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Functional characteristics of intraepithelial lymphocytes from mouse small intestine

II. *IN VIVO* AND *IN VITRO* RESPONSES OF INTRAEPITHELIAL LYMPHOCYTES TO MITOGENIC AND ALLOGENEIC STIMULI

A. McI. MOWAT, SHEILA MacKENZIE, MARIA E. BACA, MICHELLE V. FELSTEIN & DELPHINE M. V. PARROTT *Department of Bacteriology and Immunology, Western Infirmary, Glasgow*

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SUMMARY

Although isolated intraepithelial lymphocytes (IEL) have been shown to have specific and non-specific cytolytic functions, their ability to proliferate in response to T-cell mitogens or alloantigens is controversial. Here we show that IEL from mouse small intestine do not respond to mitogens such as concanavalin A and phytohaemagglutinin A or in mixed lymphocyte reactions unless an accessory spleen cell is also present. Adherent spleen cells possess the most potent helper function, but a dividing accessory cell may also be required. Supernatants from stimulated lymphocytes also assist IEL to proliferate *in vitro*, particularly in the presence of adherent accessory cells. IEL could not mediate lethal graft-versus-host disease in irradiated hosts, but could produce popliteal lymph node hypertrophy or splenomegaly in unirradiated hosts. Thus, IEL have the potential for proliferative activities characteristic of T cells, but they require accessory cells and/or factors such as interleukin-2 for their function *in vitro* and *in vivo*.

INTRODUCTION

The digestive and absorptive functions of the small intestinal epithelium are of critical importance for the health of the animal and must be maintained in the face of a constant onslaught by luminal antigens. Therefore, it is reasonable to expect that an effective local immune system has developed to protect this site, a concept supported by the large number of lymphocytes normally found within the epithelium (Ferguson, 1977). As the majority of intraepithelial lymphocytes appear to be T lymphocytes (Guy-Grand, Griscelli & Vassalli, 1978; Selby, Janossy & Jewell, 1981; Parrott *et al.*, 1982; Lyscom & Brueton, 1982; Cerf-Bensussan, Schneeberger & Bhan, 1983), it is likely that the IEL may be involved in protective cell-mediated immunity (CMI) against local pathogens. There is also a proportionate increase in the numbers of IEL in clinical enteropathies such as coeliac disease and cow's milk protein intolerance (Ferguson, McClure & Townley, 1976; Phillips *et al.*, 1979), and many of these cells have the appearance of activated lymphocytes (Marsh, 1980). Thus, it is possible that intraepithelial T cells could contribute to the tissue damage in such disorders. For these reasons, it is important that we understand the immune effector functions of IEL and the factors that regulate these activities.

Recently, it has been shown that, under appropriate circumstances, rodent IEL are capable of cytotoxic T-cell (CTL) activity (Davies & Parrott, 1981a; Klein & Kagnoff, 1984), as well as natural killer (NK) cell activity (Klein & Kagnoff, 1984; Tagliabue *et al.*, 1981, 1982; Flexman, Shellam & Mayrhofer, 1983; Mowat *et al.*, 1983) and antibody-dependent cytotoxicity (Arnaud-Battandier *et al.*, 1978), but the proliferative functions of IEL are not as clearly defined. Although lamina propria lymphocytes (LPL) can proliferate and produce interleukin-2 *in vitro* (Greenwood, Austin & Dobbins, 1983; Nauss *et al.*, 1984; Arnaud-Battandier & Nelson, 1982; Fiocchi *et al.*, 1984), some authors have found that IEL respond poorly or not at all to T-cell mitogens or in mixed lymphocyte reactions (MLR) (Greenwood *et al.*, 1983; Nauss *et al.*, 1984; Cerf-Bensussan *et al.*, 1984). In contrast, others have suggested that IEL can respond well in these assays, particularly in the presence of exogenous IL-2, and can also mediate DTH (Shields & Parrott, 1985) and alloreactivity *in vivo* (Arnaud-Battandier & Nelson, 1982; Dillon & MacDonald, 1984). In view of these discrepancies and of the potential importance of this population of cells, we thought it important to re-examine the T-cell dependent function of highly purified preparations of IEL and to define the factors that may be required for a full proliferative activity by IEL.

Correspondence: Dr A. McI. Mowat, Glasgow University Department of Bacteriology and Immunology, Western Infirmary, Glasgow G11 6NT, U.K.

MATERIALS AND METHODS

Mice

Adult CBA(H-2^k), C3H/HeOla(H-2^k), NIH(H-2^q) and (CBA × BALB/c)(H-2^{kxd}) F_1 mice of both sexes were bred and maintained in the Department of Bacteriology and Immunology and were used at 8–12 weeks of age.

Preparation of spleen cell suspensions

Spleens were removed into RPMI-1640 (Gibco, Paisley, Renfrewshire) + 5% neborn calf serum (NCS) (Gibco) and gently minced through a fine wire mesh filter. After repeated passage through a Pasteur pipette, cells were washed three times in medium before counting under phase contrast. Viabilities were normally > 75%. Splenic adherent cells were prepared by removing red cells with 0·15 M NH_4Cl at 4° for 10 min, washing three times in medium and then culturing 10^6 cells in 100 μl in the wells of a U-bottomed microtitre plate (Titertek, Flow Labs, Irvine, Ayrshire) for 2 hr at 37° in 5% CO_2 in air. Non-adherent cells were removed by two vigorous washes with RPMI/10% NCS, leaving an average of $2–5 \times 10^4$ adherent cells/well as assessed by phase-contrast microscopy. In experiments using adherent accessory cells, responder spleen cells or IEL were added to give a final concentration of 10–20% adherent cells.

Intraepithelial lymphocytes

Populations of IEL were isolated and purified as described previously (Davies & Parrott, 1981b). Briefly, the small intestine was removed into cold calcium-magnesium-free Hanks' balanced salt solution (CMF) (Gibco) and washed through with 20 ml fresh CMF. The Peyer's patches were then removed, the intestine slit longitudinally and divided into pieces 2–3 cm in length. These were washed twice in CMF and once in RPMI/2% NCS + 2 mM glutamine (Gibco) + 100 u/ml penicillin + 100 μg/ml streptomycin (Gibco) before incubating for 30 min at 37° in a shaking water-bath. The gut pieces were then shaken in warm medium and the supernatants removed and stored at 4°. This procedure was performed three times, the fragments reincubated for 30 min at 37° and the shaking procedure repeated. The supernatants were pooled and passed over a glass-wool column to remove debris before resuspending in 3·5 ml Percoll (Pharmacia, Uppsala, Sweden) of specific gravity 1·055 g. The suspension was layered onto 3 ml 1·085 g Percoll in a siliconized glass universal and 3 ml RPMI-1640 layered on top. After centrifuging at 1000 **g** for 15 min at 4°, the 1·055 g/1·085 g interface was removed and washed three times in medium supplemented with 5% NCS. The resulting suspension contained > 90% lymphocytes of > 95% viability, with the remaining cells being predominantly eosoinophils and < 1% macrophages or B cells.

Treatment of cells with mitomycin c

Cells to be treated were incubated for 30 min at 37° with 40 μg mitomycin *c* (Sigma, Poole, Dorset)/10^7 cells/ml and washed at least three times before use.

Mitogenic stimulation

Responder cells were made up to 2×10^6 cells/ml in RPMI-1640 supplemented with 10% NCS, glutamine, penicillin/streptomycin and 50 μM 2-mercaptoethanol (Sigma), and 90 μl added to the wells of U-bottomed microtitre plates. Ten μl of medium containing concanavalin A (Con A) (Sigma), phytohaemagglutinin A (PHA) (purified reagent; Wellcome Ltd, Beckenham, Kent) or lipopolysaccharide (LPS) (*E.coli* 055:B5, Sigma) were then added to give a final volume of 100 μl/well. Control wells received medium only. The cultures were incubated for a total of 96 hr at 37° in 5% CO_2/air in a humidified incubator and pulsed with 12·5 nCi ^{14}C-thymidine in 50 μl/well for the last 24 hr. The cultures were then harvested using a Skatron multi-cell harvester, and labelled cell-bound DNA was assayed by liquid scintillometry. Each experiment was carried out in triplicate. In cultures where cell mixtures were studied, equal numbers of spleen cells and IEL were added to give a total concentration of 4×10^5 cells/well, control cultures containing 4×10^5 of one type. Results are expressed as c.p.m./10^6 cells and are expressed either as the arithmetic difference (Δ c.p.m.) between the thymidine uptake in response to mitogens and the uptake by unstimulated cells, or as a stimulation index which was obtained as follows:

$$\frac{\text{thymidine uptake in presence of mitogen}}{\text{uptake by unstimulated cells}}$$

Mixed lymphocyte reactions

Ten μl of responder cells at 2×10^7/ml were added to 10 μl mitomycin *c*-treated stimulator cells at 2×10^7/ml and cultured in a final volume of 100 μl for 120 hr, with ^{14}C-thymidine being added for the last 24 hr.

Lymphocyte supernatants

Supernatants were obtained from spleen cells stimulated with Con A or in MLR, from antigen-stimulated primed lymph node cells, and from EL-4 thymoma cells stimulated with phorbol myristate acetate (PMA). In order to obtain MLR supernatants, CBA spleen cells were cultured with mitomycin *c*-treated NIH spleen cells as stimulators as described and, after 48 hr, the supernatant removed from each well. Supernatants were also obtained from 24-hr Con A-stimulated cultures of CBA spleen cells and from cultures of lymph node cells from ovalbumin (OVA)-primed mice that were stimulated with 100 μg/ml OVA for 24 hr. As an enriched source of IL-2, cultures of EL-4 mouse thymoma cells were stimulated with 10 ng PMA (Sigma)/10^6 cells/ml for 48 hr. All supernatants were centrifuged at 1000 g for 10 min to remove cell debris, filter-sterilized (0·2 μm Microflow, Flow Labs) and stored at −70° before use in cell cultures at a concentration of 20%. In these experiments, control cultures contained supernatants from unstimulated cells that had been reconstituted with appropriate amounts of Con A, PMA or OVA.

Induction of lethal graft-versus-host disease

Adult mice received 900 rads of X-irradiation from a 2·2 MeV cobalt source (beam height 100 cm at 125 rads/min) and were reconstituted within 24 hr with 10^7 IEL or spleen cells intravenously. One control group was not reconstituted in order to assess the effects of irradiation alone. After irradiation, all mice received 10 mg/l neomycin sulphate (Calbiochem, La Jolla, CA) in their drinking water.

Induction of graft-versus-host reactions in unirradiated mice

A local GvHR was induced by injecting 10^7 CBA spleen cells or IEL into one footpad of adult (CBA × BALB/c)F_1 mice and assessed 8 days later by weighing the draining popliteal lymph nodes (Ford, Burr & Simonsen, 1970). As syngeneic controls,

CBA mice were injected with CBA IEL or spleen cells and, as negative controls, all mice received 50 μl medium into the opposite footpad. The GvHR-specific activity of the parental cells was calculated as the ratio of popliteal lymph node weight produced in F_1 hosts to that produced in syngeneic CBA hosts. In order to induce a systemic GvHR, groups of six 5-day-old (CBA × BALB/c) F_1 mice were injected intraperitoneally with 10^7 CBA IEL or spleen cells, and 8 days later the intensity of the GvHR was assessed by the spleen weight assay of Simonsen (1962). Control mice received 0·1 ml RPMI-1640 intraperitoneally only, and for each experimental mouse the spleen index (SI) was given as:

$$\frac{\text{relative spleen weight (mg/10 g body wt)}}{\text{mean relative spleen weight in controls}}.$$

Statistics

Groups of means and standard deviations were compared by Student's *t*-test, while survival after irradiation was expressed as median survival times (MST), and the mortality curves were compared using Wilcoxon's Rank Sum Test.

RESULTS

Response of IEL to mitogens

In the first experiments, we examined the response of CBA IEL to different mitogens. The *in vitro* responsiveness of IEL was studied in many separate experiments, but for simplicity, the results of two representative experiments are shown in Fig. 1 and Table 1. Spleen cells showed an excellent response to Con A that was optimal at a concentration of 200–500 μg/ml Con A (Fig. 1). In the experiment shown, IEL stimulated with these concentrations of Con A also showed ^{14}C-thymidine incorporation that was significantly above background levels. However, the maximum response of IEL was minimal in comparison to that of spleen cells (Δ c.p.m. 896±141 *vs* 29,015±2669, respectively, at 200 μg/ml), and in other experiments we found no response of IEL to optimal concentrations of Con A (see below). IEL also failed to respond in extended cultures (up to 7 days) or if cultured at different cell densities (data not shown). Furthermore, IEL showed no response to optimal concentrations of PHA or LPS (Table 1).

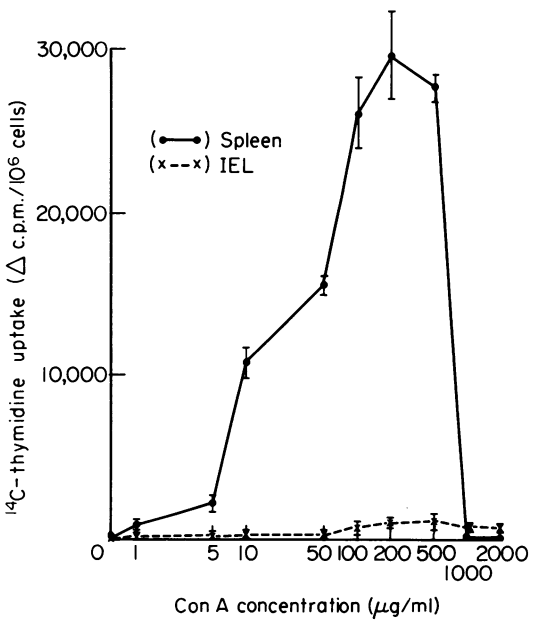

Figure 1. Proliferative response to Con A by CBA IEL and spleen cells. Lymphoid cells were cultured with different concentrations of Con A for 96 hr, and results shown are the net mean ^{14}C-thymidine uptake (Δ c.p.m./10^6 cells)±1 standard deviation for triplicate assays. Background proliferation for spleen cells and IEL in the absence of Con A were 152±51 and 104±25 c.p.m./10^6 cells, respectively.

Table 1. Uptake of ^{14}C-thymidine by intraepithelial lymphocytes and spleen cells in response to optimal concentrations of mitogens

Cells	Mitogen	^{14}C-thymidine uptake (c.p.m./10^6 cells) Experiment 1	Experiment 2
SPL	—	211±23	134±10
	Con A	13,320±2582 (62·4)	3730±1112 (28·7)
	PHA	2543±621 (12·1)	2854±425 (21·9)
	LPS	3331±478 (15·9)	424±83 (3·2)
IEL	—	135±15	161±35
	Con A	142±27 (1·05)	220±87 (1·41)
	PHA	130±26 (<1)	257±85 (1·62)
	LPS	140±11 (1·04)	231±64 (1·44)

IEL and spleen cells were cultured for 96 hr in the presence of 200 μg/ml Con A, 50 μg/ml PHA or 5 μg/ml LPS, and results shown are mean c.p.m./10^6 cells±1 standard deviation for triplicate assays. The figures in parentheses show the stimulation index calculated by dividing the response in the presence of mitogen by that found with cells alone.

Mixed lymphocyte responses by IEL

The MLR response of IEL was assessed by culturing CBA IEL (H-2^k) with mitomycin *c*-treated NIH spleen cells (H-2^q) as stimulator cells. In contrast to the vigorous response of CBA spleen cells to NIH stimulators, IEL showed no proliferative response to allogeneic cells (Table 2). Interestingly, mitomycin

Table 2. Mixed lymphocyte responses of intraepithelial lymphocytes

Responder	Stimulator	^{14}C-thymidine uptake (c.p.m./10^6 cells)	Stimulation index
CBA Spl	—	75·0±4·6	
	NIH Spl	737·5±110·2	9·8
CBA IEL	—	75·1±3·2	
	NIH Spl	46·2±3·1	0·62
NIH Spl	—	65·3±4·5	
	CBA IEL	275·4±0·0	4·2

IEL or spleen cells were cultured for 120 hr with mitomycin *c*-treated stimulator cells and the results shown are the mean c.p.m./10^6 cells±1 standard deviation for triplicate assays.

Table 3. Effect of the isolation procedure used to obtain IEL on the Con A response of spleen cells

Spleen cells	Con A	^{14}C-thymidine uptake (c.p.m./10^6 cells)	Stimulation index
Control	−	298 ± 5	11·7
	+	3505 ± 20	
Processed	−	102 ± 16	17·1
	+	1752 ± 108	

Processed spleen cells were treated by incubation and shaking as described for IEL and then cultured with 200 μg/ml Con A. Results shown are mean c.p.m./10^6 cells ± 1 standard deviation for triplicate assays. The stimulation index is the ratio of ^{14}C-thymidine uptake in the presence of Con A to that found in the absence of Con A.

c-treated CBA IEL could provide the stimulus for a moderate MLR response by NIH spleen cells, indicating the presence of MHC antigens on IEL. This response was not as great as when spleen cells were used to stimulate the MLR. In subsequent experiments, we failed to observe any MLR response by IEL in many different responder–stimulator combinations (data not shown).

Treatment of spleen cells by the method used to isolate IEL did not alter their response to Con A (Table 3), indicating that it is not the method of extraction that renders IEL incapable of responding *in vitro*.

Effect of splenic accessory cells on the *in vitro* responsiveness of IEL

Although IEL alone showed no responses *in vitro*, it has been suggested that the addition of accessory cells may enhance the mitogen response of rat lamina propria lymphocytes and human colonic IEL (Greenwood *et al.*, 1983; Nauss *et al.*, 1984). Therefore, we studied the effect of splenic accessory cells on the response of mouse IEL to mitogens and in MLR. Once again, representative results of one experiment from a series are illustrated.

In the first experiment we examined the MLR response of a 1:1 mixture of CBA IEL and spleen cells after stimulation by NIH spleen cells (Table 4). As before, IEL on their own failed to respond in the MLR (Group B), while spleen cells responded well (Group A). When CBA IEL and spleen cells were mixed a strong proliferative response to the NIH stimulators occurred, and this was significantly greater than that found with spleen cells alone (Group C *vs* Group A, $P < 0{\cdot}02$). If the IEL were treated with mitomycin *c* before culture, there was a highly significant reduction in the response of the IEL/spleen cell mixture (Group D *vs* Group C, $P < 0{\cdot}005$), indicating that IEL must contribute to the mixed response. Interestingly, the IEL/spleen cell mixture showed no response if the spleen cells had been treated with mitomycin *c* (Group E). Thus, the addition of syngeneic spleen cells does allow an MLR response by IEL, and these splenic accessory cells may have to divide to express their helper function.

In the second group of experiments, we examined the effect of spleen accessory cells on the Con A response of IEL. In the representative experiment shown, IEL did not respond to an optimal dose of Con A if cultured alone (Table 5, Group B), but if adherent cells were purified from the spleen and added to cultures of IEL at a final proportion of 15–20%, a strong proliferative response to Con A occurred (Group C). This was abolished by treating the IEL with mitomycin c (Group D). In this experiment, mitomycin *c*-treated adherent spleen cells were significantly less able to promote a response by IEL (Group E *vs* Group C, $P < 0{\cdot}02$) and, in other experiments (data not shown), mitomycin *c*-treated adherent cells were completely unable to assist a proliferative response by IEL. Although the purified adherent cells themselves did not proliferate in response to Con A (Group A), the results suggest that the accessory cells must undergo cell division at some stage of the culture to exert their helper function on IEL.

Table 4. Accessory function of spleen cells in mixed lymphocyte responses by IEL

	Responder cells		MLR response	
Group	Spleen cells	IEL	Δ c.p.m./10^6 cells	Stimulation index
A	+	−	592 ± 73	4·8
B	−	+	15 ± 34	1·1
C	+	+	836 ± 121	7·0
D	+	+(Mitoc)	504 ± 51	4·4
E	+(Mitoc)	+	31 ± 24	1·3

C3H spleen cells and/or IEL were cultured with mitomycin *c* (Mitoc)-treated NIH spleen cells as stimulators. Spleen cells responded well in MLR (Group A), while IEL alone showed no response (Group B). If spleen cells and IEL were mixed, a good MLR response occurred (Group C) and this was diminished if the IEL were treated with mitomycin *c* (Group D). Treatment of responder spleen cells with mitomycin *c* abolished the response of IEL (Group E). Results are given as the net c.p.m./10^6 cells (Δ c.p.m.), and the stimulation index obtained by subtracting or dividing the background response of spleen cells (126 ± 31 c.p.m./10^6 cells) or IEL (120 ± 25 c.p.m./10^6 cells) in the absence of stimulator cells and are the means ± 1 SD of triplicate assays.

Table 5. Accessory function of adherent spleen cells in responses of IEL to Con A

Group	IEL	Adh. spl.	Proliferative response: Δ c.p.m./10^6 cells	Proliferative response: Stimulation index
A	−	+	43±57	1·2
B	+	−	11+41	1·1
C	+	+	1023±123	7·2
D	+(Mitoc)	+	14±12	1·1
E	+	+(Mitoc)	553±98	4·3

CBA IEL were cultured alone (Group B) or with 20% syngeneic adherent spleen cells (Groups C–E) in the presence of 200 μg/ml Con A. Adherent spleen cells (Group A) and IEL showed no response to Con A alone, but an excellent response occurred when these populations were mixed (Group C), and this was abolished by pretreating the IEL with mitomycin *c* (Group D). Mitomycin *c*-treated adherent spleen cells were less able to assist IEL then untreated spleen cells (Group E *vs* Group C, $P<0{\cdot}001$). Results are shown as net c.p.m./10^6 cells (Δ c.p.m.), and the stimulation index obtained by subtracting or dividing the background proliferation of spleen cells or IEL in the absence of Con A (216±23 and 115±32 c.p.m./10^6 cells, respectively) and are the means±1 SD of triplicate assays.

Effect of lymphocyte supernatants on *in vitro* responses of IEL

The ability of spleen cells to promote mitogenic responses by IEL led us to examine the effect of lymphocyte supernatants on these functions, and our first approach was to study their effect on the Con A response of IEL. The results of one such series of experiments are shown in Table 6, where we examined the effects

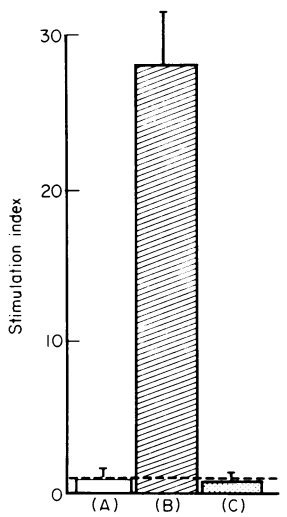

Figure 2. Effect of 20% MLR supernatant on MLR response of IEL. CBA IEL were cultured with mitomycin-treated NIH spleen cells alone (Group A) or in the presence of supernatants from a 48-hr MLR (Group B). Results shown are the mean stimulation index±1 standard deviation for triplicate assays calculated from the ratio of ^{14}C-thymidine uptake by stimulated IEL to that by unstimulated IEL (163±53 c.p.m./10^6 cells). The MLR supernatant itself has no effect on IEL in the absence of stimulator cells (Group C).

Table 6. Induction of Con A responsiveness in IEL by lymphocyte supernatants

Responder cells	Supernatant	Proliferative response: Δ c.p.m./10^6 cells	Proliferative response: Stimulation index
Experiment 1	*MLR supernatant*		
A IEL*	+	5±25	1·0
B IEL	+	675±33	6·4
C IEL adh. spl.	−	615±47	4·9
D IEL+adh. spl.	+	1080±118	9·6
Experiment 2	*Con A supernatant*		
A IEL*	+	11±31	1·1
B IEL	+	−35±50	<1
C IEL+adh. spl.	−	552±68	5·4
D IEL+adh. spl.	+	1467±123	12·7
Experiment 3	*OVA-LH supernatant*		
A IEL*	+	6±46	1·0
B IEL	+	163±51	2·5
C IEL+adh. spl.	−	221±53	3·1
D IEL+adh. spl.	+	2438+257	23·2
Experiment 4	*EL-4 supernatant*		
A IEL*	+	4±34	1·0
B IEL	+	−12±46	<1
C IEL+adh. spl.	−	1374±111	14·1
D IEL+adh. spl.	+	8455+1086	81·5

CBA IEL were cultured with 200 μg/ml Con A with or without syngeneic adherent spleen cells in the presence of 20% supernatants from an MLR (Exp. 1), Con A-stimulated spleen cells (Exp. 2), ovalbumin-stimulated primed lymph node cells (Exp. 3) or EL-4 thymoma cells stimulated with phorbol myristate acetate (Exp. 4). IEL responded to Con A in the presence of adherent cells (Groups C) and this was greatly enhanced in the presence of supernatants (Groups D). Results shown are net c.p.m./10^6 cells (Δ c.p.m.), and the stimulation index obtained by subtracting or dividing the background proliferation of IEL in the absence of Con A (range 100–130 c.p.m./10^6 cells). Results are means±1 SD for triplicate assays.

* Group A IEL were cultured in 20% supernatant in the absence of Con A.

of 20% supernatants from a 48-hr MLR (Experiment 1), from Con A-stimulated spleen cells (Experiment 2) or from OVA-stimulated, primed lymph node cells (Experiment 3) on the response of IEL to Con A. None of these supernatants was mitogenic to IEL in the absence of Con A (Group A in each experiment), and they usually had no effect on the Con A response in the absence of added adherent spleen cells (Group B). As described above, spleen adherent cells alone supported moderate Con A responses by IEL (Group C), but this was markedly enhanced by the addition of the lymphocyte supernatants (Group D). Finally, EL-4 supernatants could also induce an excellent Con A response by IEL in the presence of adherent accessory cells (Table 6, Experiment 4).

We then examined the ability of cell-free supernatants to allow IEL to respond in MLR. In the experiment shown (Fig. 2), CBA IEL showed no response to NIH stimulator cells (Group A), but, in the presence of 20% MLR supernatant, a strong MLR response occurred (Group B). Once again, the MLR

supernatant was not directly mitogenic to IEL in the absence of antigenic stimulation (Group C). In contrast to the results with Con A, the MLR response of IEL in the presence of the supernatant did not require additional accessory cells, presumably because these were already present in the spleen cell population. These stimulatory effects of lymphocyte supernatants on the response of IEL to mitogens or in MLR have been confirmed in several subsequent experiments.

Ability of IEL to cause lethal graft-versus-host disease

As IEL exhibited alloreactivity under appropriate circumstances *in vitro*, we went on to examine their function *in vivo*. Firstly, we tested the ability of IEL to induce lethal GvHD. Irradiated (CBA × BALB/c) F_1 mice that received 10^7 CBA spleen cells i.v. all died of acute GvHD within 8–9 days after cell transfer (MST 6 days), whereas 93% of recipients of syngeneic spleen cells survived indefinitely (Fig. 3). In contrast, F_1 recipients of CBA IEL had identical survival curves to irradiated, unreconstituted mice, while recipients of syngeneic IEL had poorer survival than irradiated controls (MST 11 days *vs* 21 days, $P<0.02$). Thus, IEL were unable to cause either acute, lethal GvHD or haemopoietic reconstitution of irradiated animals. Some F_1 recipients of CBA IEL were still alive >120 days after transfer, but showed no evidence of delayed GvHD.

Induction of proliferative GvHR by IEL

Acute, lethal GvHD in irradiated hosts may reflect immunological mechanisms that are different from those responsible for the mainly proliferative GvHR found in unirradiated animals. Thus, we examined the ability of parental IEL to induce local and systemic GvHR in unirradiated F_1 hybrid mice. In the experiment shown in Table 7, CBA IEL and spleen cells both produced significantly greater enlargement of the popliteal lymph node after injection into the footpad of (CBA × BALB/c) F_1 mice than in syngeneic CBA recipients. However, when the non-specific response in syngeneic mice was taken into account, it could be seen that spleen cells produced more GvHR-specific popliteal lymph node hypertrophy than IEL.

Finally, CBA IEL also induced a moderate systemic GvHR in unirradiated (CBA × BALB/c) F_1 recipients. Thus, 8 days after injecting 10^7 IEL intraperitoneally at 5 days of age (CBA × BALB/c) F_1 mice had significantly enlarged spleens compared with littermate controls (relative spleen weights 44·2 ± 6·0 and 30·0 ± 5·9 mg/10 g weight, respectively, $P=0.005$; spleen index 1·43). Interestingly, this was similar to the splenomegaly seen in F_1 recipients of spleen cells (relative spleen weight 47·9 ± 8·2, $P<0.01$; spleen index 1·55). Thus, IEL are capable of inducing both local and systemic proliferative GvHR in unirradiated hosts.

Table 7. Induction of a local GvHR by IEL

Donor cells	Host	Lymph node wt (mg)	Ratio
Medium	(CBA × BALB/c) F_1	1·15 ± 0·07	—
CBA IEL	(CBA × BALB/c) F_1	5·68 ± 0·60*	
	CBA	3·9 ± 0·91	1·46
CBA Spl	(CBA × BALB/c) F_1	3·84 ± 0·52†	
	CBA	1·38 ± 0·34	3·05

(CBA × BALB/c) F_1 or CBA mice were injected into the footpad with 10^7 CBA IEL or spleen cells and the draining popliteal lymph nodes weighed 8 days later. Results shown are means ± 1 standard deviation for six mice/group, and the ratios shown were calculated by dividing the mean lymph node weight in F_1 mice by that in syngeneic CBA mice.

* $P<0.01$ *vs* syngeneic recipients.

† $P<0.001$ *vs* syngeneic recipients.

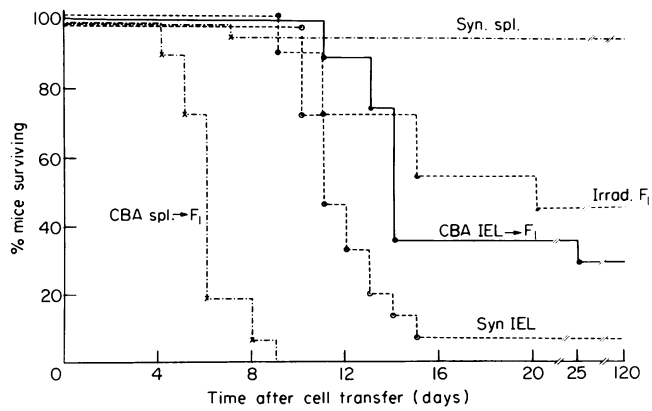

Figure 3. Comparison of IEL and spleen cells in the induction of lethal graft-versus-host disease. Lethally irradiated (CBA × BALB/c) F_1 mice were injected with 10^7 CBA spleen cells (× –·–·– ×) or IEL (●——●) to induce GvHD. F_1 and CBA mice had identical survival roles after the injection of syngeneic spleen cells (× – – – ×) or IEL (○ – – – ○) and the results are combined. One group of F_1 mice was not reconstituted after irradiation (irrad F_1 ● – – – ●). There were 12–17 mice per group.

DISCUSSION

The findings reported here show that IEL isolated from mouse small intestine have the potential to respond to T-cell mitogens *in vitro* and to allogeneic stimuli both *in vitro* and *in vivo*. Nevertheless, these responses are generally smaller than those observed with spleen cells and have an absolute requirement for accessory cells and/or their products. Thus, our study confirms that IEL comprise a heterogenous population that can have proliferative T-cell functions as well as the CTL and NK activity described previously (Davies & Parrott, 1981a; Tagliabue *et al.*, 1981, 1982; Flexman *et al.*, 1983; Mowat *et al.*, 1983; Klein & Kagnoff, 1984).

The main feature of our study was that IEL on their own showed virtually no response to either mitogens or alloantigens *in vitro*, confirming other studies (Greenwood *et al.*, 1983; Nauss *et al.*, 1984; Cerf-Bensussan *et al.*, 1984). This lack of response was not due to the technique used to isolate the IEL, nor did altering the cell density or the duration of the culture reveal any response of IEL to a wide range of mitogen concentrations. The non-responsiveness of IEL cannot be due to an absence of potentially responsive lymphocytes as it has been shown that human IEL can bind Con A normally (Greenwood *et al.*, 1983). Poor viability also cannot account for our results as the IEL populations had viabilities of >95%, and in previous work we have found that similar preparations are capable of cytotoxic

activity *in vitro* (Davies & Parrott, 1981a; Mowat *et al.*, 1983). Finally, the ability of IEL to respond in the presence of accessory cells is the strongest argument against these alternative explanations for our results.

IEL responded consistently to both mitogens and allogeneic stimuli in the presence of added spleen cells, and this was augmented by lymphocyte supernatants. In our hands, adherent spleen cells were potent accessory cells for IEL, suggesting that the cells involved may belong to the macrophage/dendritic cell series. This result is not at all surprising in view of the facts that T lymphocytes will not proliferate *in vitro* in the absence of Ia^+ accessory cells of this type (Persson *et al.*, 1978), and that adherent Ia^+ cells are not present in preparations of IEL (Davies & Parrott, 1981b; Lyscom & Brueton, 1982). One interesting feature was that, in most experiments, the accessory cells had to be capable of cell division to exert their helper activity. In other systems that are dependent on adherent accessory cells, their helper activity for T lymphocytes is not affected by mitomycin *c* (Persson *et al.*, 1978) and we cannot explain this discrepancy at present. We consider it unlikely that non-specific toxicity by mitomycin *c* accounts for its inhibitory effect on the accessory cells because our protocol causes minimal loss of viability and did not alter the ability of spleen cells to present alloantigens to other lymphoid cells (Table 2). Further work is required to elucidate the properties and phenotype of the splenic accessory cells required for IEL function.

Lymphoid cell supernatants exhibited potent augmentation of IEL responsiveness to mitogens and alloantigens but were not mitogenic for IEL. When the mitogen responses of IEL were examined, the majority of supernatants had no effect in the absence of adherent accessory cells. However, in MLR responses involving IEL and spleen cells, where accessory cells were already present, a direct effect of the supernatants could be demonstrated. Thus, the proliferation of IEL appears to require both adherent accessory cells and a soluble, lymphocyte-derived product in addition to the mitogenic or allogeneic stimulus. The nature of the soluble mediator(s) is currently under investigation, but its presence in supernatants from lymphocytes stimulated by T-dependent mitogens and antigens is consistent with IL-2 being one factor involved. This is supported by the strong helper activity of PMA-stimulated EL-4 cell supernatants which are rich in IL-2 (Farrar *et al.*, 1980), and by the finding that IL-2-rich supernatants have been shown previously to enhance the mitogenic response of IEL (Dillon & MacDonald, 1984). In addition, the inability of IEL to produce significant amounts of endogenous IL-2 (Greenwood *et al.*, 1983) is consistent with their dependence on added IL-2. Thus, it would be important to confirm these findings using purified lymphokines.

When the alloreactivity of IEL was studied *in vivo*, we found that IEL had some potential to mediate GvHR but that this was critically dependent on the nature of the host animal. Thus, parental IEL induced moderate splenomegaly in unirradiated F_1 mice and also produced a mild local GvHR in similar hosts, as others have found (Dillon & MacDonald, 1984). In contrast, IEL were totally unable to cause lethal GvHD in irradiated recipients, whereas the same number of spleen cells were potent mediators of both local and systemic GvHR. We would propose that IEL fail to cause lethal GvHD in irradiated mice due to the absence of accessory cells in the host. In contrast, unirradiated mice have normal numbers of accessory cells, and so donor IEL were able to produce the proliferative changes of splenomegaly and popliteal lymph node hypertrophy in such hosts. Thus, it would be of interest to assess the effect of added accessory cells on the alloreactivity of IEL *in vivo*.

We believe our results help to explain the discrepancies between earlier reports on the reactivity of IEL *in vitro* by identifying the critical importance of extraneous cells in this phenomenon. In two reported studies where IEL appeared to respond well to mitogens and in MLR, the preparations used were contaminated with either sIg^+ LPS-responsive cells (Dillon & MacDonald, 1984) or by both B cells and adherent phagocytic cells (Arnaud-Battandier & Nelson, 1982). In addition, Dillon & MacDonald (1984) found that IL-2 consistently augmented the mitogen response of IEL without addition of accessory cells, indicating that accessory cells may already have been present in their preparations. In the present report, and in others where IEL did not proliferate *in vitro* (Greenwood *et al.*, 1983; Nauss *et al.*, 1984; Cerf-Bensussan *et al.*, 1983), the IEL were not contaminated by significant numbers of adherent cells. Thus, we would propose that the level of contaminating accessory cells from the LP influences the level of responsiveness found in different preparations of IEL, and we would emphasize the need for rigorous purification of these cells.

There are two issues that this study was not able to address and that require clarification. Firstly, the nature of the proliferating cells among IEL has not been elucidated. Within this population there are small numbers of conventional helper/inducer and suppressor/cytotoxic T lymphocytes, and a large proportion of cells carry the suppressor/cytotoxic markers but no other T-cell antigens (Selby *et al.*, 1981; Parrott *et al.*, 1982; Lyscom & Brueton, 1982; Cerf-Bensussan *et al.*, 1983). In addition, it has been suggested that a significant proportion of rat IEL may be bone marrow-derived myeloid cells rather than T lymphocytes (Mayrhofer & Whately, 1983). Thus, it would be important to identify by FACS analysis the cell type associated with the proliferative functions we have described. The second point to be resolved is whether IEL require accessory cells to function *in situ* and, if so, the nature and origin of such cells. Although adherent Ia^+ accessory cells are not present within the epithelium, and IL-2-producing T_H are rare (Selby *et al.*, 1981; Parrott *et al.*, 1982; Lyscom & Brueton, 1982; Cerf-Bensussan *et al.*, 1983; Dillon & MacDonald, 1984), these cell types are frequent within the lamina propria (Selby *et al.*, 1981; Wilders, Sminia & Janse, 1983; Fiocchi *et al.*, 1984) and it seems probable that these could be encountered as IEL make their way into the epithelium through the LP.

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