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© ANTIGEN-SPECIFIC RESPONSE AMONG T LYMPHOCYTES FOLLOWING INTESTINAL ADMINISTRATION OF ALLOANTIGENS

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Summary. The kinetics of T lymphocyte responses to mucosally presented antigen and the appearance of antigen-reactive T cells in mucosal and systemic tissues have been investigated by measuring secondary mixed lymphocyte culture reactivity after intestinal administration of alloantigens. The data indicate that T lymphocytes in the intestine are able to respond to mucosally presented alloantigens, and that following immunisation the T cell reactivity occurs first in Peyer's patches then sequentially in mesenteric lymph nodes, thoracic duct lymph, gut lamina propria and distant mucosal sites. The response was mucosally restricted and the ultimate location of antigen-reactive cells is influenced by site of antigen administration. Evidence is also presented for the existence of systemic non-reactivity to mucosally presented antigens.

INTRODUCTION

T lymphocytes have a key role in mucosal immunity because of the relative thymus dependency of IgA responses (Ebersole, Taubman and Smith, 1979), the importance of mucosally generated T cells in regulating systemic immunity (Mattingly and Waksman, 1978) and the role of T cells in cell-mediated mucosal defence (Arnaud-Battandier *et al.*, 1978).

There is conflicting evidence regarding the existence of a pool of T lymphocytes with mucosally restricted migration behaviour, because of the heterogeneity with respect to antigen specificity and effector function of most populations studied. Cahill *et al.* (1977) and Reynolds *et al.* (1982) observed in sheep that small recirculating T cells of intestinal origin tend to recirculate preferentially through gut-associated lymphoid tissues (GALT) and suggested that these cells exhibited mucosally restricted migration behaviour, but de Freitas, Rose and Parrott (1977) were unable to substantiate these findings in mice. Evidence for mucosally restricted migration of intestinal T lymphoblasts is more convincing. Several authors have demonstrated the propensity

Abbreviations used in this paper: GALT, gut-associated lymphoid tissue; TDL, thoracic duct lymphocytes; MLN, mesenteric lymph nodes; MLC, mixed lymphocyte culture; PP, Peyer's patch; LP, lamina propria; PLN, peripheral lymph node; PBS, phosphate-buffered (pH 7·4) saline.

of T blasts from GALT or thoracic duct lymph (TDL) to migrate to the intestine (Guy-Grand, Griscelli and Vassalli, 1974; Parrott and Ferguson, 1974) and Cox and Taubman (1982) have provided evidence for the functional separation of mucosal and systemic pools of T lymphocytes. There is also indirect evidence that intestinal T cells are able to populate distant mucosal sites, suggesting the existence of a common mucosal system among mucosal T lymphocytes. Parmely *et al.* (1977) observed T lymphocytes in milk reactive to antigens experienced in the intestine and Rose, Parrott and Bruce (1978) demonstrated the ability of mesenteric lymph node (MLN) T blasts to migrate to the mammary gland.

Because of the integration of responses between mucosal sites and the interaction between mucosal and systemic immune responses to mucosally presented antigen, it is of interest to determine the kinetics of antigen-specific T lymphocyte responses to mucosally presented antigen and the migration patterns of antigen-reactive T cells. Thus, the time course of reactivity of T lymphocytes obtained from a range of mucosal and systemic lymphoid tissues has been assessed by measuring secondary mixed lymphocyte culture (MLC) reactivity following intestinal administration of alloantigens. Whereas most previous studies have investigated the migration patterns of large populations of T lymphocytes without regard to their antigen specificities or effector functions, the model employed in this study permits an analysis of the behaviour of a population of antigen-specific T cells responding to a novel antigen.

The data reported here indicate that T lymphocytes in the intestine are able to respond to mucosally presented alloantigens and exhibit a mucosally restricted migration pattern, their ultimate location being influenced by the site of antigen administraion.

Materials and Methods

Animals

Adult male and female rats of an inbred Wistar strain were used as responders for all experiments. Allogeneic cells for immunisation and as a source of stimulator cells for MLC assays were obtained from adult male and female inbred DA or PVG strain rats.

Preparation of cell suspensions

Cell suspensions from Peyer's patch (PP), MLN, peripheral lymph node (PLN, comprising popliteal and axilliary nodes) and spleen were prepared by gently passing each tissue through a small gauge stainless steel sieve into sterile RPMI-1640 culture medium (Flow Laboratories, Sydney, Australia) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 5% heat-inactivated foetal calf serum.

For preparation of cell suspensions from gut and lungs, whole organs were removed and rinsed in sterile phosphate-buffered (pH 7·4) saline (PBS). Gut was dissected free of mesentery, cut into 5 cm segments, slit open along the mesenteric attachment, again rinsed in PBS and then incubated in 1 mM dithiothreitol (Sigma Chemical Co., USA) in RPMI-1640 for 20 min at room temperature with continuous gentle shaking. Gut and lung tissues were then washed three times in RPMI-1640, chopped into small pieces and incubated in RPMI-1640 containing 25 U/ml collagenase (Worthington, USA) at a ratio of 10 ml/g of tissue at 37° in a 5% CO_2 atmosphere for 90 min. The supernatants were collected, the remaining

tissue passed through a stainless steel sieve into supplemented RPMI-1640 and the resulting suspensions added to the supernatants.

All cell suspensions were allowed to stand for 10 min at room temperature for debris to settle. Cells remaining in the supernatants were filtered through cotton wool, then washed three times in sterile PBS containing penicillin and streptomycin.

Approximate cell yields per rat for spleen, MLN and PLN preparations were 200×10^6, 100×10^6 and 30×10^6 viable lymphocytes, respectively. Lung preparations yielded approximately 8×10^6 viable lymphocytes/g of tissue with many contaminating erythrocytes and few epithelial cells. Gut preparations yielded approximately 3×10^6 viable lymphocytes/g tissue with about twice that number of contaminating epithelial cells, most of which were not viable.

TDL was obtained by cannulation of the thoracic duct as previously described (Husband and Gowans, 1978) and lymph was collected at room temperature into a sterile flask containing 5 ml sterile PBS supplemented with 20 units/ml heparin, 500 U/ml penicillin and 500 μg/ml streptomycin. Lymph was collected immediately after surgery for a period of 18 h and then the lymphocytes recovered by centrifugation and washed three times in sterile PBS containing penicillin and streptomycin. Cell recoveries were usually of the order of 400×10^6 viable lymphocytes per rat.

Cells to be used for immunisations were resuspended to 5×10^7 viable lymphocytes/ml in PBS. Cells for culture were resuspended to 2×10^6 viable lymphocytes/ml in supplemented culture medium.

Immunisations

Intestinal immunisations were performed by injection, using a 27 gauge needle, of 1 ml of a washed suspension of 5×10^7 DA spleen cells directly into the lumen of the duodenum after its exposure through a small laparotomy performed under ether anaesthesia. Care was taken to ensure that none of the inoculum was injected into the serosa or mucosa of the gut and that none escaped into the peritoneal cavity after injection. In experiments to determine the effect of site of antigen administration on the location of antigen-reactive cells, the immunising dose of cells was delivered into the lumen of a Thiry-Vella loop. Loops were prepared as previously described (Husband and Gowans, 1978) and usually contained 3 or 4 Peyer's patches. Cells were injected directly into the lumen of the loops through a small laparotomy, after sealing the exteriorised ends of the loop with plastic adhesive tape. Loops remained sealed for 24 h and were then washed through daily with sterile PBS.

Mixed lymphocyte culture

Cells from different organs of immunised Wistar rats were assayed for secondary MLC reactivity against mitomycin-treated DA spleen cells. In one experiment mitomycin-treated PVG spleen cells were used as stimulators. All stimulator cells and an aliquot of responder cells were treated with mitomycin-C (Sigma Chemical Co., USA) to a final concentration of 50 μg/ml in supplemented culture medium and incubated for 25 min at 37°. After incubation the treated cells were washed four times with supplemented PBS and the remaining untreated responder cells once. All cells were resuspended in supplemented culture medium containing 5×10^{-5} M 2-mercaptoethanol (Sigma Chemical Co., USA) to a concentration of 2×10^6 cells/ml. Triplicate 100 μl aliquots of treated and untreated responder cells were dispensed into round-bottom microtitre plates (Nunc, Denmark) and to each well was added 100 μl of the treated stimulator spleen cell suspension. For each culture, cells from the same tissues from non-immunised Wistar rats were simultaneously assayed for primary MLC reactivity against the mitomycin-treated stimulator spleen cells.

For all experiments 5 replicate plates were set up for each culture. Each day 1 plate from each experiment was pulsed with 1·25 μCi per well of ^{3}H-thymidine (specific activity 25 Ci/mM, Radiochemical Centre, Amersham). Cells were harvested 4 h later on to a Titertek filter mat (Flow Laboratories, Australia) using a Dynatech Multimash automated cell harvester. After drying, the filter disc containing harvested cells was placed in scintillant and counted in a 1215 Rackbeta liquid scintillation counter (LKB, Sweden).

The MLC reactivity is expressed as counts per minute (c.p.m). For each experiment the mean c.p.m of control cultures (mitomycin-treated responder and stimulator cells) was subtracted from that of the experimental cultures (untreated responders and mitomycin-treated stimulators). Secondary MLC reactivity was calculated by subtraction of responses observed in non-immunised controls from that observed in immunised animals in concurrent cultures for each tissue assayed.

Statistics

Differences between means were tested for significance using Student's t-test.

RESULTS

Response in Peyer's Patches

PP cell suspensions were prepared from animals at 1-7 days after intraduodenal immunisation with allogeneic cells and were cultured for 5 days. MLC reactivity was assessed each day. The results in Fig. 1 show that PP lymphocytes exhibited a poor secondary response (response in immunised rats minus response in normal rats) which was maximal at 2 days after immunisation and at this time point the MLC reactivity reached a peak after 1 day in culture.

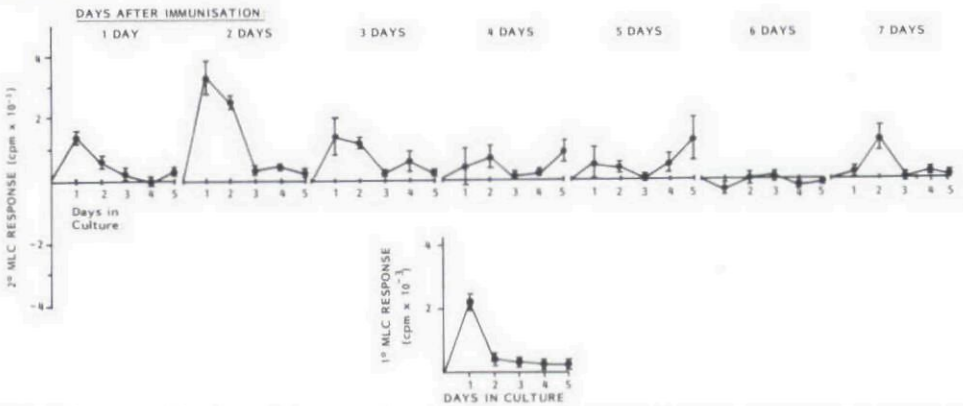

Fig. 1. Secondary MLC response (5 day cultures) of Wistar PP cells taken 1-7 days after intraduodenal immunisation with DA spleen cells. Plotted points represent mean c.p.m (primary response subtracted) from 3 animals, at each time point. Inset shows primary response, pooled data from 3 normal animals at each time point. Vertical bars represent standard errors.

Response in MLN

MLN cells were assayed for MLC reactivity in 5-day cultures at 1-9 days after intraduodenal immunisation. All cells displayed peak MLC reactivity after 3-4 days in culture but it is apparent from Fig. 2 that significant positive secondary responses at this time were only observed at days 6 and 7 after intraduodenal immunisation. The response at these times was much more substantial than that recorded at the peak of the PP response.

At 2, 5 and 9 days after immunisation the peak response appeared to be suppressed, as indicated by a negative secondary MLC response at 3-4 days in culture (after subtraction of the relevant primary reactivity).

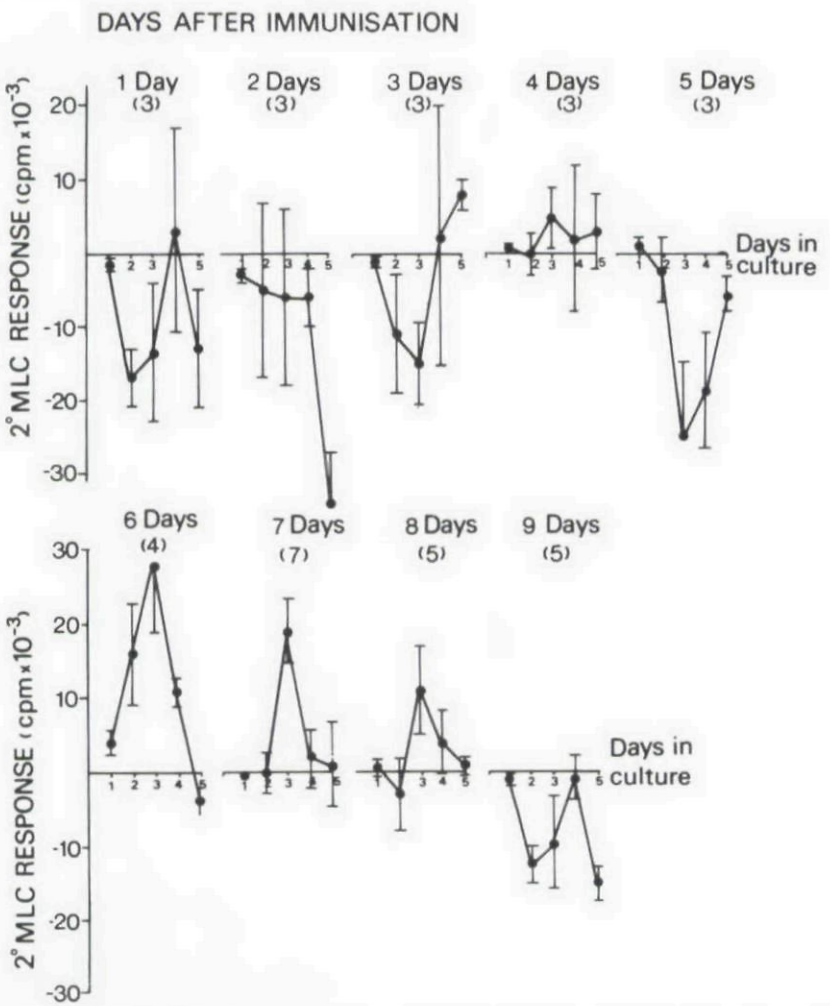

Fig. 2. Secondary MLC response (5 day cultures) of Wistar MLN cells taken 1-9 days after intraduodenal immunisation with DA spleen cells. Plotted points represent mean c.p.m (primary response subtracted). Vertical bars represent standard errors. Numbers of animals killed at each time point are shown in parentheses.

Antigen specificity of the secondary response in MLN

In order to ensure that the observed secondary responses in MLN at day 7 were antigen-specific, that is that they indeed reflected a secondary response to DA antigens, MLN cells from 6 Wistar rats which had been intraduodenally immunised with DA spleen cells 7 days previously were cultured with either mitomycin-treated DA spleen cells or mitomycin-treated PVG spleen cells. MLN cells from an additional 3 non-immunised Wistar rats were also cultured with these stimulators to provide comparable primary reactivity data. The results in Fig. 3 demonstrate that in immunised rats the MLC response to DA stimulators was greater than to PVG stimulators and reached a peak

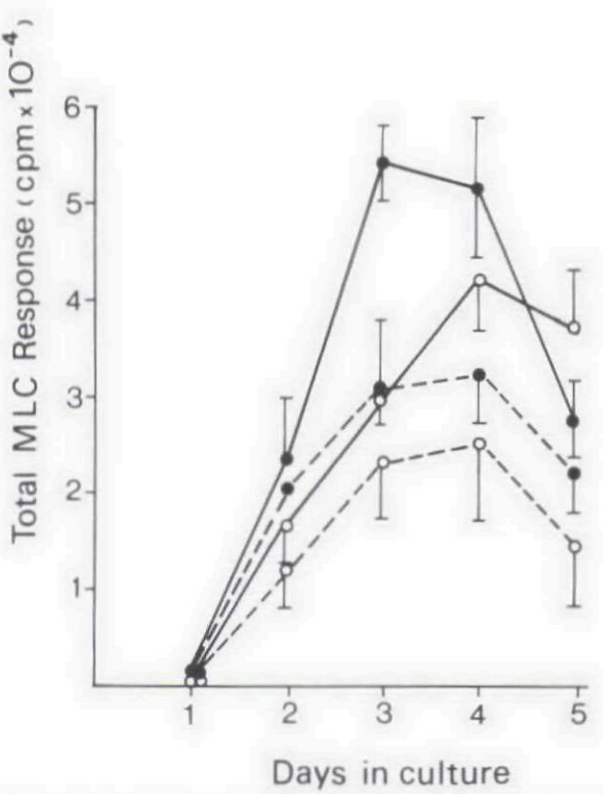

Fig. 3. Total MLC response of immunised Wistar MLN cells taken 7 days after intraduodenal immunisation with DA spleen cells and cultured for 5 days in the presence of mitomycin-treated DA (●——●) or PVG (○——○) stimulator cells, or non-immunised Wistar MLN cells cultured with DA (●- - -●) or PVG (○- - -○) stimulator cells. Plotted points represent mean c.p.m from 6 immunised or 3 non-immunised rats. Vertical bars represent standard errors.

one day earlier (day 3) at which time it was significantly greater than any of the other responses ($P<0.01$). The response of immunised rats to PVG stimulator cells was similar in kinetics to the primary response of non-immunised rats to either stimulator cell type, but the magnitude of the response to PVG cells at the peak (day 4) was slightly greater in immunised than non-immunised rats. Although this was not statistically significant, it probably reflects the presence of shared or cross-reacting antigens on PVG and DA spleen cells.

Response in TDL

Secondary MLC responses in TDL at 3-9 days after intraduodenal immunisation was assessed by comparing MLC reactivity in cultures of TDL lymphocytes collected from 3 immunised and 3 normal rats at each time interval (Fig. 4). A significant secondary MLC response was not detected until day 5 and persisted until day 9 with the highest and most consistent response occurring at day 8. On days 5, 6, 7 and 9 positive secondary

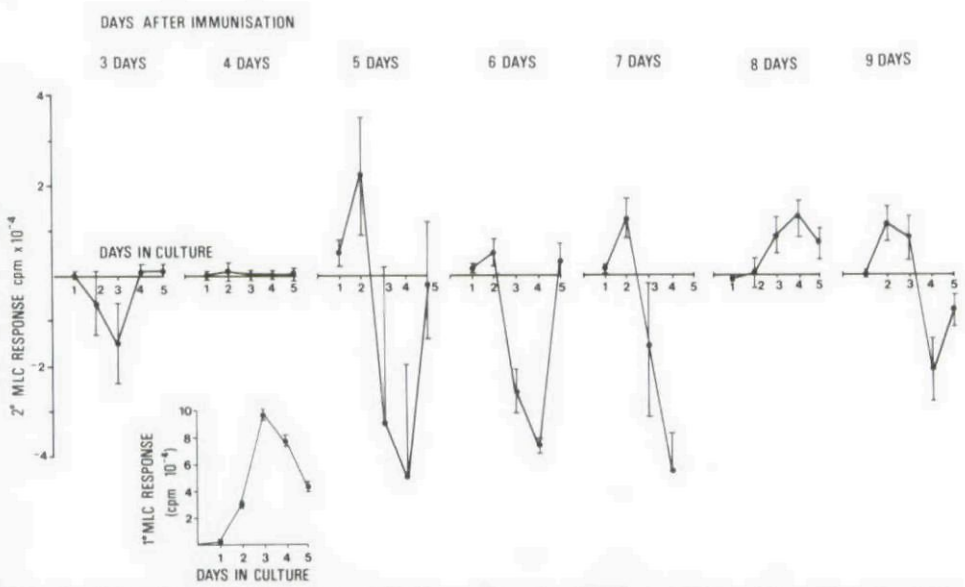

Fig. 4. Secondary MLC response (5 day cultures) of Wistar TDL cells, collected within 18 h of cannulation, on days 3-9 after intraduodenal immunisation with DA spleen cells. Plotted points represent mean c.p.m (primary response subtracted) from 3 animals at each time point. Inset shows primary response, pooled data from 3 normal animals at each time point. Vertical bars represent standard errors.

responses were recorded up to 2-3 days in culture, but after this time the MLC reactivity of cells from immunised animals declined sharply to become less than the primary reactivity, resulting in a negative secondary response towards the end of these cultures.

Comparison between systemic and mucosal responses

The above data establish that gut mucosa-associated T lymphocytes are able to respond to intraduodenally administered alloantigens. To determine whether this response was mucosally restricted, secondary MLC responses were assessed simultaneously in MLN, gut lamina propria (LP), PLN and spleen at 7 days after intraduodenal immunisation. Figure 5 shows that after 3 days in culture (at which time the MLN response was at its peak) and after 4 days in culture there was a significant response among gut LP lymphocytes, although slightly lower than for MLN cells, but that spleen and PLN lymphocytes displayed a negative secondary MLC reactivity (after subtraction of primary response). The differences between the responses displayed in mucosal and systemic tissues at these times were highly significant ($P<0{\cdot}001$ for MLN or gut LP versus PLN or spleen). Similar negative secondary responses were observed when PLN cells were assayed at 8, 9 or 10 days after immunisation (data not shown).

Effect of site of antigen administration on location of antigen-reactive cells

To determine whether the location of antigen-reactive cells in GALT was influenced by the site of antigen administration, isolated Thiry-Vella intestinal

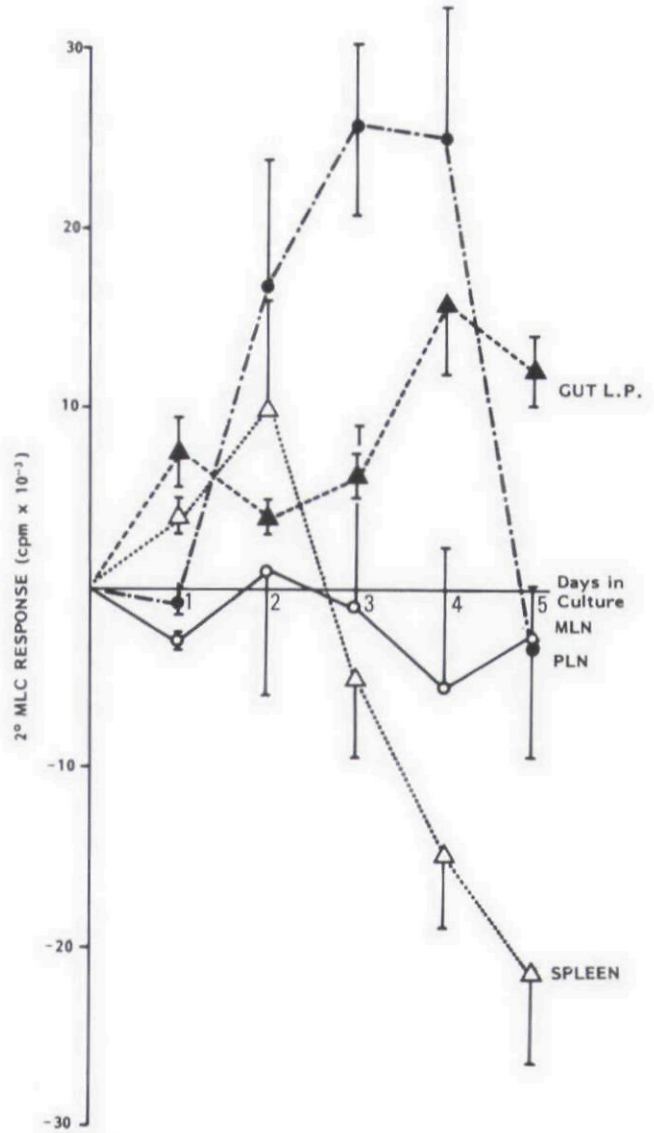

Fig. 5. Secondary MLC responses (5 day cultures) of Wistar MLN (● -.-.- ●), PLN (○——○), spleen (△.....△) and gut LP (▲ - - - ▲) cells taken at 7 days after intraduodenal immunisation with DA spleen cells. Plotted points represent mean c.p.m (primary response subtracted). Vertical bars represent standard errors. Data were obtained from 7 rats but gut LP cells were prepared from only 3 of these animals.

loops were immunised with allogeneic cells and a comparison made between responses in MLN draining loops and MLN draining the remaining intact intestine, and between responses in the loop LP cells and intact gut LP cells. Table 1 shows that there was a significantly greater secondary MLC response in MLN draining the immunised loop and among LP cells from the immunised

loop than in tissues associated with the non-immunised intact intestine. Indeed, the secondary MLC response was suppressed (relative to the primary response for MLN cells) in cells from MLN draining the non-immunised intact intestine.

TABLE 1

*Peak secondary MLC response (after 3 days in culture) of lymphocytes from MLN draining an immunised gut loop or MLN draining the remaining intact gut and from immunised loop LP or remaining gut LP at 7 days after intra-loop immunisation.**

MLN (5)		Lamina propria (3)	
Loop	Gut	Loop	Gut
18026 ±7324	−7194 ±1115	824 ±57	179 ±40
$P<0.001$		$P<0.01$	

* Values are mean c.p.m ± standard error (primary response subtracted). Number of animals is shown in parenthesis.

Responses at a distant mucosal site

Because of the documented ability of mucosally generated lymphocytes to migrate to and populate distant mucosal sites (Bienenstock and Befus, 1980), the time course of MLC reactivity at 6-10 days following intraduodenal administration of allogeneic cells was compared in gut LP and lungs

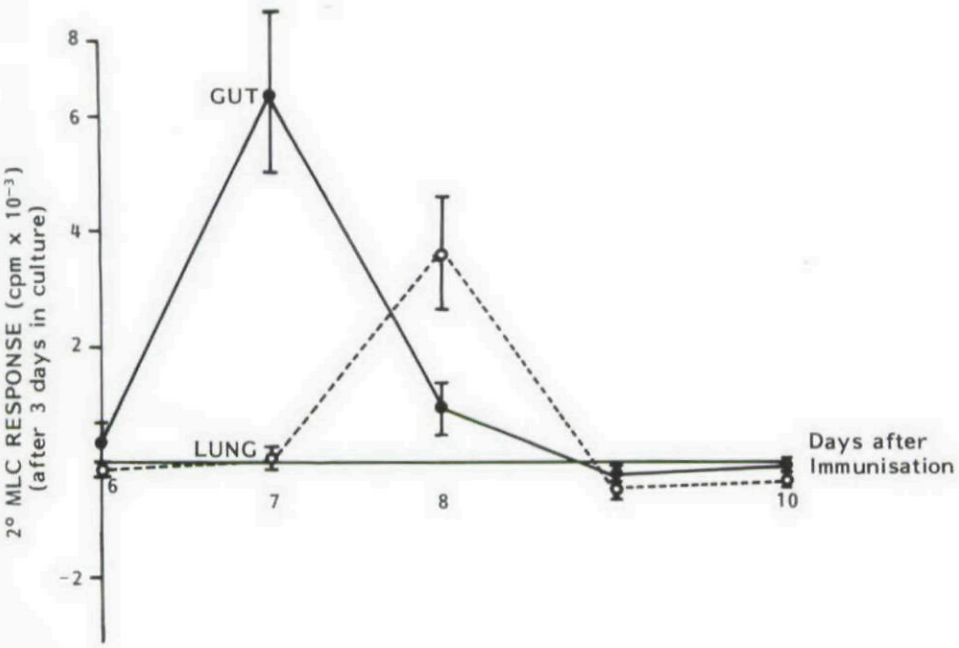

Fig. 6. Peak secondary MLC response (after 3 days in culture) of Wistar gut LP and lung cells taken 7-10 days after intraduodenal immunisation with DA spleen cells. Plotted points represent mean c.p.m (primary response subtracted). Vertical bars represent standard errors. Data were obtained from 3 rats at each time point.

to determine whether the time course of appearance of antigen-reactive T cells in these tissues was consistent with a similar pattern of migration. Since these cultures all exhibited peak MLC reactivity after 3 days in culture, the 3-day response only is shown for each time point after immunisation (Fig. 6). The results indicate a transient response in lungs at 8 days after intraduodenal immunisation, slightly later than the maximal response among gut LP cells (which occurred at 7 days) and of a lower magnitude.

DISCUSSION

The experiments reported in this paper describe the induction and tissue distribution of antigen-specific T cells reacting to alloantigens using secondary MLC reactivity to assess the proliferative response among T cells following intestinal administration of allogeneic cells. The early appearance of MLC-reactive cells in PP (Fig. 1) and later in MLN (Fig. 2), TDL (Fig. 4) and gut LP (Fig. 6) could be interpreted as evidence for a migration pathway for these cells analogous to that described for antigen-specific B lymphoblasts generated in response to intestinal antigen, i.e., PP, MLN, thoracic duct, gut LP (Husband, Monié and Gowans, 1977) and is consistent with findings of Guy-Grand *et al.* (1974) with respect to T blasts identified by immunofluorescence. It should be noted that, since no attempt was made to isolate a lymphocyte population from the LP cell suspensions, the LP responses recorded here may have been underestimated because of the presence of contaminating non-lymphoid cells in the cultures. Alternatively, it could be argued that the sequential appearance of reactivity in these tissues represents relocation of absorbed antigen. But the negative MLC reactivity in systemic lymphoid tissue (e.g. PLN, Fig. 5) indicates that antigen is probably localised within GALT and does not reach the circulation and, in any case, the appearance of antigen-reactive T cells among TDL indicate that at least a proportion of the reactive cells emanating from GALT are migratory cells.

The demonstration of transient MLC reactivity in lung tissues (Fig. 6) suggests that intestinally derived antigen-reactive T cells appear in mucosal sites other than the intestine and these data provide evidence for the existence of a common mucosal immune system among T cells, analogous to that demonstrated for IgA-producing B cells (Bienenstock and Befus, 1980). It should be noted, however, that the responses recorded in the lung cell suspensions may also be an underestimate of actual mucosal reactivity, since whole unperfused lungs were used to prepare the suspensions and large numbers of contaminating non-lymphoid cells and T lymphocytes of systemic origin would have been present to dilute the numbers of cells derived from the bronchial mucosa and perhaps to suppress their reactivity. This finding supports the conclusion of Parmely *et al.* (1977) and Rose *et al.* (1978) that the observed migration of intestinal T lymphocytes to mammary tissues is consistent with the concept of a common immune system among mucosal T lymphocytes.

The failure to detect significant secondary MLC responses among cells prepared from systemic lymphoid tissues (PLN and spleen, Fig. 5) suggests that antigen-reactive cells do not appear in these tissues in substantial numbers and is evidence for a mucosally restricted response pattern. Negative secondary responses recorded for PLN and spleen cells, after subtraction of the relevant primary response (Fig. 5), may reflect either active antigen-specific suppression in these tissues or depletion of antigen-reactive cells by recruitment to the site of interaction with antigen in GALT in immunised rats. The latter possibility would be unlikely if separate systemic and mucosal T cell pools exist, but time course studies within systemic tissues would be required to resolve this issue.

Previous studies have established that the migration and extravasation of IgA plasma cell precursors arising in GALT in response to intestinal immunisation is antigen-independent, but that the subsequent persistence of these cells in the LP is dependent on the site of antigen administration (Husband and Gowans, 1978; Husband, 1982). It was of interest to determine whether this concept could be applied to intestinal T lymphocytes. The influence of site of administration of allogeneic cells on the subsequent appearance of antigen-reactive T lymphocytes in the gut LP was investigated by comparing secondary MLC reactivity among LP lymphocytes prepared from immunised intestinal Thiry-Vella loops with that in the non-immunised intact intestine. The results (Table 1) parallel those if IgA B cells in that secondary MLC reactivity was significantly greater among immunised loop LP cells than LP of non-immunised intestine. However, this response was much less than that observed in gut LP when the whole intestine was immunised (Fig. 6) and this probably reflects the reduced length of intestine exposed to antigen in the immunised loop model. It was also demonstrated that substantial secondary MLC reactivity only occurred in MLN draining the immunised loop and not in MLN draining non-immunised intact intestine (Table 1), which is further evidence that initial interaction with antigen and the recruitment of antigen-reactive T cells occur locally at the site of antigen administration or in lymphoid tissue draining that site.

The influence of antigen on T lymphocyte migration has been demonstrated in other experimental systems with respect to systemic antigen exposure. Sprent and Miller (1976) showed that H-2 activated thoracic duct T cells tended to home to specific allografts and Emeson (1978) showed that alloantigen-reactive T lymphocytes were selectively recruited to lymphoid cell allografts and their draining lymph nodes.

In practical terms, the intestinal sensitisation to ingested allogeneic cells demonstrated here has particular relevance to the suckling neonate which represents a situation where the gut is exposed to allogeneic maternal cells via milk over an extended period, and may therefore mount a T cell response in the intestine and possibly at other distant mucosal sites. While the ingested cells may provide a source of passive immunological protection (Ogra, Weintraub and Ogra, 1977), there are serious deleterious implications

because of the possibility of graft versus host or autoimmune reactions (Head and Beer, 1979).

The data presented in this paper provide evidence for a degree of specialization among mucosal T lymphocytes analogous to that displayed by mucosal B lymphocytes. The results indicate that the intestinal mucosa is capable of mounting a T lymphocyte response to mucosally presented alloantigens and that this response is mucosally generated. From time course studies these cells appear to originate in PP tissues, then later appear in MLN, TDL and subsequently gut LP, lungs, and may disseminate to a variety of mucosal sites, where their ultimate location is influenced by the site of antigen administration. These cells display a mucosally restricted response pattern and evidence is presented for induction of systemic non-reactivity following intestinal alloantigen administration.

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