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Migration inhibition of lymph node lymphocytes as an assay for regional cell-mediated immunity in the intestinal lymphoid tissues of mice immunized orally with ovalbumin

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Summary. A migration inhibition assay, using lymph node lymphocytes, has been used as an *in vitro* assay for cell-mediated immunity (CMI) to ovalbumin in the mesenteric lymph nodes of mice fed ovalbumin. Migration inhibition developed only if an ovalbumin feed was preceded by cyclophosphamide administration; sensitization developed within 24 hr of a single ovalbumin feed, persisted for 14 days and could be recalled on secondary oral challenge with ovalbumin.

The intestinal CMI occurred in the absence of detectable systemic immunity and was found only in mice given cyclophosphamide before oral immunization. These results confirm earlier reports on induction of CMI to ovalbumin in the intestinal mucosa, and support the hypothesis that abrogation of a gut-associated suppressor system is necessary to allow induction of intestinal CMI to a dietary protein.

Abbreviations: FCA, Freund's complete adjuvant; CMI, cell-mediated immunity; CY, cyclophosphamide; DTH, delayed-type hypersensitivity; GALT, gut-associated lymphoid tissues; HSA, human serum albumin; MI, migration index; MLN, mesenteric lymph node; OVA, ovalbumin.

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INTRODUCTION

Although it has been proposed that local cell-mediated immunity (CMI) to dietary proteins may be responsible for enteropathy associated with food hypersensitivity (Ferguson & Mowat, 1980), such reactions are rare. It is probable that this is because the usual consequences of feeding proteins to naive animals include the induction of unresponsiveness for systemic delayed-type hypersensitivity (DTH; Miller & Hanson, 1979; Challacombe & Tomasi, 1980). In earlier work we have shown that pretreatment of mice with cyclophosphamide (CY) abrogated the state of tolerance for systemic DTH which normally results from feeding small amounts of ovalbumin (OVA; Mowat, Strobel, Drummond & Ferguson, 1982). In addition, this regime allowed the development of mucosal changes associated with local CMI when orally immunized mice were challenged orally with OVA (Mowat & Ferguson, 1981).

The mucosal changes observed are indirect parameters of intestinal CMI and also could only be elicited on *in vivo* challenge of immunized mice. If the mechanisms underlying the development of intestinal CMI to dietary antigens are to be elucidated fully, it would be important to have a reliable means of assessing these responses *in vitro*. In addition, it would be useful to be able to do so after primary immunization only. Systemic CMI rarely occurs after feeding

protein antigens to adult animals (Goldberg, Kraft, Peterson & Rothberg, 1971) while humoral immunity in the intestine is dissociated from the systemic antibody response (Dolezel & Bienenstock, 1971). Assays of systemic CMI are therefore unlikely to be reliable measures of CMI occurring locally in the small intestine. In the accompanying paper we demonstrate that migration inhibition of lymph node cells from parenterally immunized mice correlated with CMI in these animals (Mowat & Ferguson, 1982) and in an earlier report we demonstrated that migration of mesenteric lymph node (MLN) cells from CY-treated OVA fed mice was inhibited in the presence of OVA, lymph node cells being taken after a further oral challenge with OVA (Mowat & Ferguson, 1981). In this present paper we have extended our earlier preliminary work on mesenteric lymph node reactivity to OVA, to investigate induction of intestinal CMI in MLN of mice fed a single dose of OVA. In addition, we have tested the hypothesis that local CMI to a dietary antigen can occur in the absence of systemic CMI.

MATERIALS AND METHODS

Animals
Female BALB/c mice aged 6–8 weeks were used throughout.

Antigens
Ovalbumin (Sigma Fraction V) and human serum albumin (HSA; Sigma Fraction V) were dissolved in sterile saline for use.

Oral immunization
Unanaesthetised mice were fed 2 mg OVA in 0·2 ml of saline by means of a rigid steel tube with a rounded end, placed in the oesophagus. For secondary challenge, mice received 2 mg OVA/100 ml of their drinking water for a period of 10 days, beginning 28 days after the initial feed. Since the mice drank approximately 5 ml/day this corresponded to 0·1 mg OVA/mouse/day.

Cyclophosphamide
Cyclophosphamide (Endoxana-WB Pharmaceuticals) was dissolved in water and 100 mg/kg injected i.p. 2 days before oral immunization.

Local CMI in the MLN of orally immunized mice
MLN were removed from mice at intervals after the first feed of OVA and on completion of the 10 day challenge period. Cells from three–four mice in each group were pooled for each experiment and were used in the migration inhibition assay described previously (Mowat & Ferguson, 1982). Migration was assessed in the presence of 0·1 or 1 mg/ml OVA.

Three groups of mice were used in these experiments: CY/OVA mice received CY 2 days before feeding of OVA, while controls were fed OVA alone or were given CY alone.

Systemic DTH responses
The group of orally immunized mice were assessed for systemic DTH by an intradermal skin test. Mice were shaven on both flanks and the increment in double skinfold thickness measured 24 hr after an intradermal injection of 100 μg OVA in saline using skinfold calipers (Carobronze Ltd). Antigen-specific increments in millimetres were obtained by subtracting the non-specific response to saline in immunized mice. A group of positive controls were included in these experiments, and were tested for DTH 21 days after immunization with 100 μg OVA in FCA i.d.

Statistics
Results are expressed as means $\pm$ 1 standard deviation and groups were compared by Student's t test.

RESULTS

Migration inhibition of MLN cells after ovalbumin feeding

Migration inhibition tests were performed with 0·1 mg/ml OVA, in mesenteric lymph node cells taken at intervals from 1–22 days after a feed of 2 mg OVA, in mice given CY 2 days before an OVA feed, and in mice given CY alone. Results illustrated in Fig. 1 show that animals fed OVA alone, or given CY alone, had no migration inhibition at any time. However, animals given OVA preceded by CY had significant inhibition of migration at 24 hr after the feed of OVA, and a similar degree of migration inhibition persisted until 13 days after feeding [migration index (MI) at 13 days = 0·65].

Migration inhibition of MLN cells from OVA immunized animals did not occur when the cells were incubated in the presence of HSA, nor did OVA itself inhibit migration of MLN cells from unimmunized controls (Table 1).

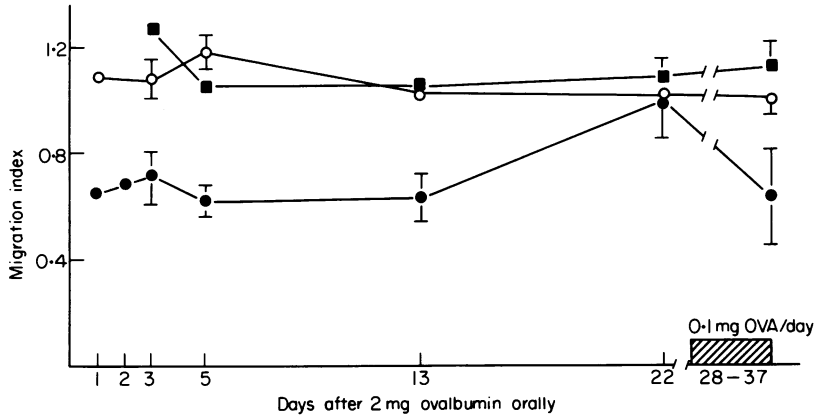

Figure 1. Development of migration inhibition in the MLN of mice fed 2 mg OVA after CY pretreatment, in mice fed OVA alone, and in mice given CY alone. Cells were assayed with 0·1 mg/ml OVA and, where shown, bars represent mean ± 1 SD of three experiments (OVA/CY groups *v.* others $P < 0{\cdot}01$). Other results are for one experiment and statistical analysis between groups is not possible. However, within each experiment at times up to 13 days, significant migration inhibition was observed in OVA/CY mice ($P < 0{\cdot}02$ *v.* control wells). (●—●) OVA/CY; (○—○) OVA alone; (■—■) CY alone.

A local CMI response in the MLN of mice immunized and challenged orally with OVA

Animals given OVA and CY, OVA alone or CY alone as above, were, 28 days after the first OVA feed, challenged with OVA at a dose of 0·1 mg/mouse/day. MLN cells taken on day 37 showed no migration inhibition in the OVA fed group, or the CY alone group but there was significant inhibition of migration of MLN cells from mice treated with CY and fed OVA, these mice having subsequently been challenged orally with OVA (MI = 0·63 ± 0·18, $P < 0{\cdot}01$; Fig. 1).

Systemic DTH responses in mice immunized and challenged orally with ovalbumin

The presence of systemic DTH was investigated either 5 or 21 days after a single feed of OVA or on completion of the 10 day oral challenge programme. DTH was assayed by the increase in double skinfold thickness 24 hr after 100 μg OVA intradermally.

A group of mice were immunized with 100 μg OVA in FCA intradermally as positive controls in these studies and when skin tested 21 days later, these mice had excellent DTH responses as measured by skin testing (Fig. 2). However, no DTH could be elicited in any of the groups of orally immunized mice. In particular, CY-treated OVA-fed mice had no systemic DTH either 5 days after oral priming nor on completion of the challenge protocol, despite the presence of local CMI in the gut-associated lymphoid tissue (GALT) at these times.

Table 1. Specificity of migration inhibition of MLN cells

Cell source	No. of experiments	Antigen	MI
Normal MLN	3	OVA (0·1 mg/ml)	1·01 ± 0·08
	3	OVA (1 mg/ml)	1·04 ± 0·06
MLN-OVA alone	2	HSA	0·95 ± 0·06
MLN-OVA/CY	3	HSA	0·92 ± 0·07

Migration indices (means ± 1 standard deviation) of normal MLN cultured with OVA and of MLN from OVA immunized mice cultured with 0·1 mg/ml HSA. MLN were obtained from immunized mice 5 days after feeding OVA.

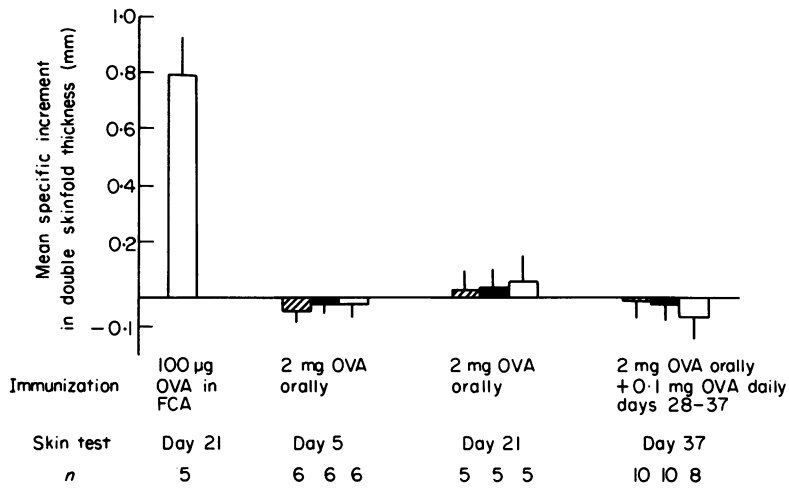

Figure 2. Systemic cell-mediated immunity after primary and secondary oral immunization with OVA in mice given OVA ± CY or CY alone. A group of mice was also immunized with 100 μg OVA in FCA as positive controls. Results are mean specific increment in double skinfold thickness, 24 hr after 100 μg OVA in saline intradermally + 1 SD (five–eight mice/group). (■) CY-treated OVA-fed mice; (■) OVA-fed mice; (□) CY-treated mice.

DISCUSSION

This study has shown that a single feed of ovalbumin is able to induce a state of CMI in the mesenteric lymph node and that this response could be recalled by secondary oral challenge with OVA. However, intestinal CMI developed only if mice received cyclophosphamide before oral immunization and occurred in the absence of systemic immunity.

The results thus confirm and extend our previous study which showed that CMI could be induced in the intestinal mucosa and MLN of CY-treated OVA-fed mice after secondary challenge with OVA (Mowat & Ferguson, 1981). We now report that the MLN may be sensitized within 24 hr of feeding OVA to CY-treated mice and that this persists for up to 2 weeks after feeding. The development of CMI in the MLN is surprisingly rapid after oral immunization. However, after feeding OVA, activation of suppressor and helper T cells occurs in the GALT within 24 hr (Richman, Graeff, Yarchoan & Strober, 1981) and it is likely that induction of specific effector T cells may also occur there very rapidly. In our earlier work on the induction of CMI to OVA in the intestinal wall, we did not examine the mucosal changes in mice challenged orally within a short time after sensitization. It would be of interest to learn if these too, could be elicited as rapidly. Sensitization was lost from the MLN 14 days after feeding and this is also of interest, since migration inhibition of MLN cells and mucosal CMI could be elicited in mice challenged orally 28 days after feeding OVA (Mowat & Ferguson, 1981). The loss of sensitization could reflect the disappearance of a transient, local immune response or migration of committed lymphocytes from the MLN to other parts of the intestine or GALT. The ability to mount a secondary response in both the MLN and mucosa and the abrupt rather than gradual loss of sensitization would argue against complete abrogation of the immune state in these mice. Rather, we would propose that after oral immunization of CY-treated mice T-cell sensitization in the GALT is followed by emigration of specific effector cells to the mucosa. In addition, memory cells must develop within the GALT in response to fed antigen although the subsequent localization patterns and sites of activation of such cells is unclear. A gut-committed pool of small, recirculating T cells has been described in the sheep (Cahill, Poskitt, Frost & Trnka, 1977) but not in the mouse (Freitas, Rose & Rocha, 1980). It would be logical to assume, however, that memory cells primed by the oral route should localize in tissues where they are most likely to re-encounter the antigen to which they are committed.

In contrast to the intestinal CMI induced in these mice, systemic DTH could not be elicited in CY-treated mice at any time after primary or secondary oral immunization. Furthermore, we have already

shown that serum antibodies are not present after a similar regime of oral immunization and challenge with OVA (Mowat & Ferguson, 1981). Intestinal T cells represent a pool of lymphocytes which is separate from their peripheral counterparts (Rose, Parrott & Bruce, 1976; Cahill *et al.*, 1977; Guy-Grand, Griscelli & Vassalli, 1978) and systemic CMI rarely occurs after oral immunization of animals (Goldberg *et al.*, 1971). It is entirely conceivable therefore that fed protein antigens may induce CMI in the GALT with subsequent migration of committed T cells to the gut, in the absence of systemic sensitization. This idea is supported by the finding that CMI may be induced in the respiratory tract without systemic CMI after inhalation of antigen (Henney & Waldmann, 1970) and by the dissociation between intestinal and systemic antibody responses (Dolezel & Bienenstock, 1971). Our results are thus further evidence in favour of the segregation of mucosal and systemic T cells and CMI. In addition, the findings of this study demonstrate that migration inhibition of lymph node cells can be applied to intestinal as well as peripheral lymphoid organs and confirm our hypothesis that this is an assay for locally defined CMI (Mowat & Ferguson, 1982).

Intestinal CMI has been detected by the production of lymphokines by intestinal mucosa or mucosal lymphocytes after oral immunization of normal animals with vibrio cholera (Gadol, Waldman & Clem, 1976) and transmissible gastroenteritis virus (Frederick & Bohl, 1976). In addition, there is a single report of CMI developing in the MLN and intestinal mucosa of pigs immunized orally with hapten-protein conjugates (Huntley, Newby & Bourne, 1979). In our experiments, intestinal CMI has been found only in mice given CY before feeding antigen. Our study is not directly comparable with that of Huntley *et al.* (1979) however, since they had to use repeated doses of soluble antigen to demonstrate intestinal CMI, and the anatomy of the pig GALT is substantially different to other animals. Further studies are required to resolve this discrepancy.

Feeding protein antigens normally induces a suppressor T-cell-dependent unresponsiveness of subsequent systemic DTH responses and this may be abrogated by CY (Miller & Hanson, 1979; Challacombe & Tomasi, 1980; Mowat *et al.*, 1982). The present results thus support our hypothesis that induction of intestinal CMI to dietary antigens requires abrogation of a gut-associated suppressor cell system (Mowat & Ferguson, 1981). CY represents only one way of modulating this homeostatic mechanism and we would propose that migration inhibition of MLN lymphocytes presents a simple assay by which the induction of intestinal CMI to dietary antigens may be reliably measured.

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