. route) results in site of origin effects. The data in Table 3 indicate that, regardless of the site of priming, there was a similar distribution of IgA-ACC following challenge.

This indicates that in the experimental system used here, there is no site of origin effect, at least with respect to ACC originating in and returning to the small intestine. This is contrary to expectations arising from the data of Pierce & Cray (1982), although those experiments compared site of origin effects between small and large bowel, and employed a model based on enteric rather than i.p. priming. It should be emphasised, however, that enteric primary is only successful if active cholera toxin is used (Pierce & Koster, 1980), presumably because of its ability to bind to epithelial cell membranes and activate adenyl cyclase (Pierce, 1978). Enteric priming could not be achieved with toxoid (Pierce & Gowans, 1975; Pierce, 1978) or other soluble protein antigens (Husband, 1978) although, given i.p. in FCA, these antigens are quite effective at priming the small intestine (Pierce & Gowans, 1975; Husband & Gowans, 1978; Husband, 1978). For this reason, the i.p. priming model has been used in the experiments reported here to enable study of ACC responses to antigens other than cholera toxin.

Taken together, the results indicate that when intestinal immunization is achieved by priming with antigen in FCA followed by intralumenal challenge, there is no site of origin effect on ultimate plasma cell location within the small intestine, whether whole gut priming and segmental challenge or segmental prim-

ing and whole gut challenge are used. While site of origin may affect extravasation, the overriding influence on ultimate distribution patterns is the site of antigen challenge inducing local retention and proliferation.

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