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Intra-epithelial lymphocytes: interferon-gamma production and suppressor/cytotoxic activities

E. C. EBERT *Department of Medicine, UMDNJ–Robert Wood Johnson Medical School, New Brunswick, NJ, USA*

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SUMMARY

Human intraepithelial lymphocytes (IEL) proliferate minimally in response to phytohaemagglutinin (PHA), but produce as much interleukin-2 (IL-2) as do peripheral blood lymphocytes (PBL). The addition of sheep erythrocytes during activation of IEL with PHA markedly augments both T cell functions. This study evaluates the ability of IEL to produce interferon-gamma (IFN-γ) and to develop suppressor and cytotoxic activities when stimulated with mitogens in the presence or absence of sheep erythrocytes. PHA-activated IEL produced as much IFN-γ as did PHA-activated peripheral blood $CD8^+$ T lymphocytes. IEL activated by concanavalin A (Con A) demonstrated less suppressor activity directed against T cell proliferation than did Con A-activated peripheral blood $CD8^+$ T lymphocytes. IEL generated less mitogen-induced cellular cytotoxicity and lymphokine-activated killer cell activity than did peripheral blood $CD8^+$ T lymphocytes. The addition of sheep erythrocyte lysates during mitogen stimulation of IEL markedly enhanced their proliferation and lymphokine production but did not affect their suppressor or cytotoxic activities.

Keywords intra-epithelial lymphocytes interferon-gamma lymphokine-activated killer cells

INTRODUCTION

Human intra-epithelial lymphocytes (IEL) are T cells, predominantly of the $CD2^+CD3^+CD4^-CD8^+$ phenotype, located between intestinal epithelial cells. These lymphocytes are one of the first components of the mucosal immune system to encounter orally ingested antigens or intestinal pathogens. Their reaction to such antigens may help to determine whether the resulting immune response is predominantly one of tolerance or sensitization to foreign substances.

IEL from humans or rodents differ from lymphocytes in the peripheral blood (PB) or the spleen of these respective species in their low proliferative responses to T cell mitogens, such as phytohaemagglutinin (PHA) or concanavalin A (Con A) (Greenwood, Austin & Dobbins, 1983; Cerf-Bensussan *et al.*, 1984). Similarly, IEL from humans proliferate poorly in response to antibodies directed against the CD3 receptor. The low proliferation of IEL in response to mitogen is due to incomplete lymphocyte activation as demonstrated by a low expression of the CD25 (Tac) antigen representing the interleukin-2 (IL-2) receptor. Yet, these IEL produce as much IL-2 as do PB $CD8^+$ T lymphocytes. In contrast, IEL, stimulated by both PHA and sheep erythrocytes display marked proliferation, CD25 antigen expression, and IL-2 production, equivalent to those demonstrated by PB $CD8^+$ T lymphocytes activated in the same manner. The sheep erythrocytes enhance mitogen-induced activation of lymphocytes by attaching to their CD2 receptor. Similarly, IEL demonstrate brisk proliferation in response to antibodies that activate lymphocytes also by attaching to their CD2 receptors. These studies indicate that human IEL proliferate better in response to stimuli of the CD2 receptor than to stimuli of the CD3 receptor or to T cell mitogens (Ebert, 1985, 1989).

Since IEL proliferate only to select stimuli, their ability to carry out other T cell functions may also be restricted. To evaluate this possibility, IEL were tested for their ability to produce interferon-gamma (IFN-γ) and for their suppressor and cytotoxic capabilities after stimulation either with mitogen or with mitogen combined with sheep erythrocytes.

Correspondence: Ellen C. Ebert, MD, UMDNJ–Robert Wood Johnson Medical School, One Robert Wood Johnson Place, CN-19, New Brunswick, New Jersey 08903-0019, USA.

MATERIALS AND METHODS

Isolation of lymphocytes

Peripheral blood lymphocytes (PBL) were isolated from the heparinized whole blood of healthy individuals undergoing gastric bypass operations using density gradient centrifugation with Ficoll (Bionetics Laboratory Products, Kensington, MD). T cells were isolated from PBL by nylon wool columns, and PB $CD8^+$ T cells were obtained by anti-CD4 antibody and complement lysis of T cells as described previously (Ebert, 1989).

Jejunal mucosa from gastric bypass patients was dissected from the submucosa, rinsed vigorously, and minced. Tissue

occupying up to a 5-ml volume was placed in a 50-ml plastic tube (Corning Glass Works, Corning, NY) and treated for 20 min at 37°C with 10 ml of 1 mM dithiothreitol (DTT, Sigma Chemical Co., St Louis, MO) mixed freshly with RPMI 1640 (GIBCO, Grand Island, NY) containing 5% fetal calf serum (FCS, Microbiological Associates Bioproducts, Walkersville, MD), 1% antibiotic–antimycotic solution (GIBCO), and 10 mM HEPES (Sigma). The tissue was then incubated in 5 ml of 0·75 mM EDTA (Sigma) solution in calcium- and magnesium-free HBSS (CMF-HBSS, GIBCO) containing 5% FCS, for a total of 4·5 h in a 37°C shaking water bath (160 oscillations/min) (Precision Instruments, GCA Corporation, Chicago, IL). This incubation was interrupted every 45 min to wash the tissue with CMF-HBSS, removing loosely bound cells. The cells released by the first three treatments were saved to obtain the IEL, while cells from the last three treatments were discarded. Finally, the tissue was digested for 3 h at 37°C with 10 ml of a solution containing 0·01% deoxyribonuclease and 20 U/ml collagenase (Worthington Biochemicals, Freehold, NJ) in serum-supplemented RPMI. Following this treatment, a wire mesh was placed over the tissue, pressed, and rubbed with forceps. The resulting cell suspension served as the source of lamina propria lymphocytes (LPL).

These crude preparations were kept overnight at 4°C in serum-supplemented RPMI medium and then warmed in a 37°C incubator for 90 min before Percoll separation. This procedure improved the purity of lymphocytes obtained from the density gradient centrifugation. The cell suspensions were passed through a 100-mesh sieve (Thomas Scientific Co., Philadelphia, PA) to remove cell clumps and debris. Cell pellets of up to 0·5 ml were resuspended in 2 ml of 100% Percoll, containing nine parts Percoll (Pharmacia Fine Chemicals, Piscataway, NJ) and one part 10 × HBSS (GIBCO) adjusted to pH 7·4 and osmolality of 290 mOsm/kg. Above this were successively layered 2 ml each of 60%, 40%, and 30% Percoll, all prepared from 100% Percoll diluted with RPMI 1640 containing HEPES. The tube was spun at 400 *g* for 20 min at 25°C, and the cells immediately above the 60% layer were collected and washed to obtain purified IEL and LPL. These preparations contained over 85% $CD2^+$ cells that were at least 98% viable by trypan blue exclusion.

Measurement of IFN-γ activity

The amount of IFN-γ in supernates collected from 3-day cultures of lymphocytes (1×10^6/ml) with medium, with PHA (μg/ml, Burroughs-Wellcome, Greenville, NC), with sheep erythrocyte lysates, or with a combination of PHA and sheep erythrocyte lysates was measured using a radioimmunoassay (Centecor, Malvern, PA). The lysates were prepared from sheep erythrocytes (GIBCO) that were treated with neuraminidase, resuspended in H_2O, and then added to cultures at a 50:1 ratio of erythrocytes-to-lymphocytes (Ebert, 1985).

Con A-induced suppressor assay

Lymphocytes (1×10^6/ml) were incubated with Con A (18 μg/ml, ICN Pharmaceuticals, Cleveland, OH), with sheep erythrocyte lysates, with both Con A and lysates, or with medium alone for 2 days at 37°C, 95% air/5% CO_2. The cells were then treated with 66 μg/ml of mitomycin C (Sigma) for 30 min at 37°C and washed thoroughly. Each cell type (1×10^5 viable lymphocytes/0·1 ml) was then cultured with fresh allogeneic PBL (1×10^5/0·1 ml) with or without Con A (6 μg/ml) for 4 days. The cultures were pulsed with 1 μCi ^{3}H-TdR for the last 18 h of culture, harvested, and the radioactivity (ct/min) was measured. The percentage suppression of proliferation was calculated as follows:

$$1-\left(\frac{\text{ct/min } (T_{sm}+T_F+\text{Con A})-\text{ct/min } (T_{sm}+T_F)}{\text{ct/min } (T_{cm}+T_F+\text{Con A})-\text{ct/min } (T_{cm}+T_F)}\right)\times 100$$

where T_{sm} represents cells initially cultured in Con A in the presence or absence of sheep erythrocyte lysates and then treated with mitomycin-C (suppressor cells); T_{cm} represents cells initially cultured in medium alone or with sheep erythrocyte lysates and then treated with mitomycin C (control cells); and T_F represents fresh allogeneic PBL (indicator cells).

Cytotoxicity assays

Lymphocytes (2×10^6/ml) were activated by PHA (1 μg/ml), by Con A (18 μg/ml), by recombinant IL-2 (6 U/ml, Amgen Corporation, Thousand Oaks, CA), or by medium alone, each with or without sheep erythrocyte lysates, for 3 or 7 days. The lymphocytes were washed, counted, and mixed with ^{51}Cr-labelled K562, Daudi, DLD-1, CAPAN, 5637 or JAR cells (American Type Culture Collection, Rockville, MD) at a 25:1 effector-to-target cell ratio. Those targets growing in suspension (K562 and Daudi) were labelled with 200 μCi sodium chromate (New England Nuclear, Boston, MA) on the day of the assay and mixed with effector cells in round-bottomed microwells. Those targets growing in monolayers (DLD-1, CAPAN, 5637, and JAR) were removed from the culture flask by trypsin–EDTA (GIBCO), labelled with sodium chromate, and then allowed to re-adhere to flat-bottomed microwells for 18 h before the addition of effector cells.

After mixing effector and target cells, the plate was incubated at 37°C for 4 h at 95% air/5% CO_2. The experimental release of chromium was then determined by counting the radioactivity in the supernatant. The spontaneous and maximal releases were determined by counting the radioactivity in the supernates after incubating targets with either medium or 4% cetrimide solution (Fisher, Springfield, NJ). The spontaneous release was always less than 20% of the maximal release.

The arithmetic mean of the radioactivity from three wells of each cell combination was calculated. The percentage of cytotoxicity was determined as:

$$\frac{\text{Experimental}-\text{Spontaneous release}}{\text{Maximal}-\text{Spontaneous release}}\times 100.$$

Statistical analysis

For each set of data, an arithmetic mean and s.d. were calculated. Two sets of data were compared with Student's *t*-test.

RESULTS

PHA-induced production of IFN-γ by IEL

IEL produced the same amount of IFN-γ as did PB $CD8^+$ T lymphocytes in response to PHA (Table 1). Stimulation of IEL with both PHA and sheep erythrocyte lysates increased their synthesis of this lymphokine by eight-fold, similar to the response of lymphocytes in the PB. However, lymphocytes cultured with either medium or lysates alone, without mitogen, produced little IFN-γ.

Table 1. PHA-induced production of interferon-gamma (IFN-γ) by intra-epithelial lymphocytes (IEL) and peripheral blood CD8+ T lymphocytes with or without sheep erythrocyte lysates

Stimulus	IEL	CD8+ T lymphocytes
PHA	19±16 (5)*	23+15 (5)*
PHA+lysates	148±55 (5)	173±40 (5)
Medium	3±2 (3)	2±2 (3)
Lysates	3±2 (3)	3±3 (3)

Culture medium, collected after a 3-day incubation of 1×10^6 lymphocytes/ml with each of the stimuli was filtered and tested for IFN-γ production using a radioimmunoassay.

The number of experiments performed is in parentheses.

* Stimulation of lymphocytes with both PHA and sheep erythrocyte lysates resulted in more IFN-γ production than stimulation with PHA alone ($P<0.05$).

PHA, phytohaemagglutinin.

Table 2. Con A-induced suppressor activity by intra-epithelial lymphocytes (IEL), lamina propria lymphocytes (LPL), peripheral blood lymphocytes (PBL) and peripheral blood CD8+ T lymphocytes with or without sheep erythrocyte lysates

Stimulus	IEL	LPL	PBL	CD8+ T lymphocytes
		(% suppression)		
Con A	26±21 (10)*	53±28 (8)	52±18 (10)	52±10 (4)
Con A+ lysates	−14±84 (5)	40±10 (3)	78±13 (14)	65±15 (3)

Lymphocytes (1×10^6/ml) were cultured with Con A (18 μg/ml), with sheep erythrocyte lysates, or with medium alone for 2 days. These lymphocytes were then washed thoroughly, treated with mitomycin C, and then added at a 1:1 ratio to fresh allogeneic PBL in the presence or absence of Con A (6 μg/ml). Proliferation was measured by ^{3}H-TdR uptake on day 4, and suppressor activity calculated as described in Materials and Methods.

The number of experiments performed are in parentheses.

* This value is significantly less than the suppression generated by LPL, PBL or peripheral blood CD8+ T lymphocytes in response to Con A ($P<0.05$).

Con A, concanavalin A.

Con A-induced suppressor activity by IEL

Mitogen-induced IEL were then tested for their ability to suppress the proliferation of fresh allogeneic PBL in response to mitogen. To determine this suppressor activity, IEL cultured for 2 days with Con A or with medium alone were washed, treated with mitomycin C, and then mixed at a 1:1 ratio with fresh allogeneic PBL stimulated with a submaximal concentration of Con A. Proliferation of PBL was measured 4 days later after being cultured either alone or with IEL previously activated with Con A or medium. The decrease in the proliferation of PBL resulting from the presence of Con A-activated IEL was calculated as described, giving a percentage of suppression due to the IEL. IEL incubated in medium alone had no effect on the Con A-induced proliferation of PBL. IEL activated by Con A reduced the proliferation of PBL by 26±21%. However, this suppressor activity was less than that generated by Con A-stimulated LPL, PBL, or PB CD8+ T lymphocytes ($P<0.05$, Table 2). The suppressor activities generated by LPL, PBL, or PB CD8+ T lymphocytes were equivalent, suggesting that this function does not depend on the numbers of CD8+ T cells, as demonstrated previously (Damle & Gupta, 1982).

When IEL were stimulated with both Con A and sheep erythrocyte lysates, their effects on mitogen-induced proliferation of PBL were highly variable, ranging from enhancement to suppression, perhaps due to carry-over of the lysates to the indicator culture. However, IEL cultured with sheep erythrocyte lysates alone did not alter the proliferation of PBL in the indicator culture (not shown).

Table 3. Cytotoxic activity of intra-epithelial lymphocytes (IEL) or peripheral blood CD8+ lymphocytes after stimulation with phytohaemagglutinin (PHA) or interleukin-2 (IL-2)

Targets	Stimulus for effectors	Effectors (% cytotoxicity) IEL	CD8+ T lymphocytes
Daudi	Medium	0±0 (3)	0±0 (3)
	PHA	1+1* (4)	13±7 (3)
	IL-2	0±0* (5)	35±14 (4)
K562	Medium	1±2* (4)	9±4 (3)
	PHA	7±5* (7)	40±15 (4)
	IL-2	5±5* (4)	36±10 (4)
DLD-1	Medium	6±7 (4)	15±9 (4)
	PHA	31±6 (3)	40±11 (3)
	IL-2	29±11* (5)	54±13 (5)

IEL or CD8+ T lymphocytes were cultured for 3 days with medium alone, with PHA (1 μg/ml), or with IL-2 (6 U/ml), washed, recounted, and tested for cytotoxic activity against the targets listed using a viable effector-to-target cell ratio of 25:1.

The number of experiments performed is in parentheses.

* These values are significantly less than the comparable cytotoxic activities of CD8+ T lymphocytes, using the paired Student's *t*-test ($P<0.05$).

Cytotoxic activity of mitogen- or IL-2-stimulated IEL directed against a variety of target cell types

IEL and PB CD8+ T lymphocytes were cultured for 3 days with medium alone, with PHA (1 μg/ml), or with IL-2 (6 U/ml) and tested for cytotoxic activity directed against the Daudi, the K562, and the DLD-1 tumour cell lines derived from a Burkitt lymphoma, a chronic myelogenous leukaemia, and a colonic adenocarcinoma, respectively (Table 3). IEL activated by either

Table 4. Lymphokine-activated killer (LAK) cell activity of intra-epithelial lymphocytes (IEL), lamina propria lymphocytes (LPL), peripheral blood lymphocytes (PBL) and peripheral blood CD8+ T lymphocytes

	Effectors (% cytotoxicity)			
Targets	IEL	LPL	PBL	CD8+ T lymphocytes
Daudi (Burkitt lymphoma)	1±2* (6)	6±3† (5)	32±16 (5)	51±15 (3)
K562 (chronic myelogenous leukaemia)	17±17* (8)	18±7† (8)	47±24 (7)	74±10 (3)
DLD-1 (colonic adenocarcinoma)	22±13* (12)	10±7 (9)	14±10‡ (9)	57±12 (6)
CAPAN (pancreatic adenocarcinoma)	11±6* (3)	11±11 (3)	14±18‡ (3)	48±9 (3)
5637 (bladder epidermoid carcinoma)	20±5* (3)	25±12 (3)	17±18‡ (3)	60±2 (3)
JAR (choriocarcinoma)	5±6* (3)	5±8 (3)	3±3‡ (3)	34±10 (3)

Lymphocytes were cultured with IL-2 for 7 days, washed, recounted, and then tested for cytotoxic activity against the targets listed using a viable effector-to-target cell ratio of 25:1.

The number of experiments performed is in parentheses.

* LAK activity of IEL was significantly less than that of peripheral blood CD8+ T lymphocytes ($P<0.001$ for the Daudi and K562 targets; $P<0.05$ for the other targets). LAK activity of IEL was significantly less than that of LPL against the Daudi cells ($P<0.05$). LAK activity of IEL was significantly less than that of PBL against the Daudi and K562 cells ($P<0.05$).

† LPL demonstrated less LAK activity against the Daudi and K562 targets than did PBL ($P<0.01$).

‡ PBL demonstrated less LAK activity against the DLD-1, the CAPAN, the 5637, and the JAR targets than did peripheral blood CD8+ T lymphocytes ($P<0.001$).

PHA or IL-2 demonstrated no cytotoxic activity against the Daudi cells, and only partial activity against the K562 cells, compared with that of PB CD8+ T lymphocytes activated in a similar manner. In contrast, PHA-activated IEL displayed the same cytotoxic activity as did PB CD8+ T lymphocytes when directed against the DLD-1 colonic adenocarcinoma cell line, while IL-2-activated IEL demonstrated significantly reduced activity compared with their counterparts in the PB.

The LAK activity of IEL was then measured after a 7-day stimulation with IL-2 (Table 4), since the proliferation and LAK activity of IEL cultured with IL-2 peaks on this day. Again, the cytotoxic activity of IEL was significantly lower than that of PB CD8+ T lymphocytes against all target cells tested. In addition, LAK activity of IEL was significantly lower than that of LPL against the Daudi target, and lower than that of PBL against the Daudi or K562 targets. However, LAK activity of IEL was equivalent to that of either LPL or PBL against all solid tumours tested: the DLD-1, the CAPAN (pancreatic adenocarcinoma), the 5637 (bladder epidermoid carcinoma), and the JAR (choriocarcinoma).

The effects of sheep erythrocyte lysates on the cytotoxic activity of IEL were then measured. Adding the lysates during the stimulation of IEL with PHA, Con A, or IL-2 did not affect the mitogen-induced cellular cytotoxicity or LAK activities of these lymphocytes (not shown).

DISCUSSION

IEL represent a poorly understood but potentially important compartment of T cells. Their location between epithelial cells throughout the gastrointestinal tract suggests that they are among the first mucosal cells to encounter orally ingested antigens. Their reaction to such antigens may affect the subsequent mucosal immune response. For example, IL-2 production by IEL may trigger the development of LAK cells, while IFN-γ production may augment HLA-DR expression by epithelial cells. Suppressor activity by IEL may initiate tolerance to orally administered antigens, while cytotoxic activity may mediate the death of transformed or virally infected epithelial cells. The response of IEL is certainly complex, depending on environmental influences as well as the antigenic stimulus. However, an understanding of their capacity to carry out these various T cell functions in response to mitogen may indicate which of these reactions predominate *in vivo*.

The present study evaluates the IFN-γ production as well as certain suppressor and cytotoxic capabilities of IEL activated by mitogen or by mitogen and sheep erythrocytes, agents that induce either minimal or brisk proliferation of IEL, respectively.

The capacity of human IEL to produce IFN-γ has never been demonstrated. However, mitogen-activated murine IEL produce IFN-γ (Dillon, Dalton & MacDonald, 1986), that induces the expression of HLA-DR antigens on epithelial cells (Cerf-Bensussan *et al.*, 1984). Here we have shown that mitogen-activated human IEL produce as much IFN-γ as do PB CD8+ T lymphocytes. Similarly, mitogen-stimulated IEL produce as much IL-2 as do PB CD8+ T lymphocytes (Ebert, 1989). Whether this is true of other lymphokines remains to be determined.

Suppressor activity by human IEL is also incompletely defined. The present study shows that even Con A-activated IEL generate less suppressor activity directed against lymphocyte proliferation than do Con A-activated PB CD8+ T lymphocytes. This finding indicates that the minimal mitogen-induced proliferation by IEL is not due to intense suppression. Suppressor activity undoubtedly depends not only on the type of activity measured but also on the stimulus. Although human IEL do not suppress proliferation of PBL, they can either enhance or depress the pokeweed mitogen-induced synthesis of immunoglobulin by PBL, depending on the number of cells added (Greenwood *et al.*, 1983). In addition, human epithelial cells,

which cause PBL to develop potent suppressor activity directed against B cell immunoglobulin synthesis (Mayer & Shlien, 1987), may prove to affect IEL in a similar manner.

IEL from a variety of animal species are potent cytotoxic cells displaying spontaneous cytotoxicity (Arnaud-Battandier *et al.*, 1978; Tagliabue *et al.*, 1981), mitogen-induced cellular cytotoxicity activity (Arnaud-Battandier *et al.*, 1978), antibody-dependent cellular cytotoxicity (ADCC) (Flexman, Shellam & Mayrhofer, 1983), and cytotoxic T lymphocyte activity (Ernst *et al.*, 1986). In contrast, human IEL are capable of little spontaneous cytotoxicity against the K562 target (Cerf-Bensussan, Guy-Grand & Griscelli, 1985) and little ADCC activity. However, they possess potent mitogen-induced cellular cytotoxicity activity against Chang cells but not against chicken erythrocytes (Chiba *et al.*, 1981), suggesting target cell specificity. The present report shows that IEL are capable of some mitogen-induced cellular cytotoxicity and LAK activities against the K562 targets and a variety of solid tumour lines but not against the Daudi cells, again suggesting target cell specificity.

Human LPL have certain functions in common with IEL. Both types of mucosal lymphocytes produce IFN-γ in response to PHA (Lieberman *et al.*, 1988) and both possess LAK activity (Fiocchi, Tubbs & Youngman, 1985). In addition, both demonstrate minimal spontaneous cytotoxicity against the K562 target (Hogan, Halpel & Doe, 1985). However, LPL demonstrate greater mitogen-induced proliferative responses and suppressor cell generation than do IEL.

The actions of IEL after culture with both mitogen and sheep erythrocytes were evaluated since this combination of stimuli cause IEL to display marked proliferation, CD25 antigen expression, and IL-2 production, similar to those demonstrated by PB $CD8^+$ T lymphocytes (Ebert, 1989). The production of both IFN-γ and IL-2 by IEL, which was equivalent to that of PB $CD8^+$ T lymphocytes, increased markedly with the addition of sheep erythrocytes. However, the suppressor and cytotoxic activities by IEL, which were less than that of PB $CD8^+$ T lymphocytes, were not enhanced by the addition of sheep erythrocytes. This suggests that the suppressor and cytotoxic activities of IEL do not correlate with the degree of activation and proliferation induced by a stimulus. Whether there is a factor *in vivo* that has the same lymphocyte-stimulating effect as do sheep erythrocytes is unknown. Such a factor might bind epithelial cells as do sheep erythrocytes (Ebert, 1986), and so be in an ideal position to stimulate IEL.

Mitogen-stimulated human IEL are capable of carrying out certain T cell functions, particularly lymphokine production, despite their low proliferative response. Which of these activities is important in the epithelium during a normal immune response or in disease states remains to be studied.

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