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Analysis of natural killer effector and suppressor activity by intraepithelial lymphocytes from mouse small intestine

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SUMMARY

Intraepithelial lymphocytes (IEL) are morphologically similar to NK cells in other tissues and we have studied the NK activity of IEL isolated from mouse small intestine. In contrast to spleen NK cells, IEL showed little activity against YAC-1 over 4 h but had high levels of NK activity when the assay was extended to 18 h. IEL from nude mice did not show the enhanced NK activity found in other tissues. IEL were also found to suppress the NK activity of spleen cells and this suppressor function was not mediated by T lymphocytes or macrophages. The results indicate that the intestinal epithelium contains a population of potent NK cells which may represent a type of NK cell different to that found in other tissues. In addition, there are also cells capable of regulating NK cell function in the epithelial layer.

INTRODUCTION

The small intestine is the site which probably receives the greatest and most continuous antigenic stimulation in the body. It is reasonable to assume therefore that the intestinal mucosa would contain large numbers of effector and regulatory lymphocytes, including natural killer cells.

The exact function of natural killer (NK) cells is not known, but it has been proposed recently that NK cells may have both cytotoxic and immunoregulatory roles *in vivo* (Beverley, 1981). We were therefore interested to examine NK activity in the small intestinal mucosa and in particular to study NK activity of the lymphoid cells in closest contact with the intestinal lumen, the intraepithelial lymphocytes (IEL).

To date, work done on cytotoxic reactivity in the intestine of experimental animals and man has given rather equivocal results. In humans, K cells, mediating antibody-dependent cellular cytotoxicity (ADCC), are only detectable with certain target cells (MacDermott, Franklin *et al.*,1980). In guinea-pigs, both NK and K cell activities are detectable in small intestine IEL (Arnaud-Battandier *et al.*, 1978), and in mice, both cytotoxic T cell activity (Davies & Parrott, 1980, 1981a) and NK activity (Tagliabue *et al.*, 1981) are detectable in LPL and IEL. In data to be presented here, however, we have found that there is very little NK activity in IEL using a standard 4 h assay. Assays with longer incubation times and co-culture experiments with spleen lymphocytes and IEL have led us to believe that the NK activity of IEL might nevertheless be quite high and that it may be regulated by a suppressive mechanism which is particularly apparent during the shorter assay periods.

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MATERIALS AND METHODS

Animals. C57Bl/10ScSn (H-2^b), C3H/HeB (H-2^k), CF/NIH (H-2^q) and BALB/c (H-2^d) mice 6–8 weeks old were obtained from department stocks. Within any one experiment, age and sex matched animals were used. CBA nu/nu and nu/+ littermates were obtained from Olac Ltd. C57Bl/6 mice carrying the beige mutation (bg/bg) were a gift from Dr H.S. Micklem, Department of Zoology, University of Edinburgh.

Cell preparations. Spleen, mesenteric lymph node (MLN), peripheral lymph node (PLN) and thymus cell suspensions were prepared by standard techniques. All cell suspensions were filtered through columns of loosely packed sterile glass wool, washed at least three times and stored at 4°C before use. Spleen cells were treated with isotonic ammonium chloride to remove red blood cells. This treatment did not affect NK activity in our system. Intraepithelial lymphocytes were prepared by a technique described previously (Davies & Parrott, 1981b) and were purified on discontinuous density steps of Percoll (Pharmacia). Viabilities of cell suspensions, as assessed by the exclusion of 0·2% eosin were in excess of 80% for spleen, MLN and PLN and in excess of 90% for thymus and IEL.

Tumour cells. YAC-1 tumour cells were grown *in vitro* in RPMI 1640 + 10% fetal calf serum (FCS) and were always subcultured 24 h before use. For use in the cytotoxicity assay, tumour cells were labelled at 5×10^6 cells/50μCi ^{51}Cr/ml of medium for 60 min at 37°C. The cells were then washed five times and resuspended at 2×10^5 cells/ml for use in the assay.

Cytotoxic assay. The cytotoxic assay was set up in V bottomed microtitre plates as described previously (Davies & Parrott, 1980) for cytotoxic T cell assays except that the incubation time was 4 or 18 h at 37°C. Effector to target cell ratios of 25:1, 50:1 and 100:1 were used. For assessing the spontaneous release of ^{51}Chromium, tumour cells were incubated with thymus cells, which have no NK activity, at the appropriate cell densities. When cell mixtures were used, the effector:target ratio given was always the spleen:target ratio and the contribution of the additional cell type in the mixture was 1:1 with spleen cells.

Calculation of results. The percentage cytotoxicity induced by each cell type was calculated using the following formula:

$$\text{Percentage specific cytotoxicity} = \frac{\text{Release in presence of effector cells} - \text{spontaneous release}}{\text{Maximum release} - \text{spontaneous release}} \times 100$$

Maximum release of ^{51}Cr was assessed by treating labelled cells with 10% Triton X-100. In each experiment the results are expressed as the mean of quadruplicate cultures of cells derived from a pool of 2–4 mice. The results shown in the tables are expressed as the median and range of a number of separate experiments.

Depletion of T lymphocytes. Ten million lymphoid cells/ml were incubated for 30 min at room temperature in 1:1,000 monoclonal anti-Thy 1.2 antiserum (F7D5-Olac 1976) and then for 45 min at 37°C in the presence of 1:10 fresh guinea-pig serum as a source of complement (C^1). Cells were washed three times before use. Control cells were incubated in medium + C^1 alone.

Depletion of macrophages. Cell suspensions were depleted of macrophages by incubation in a Sephadex G10 column (Pharmacia) for 30 min at 37°C. Depleted cell suspensions were washed three times before use.

Gluteraldehyde treatment of cells. Ten million lymphoid cells/ml were incubated for 20 min at room temperature in 1% gluteraldehyde in RPMI 1640 and washed three times before use.

RESULTS

The natural killer activity of intraepithelial lymphocytes (IEL) from various strains of mice

The natural cytotoxicity of IEL from C57Bl, C3H, NIH and BALB/c mice was measured against YAC-1 tumour cells. In these four strains tested, IEL exhibited minimal cytotoxicity compared with spleen cells, although slightly higher levels were seen in NIH mice than in the other three (Table 1).

Table 1. NK activity of intraepithelial lymphocytes, spleen and lymph node cells from different strains of mice. Results are median and range of % cytotoxicity with numbers of experiments in parenthesis*.

Strain	IEL	Spleen	Lymph Nodes
C57Bl	1·6 (7) (0–4·4)	19·0 (8) (13·1–44·4)	3·3 (3) (1·3–4·4)
C3H	2·7 (4) (0·7–4·7)	26·5 (7) (11·4–42·6)	nd
NIH	6·1 (4) (2·3–8·4)	9·7 (4) (5·1–14·8)	0·2 (3) (0–1·4)
Balb/c	3·5 (4) (1–6·0)	15·5 (7) (7–23)	6·9 (7) (2–14)
Beige	0 (4)	0 (4)	0 (4)
CBA nu/+	0·8 (3) (0–7·3)	20·0 (3) (16·3–20·7)	0·7 (3) (0–1·2)
CBA nu/nu	2·4 (4) (0–4·9)	66·0 (3) (47–71)	28·0 (3) (8–37·6)

* Effector:target ratio 50:1 4 h incubation.

The IEL from beige mice had no NK activity at all but this was not unexpected because beige mice have few NK cells in any other tissues. Nude mice have consistently more NK activity than other strains of mice (Kiessling, Klein & Wigzell, 1976; Warner, Woodruff & Burton, 1977). There was no difference (Table 1) in the NK activity of IEL from CBA nu/+ or CBA nu/nu despite the large amount of NK activity in the spleen and peripheral nodes in the CBA nu/nu when compared with CBA nu/+ mice.

Absence of NK activity in IEL was not due to any harmful effects of the isolation method used to prepare these cells, since treatment of spleen cells in the same way did not alter their NK activity (results not shown).

These results would indicate that mouse IEL are deficient in conventional NK activity. We decided to investigate the possibilities that NK activity could either be revealed by prolonging the assay time, or that a suppressor mechanism could account for the observed lack of NK activity.

The suppressor activity of IEL on spleen NK activity

Table 2 shows the results of a representative experiment. Suspensions of IEL, thymus or mesenteric lymph node cells were added to C57Bl spleen cells at different E:T ratios and assayed against YAC-1 over 4 h. Addition of MLN cells had little or no effect but the IEL had a profound inhibitory

Table 2. Suppressive effect of IEL on spleen NK activity. Spleen cells were cultured at the effector:target ratios shown and equal numbers of the second cell population added. Results are % cytotoxicity using C57Bl/10 cells

	50:1	25:1	12·5:1
Spleen	19·0	12·2	10·1
IEL	0·0	0·4	0·5
MLN	5·5	3·8	2·1
Spl*+Thy	19·8	14·1	9·5
SPl*+IEL	6·8	5·9	5·1
Spl*+MLN	23·4	17·7	10·7

* Effector:target ratios shown refer to spleen:target cell ratio.

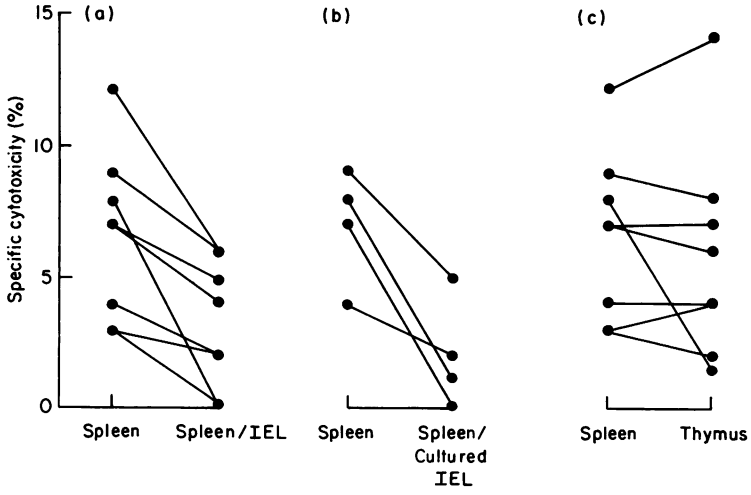

Fig. 1. Suppressor activity of lymphoid cell populations on spleen NK activity. C57Bl/10 spleen cells were mixed in equal proportion with (a) IEL; (b) IEL which had been pre-cultured for 18 h and (c) thymocytes and assayed for 4 h *vs* YAC-1 at 25:1 effector:target ratio.

action, especially at higher effector to target ratios. The suppressive effect is seen clearly in Fig. 1a where the results of eight experiments are summarized. At a spleen:target ratio of 25:1, addition of IEL was found to produce a mean suppression of 55·4%. Pre-culture of IEL for 18 h before mixing with spleen cells did not eliminate their suppressive activity (Fig. 1b; mean 79·8% suppression). Comparable suppressor activity was also found in IEL from BALB/c, C3H and NIH mice (results not shown).

Addition of thymocytes had variable effects on spleen NK activity. In some experiments, substantial suppression was observed, while in others, little or no effect was found (Fig. 1c).

Effect of prolonging the assay time on NK activity of IEL

Extension of the assay period from 4 to 18 h demonstrated enhancement of NK activity in preparations of both spleen cells and IEL, but the increases were much greater in IEL than spleen (Table 3). The enhanced activity by IEL was not altered by depletion with anti-Thy 1·2+C^1, indicating that the results did not reflect an early appearance of cytotoxic T cell activity.

In view of the suppressor activity of IEL in 4 h assays it was important to know whether the

Table 3. The effect of increasing the assay time on NK activity of IEL and of IEL induced suppression of spleen NK activity in C57Bl/10 mice. Results are the median and range % cytotoxicity obtained after 18 h culture. Numbers of experiments in parenthesis

	Effector:target ratio	
Tissue	50:1	25:1
Spleen	23·0 (7) (10–43·2)	14·5 (7) (6·4–31·6)
IEL	29·7 (8) (9·1–63·0)	10·0 (7) (1–17)
Spl/IEL	14·8 (4) (9–25·5)	14·4 (6) (7·8–30·5)
Anti-Thy IEL	37·0 (3) (20–48)	13·0 (3) (0–25)

enhanced NK activity of IEL at 18 h was due to loss of this suppression. Table 3 shows that IEL still extended marked suppression of spleen NK activity at 50:1 effector:target cell ratio but had little effect at 25:1 effector:target ratio. These results would suggest that IEL are still capable of suppressor activity over 18 h and that loss of suppression does not account for the enhanced NK activity of IEL themselves in these assays.

The effect of removing macrophages and anti-Thy sensitive cells on the suppressor effects of IEL

The proportion of macrophages contaminating an IEL preparation is not more than 1% (Davies & Parrott, 1981b). Nevertheless since it has been reported that macrophages may suppress NK activity (Cudkowicz & Hochman, 1979) we have incubated IEL preparations with Sephadex G10 before adding them to the mixture of spleen effector plus target cells. No reduction in suppression was observed (Table 4). Similar results were obtained when IEL were pretreated with carbonyl iron to remove macrophages (data not shown). Suppression of NK activity by T cells has also been reported (Osband & Parkman, 1978) and T cells are the predominant cell type in IEL. Pre-treatment of IEL with anti-Thy 1.2 and complement which killed 63% of the cells present brought an already low level of NK activity in IEL nearer to zero but addition of anti-Thy treated IEL to spleen cells did not alter the suppressor effect (Table 4).

Investigation of competition effects

The greater suppressor effect of IEL on spleen cells at higher effector:target ratio suggested that IEL might be competing with spleen cells and binding to YAC-1 targets without lysing them. This

Table 4. The effect of removing macrophages or T cells on the suppressor effect of IEL. IEL from C57Bl/10 mice were depleted of macrophages by incubation with Sephadex G10 (a) and of T cells using anti-Thy 1.2+C^1 (b). Results are percentage cytotoxicity obtained in 4 h assays using 50:1 effector:target ratio.

(a) Incubation with Sephadex G10			(b) Elimination of T cells with anti-Thy 1.2+C	
Expt	*1*	*2*		
Spleen	18·7	12	Spleen	21·7
IEL	0	0·9	IEL	1·7
Depleted IEL	nd	2·2	Anti-Thy IEL	0·5
Spleen+IEL	14·2	9	Spleen+IEL	8·2
Spleen+depl. IEL	8·7	10·7	Spleen+anti-Thy IEL	10·4

Table 5. The effect of gluteraldehyde on suppression of spleen NK activity by IEL and thymocytes

Untreated		Gluteraldehyde treated	
Cell source	% Cytotoxicity	Cell source	% Cytotoxicity
Spleen	10·0 (8–12·1)		
IEL	1·5 (0–6·0)	gIEL	0 (0–0·3)
Spleen+IEL	1·0 (0–3·4)	Spleen+gIEL	4·0 (0–6·0)
Spleen+Thy	7·5 (5·0–8·0)	Spleen+gThy	6·8 (1·5–11)

Median and range of four separate experiments using C57Bl/10 spleen cells at 25:1 effector:target ratio in gIEL and gThy treated with 1% gluteraldehyde for 20 min.

was investigated by pre-treating the IEL with gluteraldehyde which fixes cells but leaves cell membranes intact and impermeable to eosin. Pre-treatment of IEL with 1% gluteraldehyde reduced their low NK activity but did not eliminate their suppressor effect on spleen cell NK activity. Gluteraldehyde also had no effect on the suppressive effect which thymocytes had in these experiments (Table 5).

Finally, we examined whether IEL could act as competitive targets for the action of spleen NK cells, as has been reported for thymocytes (Nair *et al.*, 1981). Spleen cells were therefore incubated for 4 h in cytotoxicity assays in which ^{51}Cr labelled IEL were employed as target cells. We have been unable to detect cytotoxicity against IEL in these assays.

DISCUSSION

The results presented in this paper show clearly that intraepithelial lymphocytes from mouse small intestine are capable of natural killer activity, but unlike NK cells from spleen or lymph nodes intraepithelial NK cells show minimal lysis of YAC-1 cells in a 4 h assay. In addition, normal IEL exerted a potent suppressive action on spleen NK activity.

Our findings thus confirm earlier work describing NK activity in guinea-pig (Arnaud-Battandier *et al.*, 1978) and mouse IEL (Tagliabue *et al.*, 1981), using an overnight assay. In addition, our results are consistent with recent work which identifies NK effector cells as large, granular lymphocytes (Reynolds, Timonen & Herberman, 1981; Timonen, Ortaldo & Herberman, 1981), since 30–40% of IEL are of this type (Parrott *et al.*,1982). Previous workers using intestinal NK cells did not test for NK activity in a conventional 4 h assay and it is apparent from our study that IEL have little or no NK activity when tested in this manner. In contrast, spleen cells showed similar or decreased activity when assayed over 18 h rather than 4 h. There are several possible reasons for this discrepancy. It is conceivable that an NK 'receptor' has been modified *in vivo* and that overnight culture is required for regeneration of this structure. Any effect of the procedure used to isolate the IEL can be excluded, since treatment of spleen cells in this way did not alter their 4 h activity. Overnight culture of IEL in the absence of target cells did not enhance NK activity by these cells in a subsequent 4 h assay and it may be that prolonged contact between the IEL and their targets is required for lysis to occur. It is conceivable that the IEL are effectively trypsinized *in vivo* due to their site in the intestine and that recovery occurs during overnight culture with target cells lysis then being observed. It is known that trypsinization of spleen cells reduces their NK function due to interference with the NK target cell receptor (Roder *et al.*, 1978). After 3–4 h, NK activity of spleen cells regenerates, only to disappear again on further culture. Our failure to detect enhanced activity of IEL in the absence of target cells may thus reflect subsequent loss of the regenerated receptor. Further studies are intended to investigate the influence of intestinal enzymes on the NK function of IEL.

Prolonged contact between IEL and target cells appears essential for lysis to occur and the failure to lyse the target cell at 4 h may thus be due to a defective lytic mechanism in the IEL themselves. It may be that this is overcome by the production of stimulatory factors which is induced by the target cells themselves, (Trinchieri, Santoli & Knowles, 1977). A defect of this nature has been described in the NK deficient beige mouse strain in which NK activity can be enhanced by interferon (Beck & Henney, 1981). It remains to be substantiated whether NK lysis by the IEL can be stimulated in a similar way.

Considerable heterogeneity within NK cells has been demonstrated in the mouse (Minato, Reid & Bloom, 1981; Burton *et al.*, 1981) and it is conceivable that the intraepithelial and splenic NK cells belong to different subpopulations. Our failure to detect enhanced NK activity by IEL of nude mice and the high level of cytotoxicity at higher effector: target ratios by IEL compared with spleen cells are consistent with this hypothesis. Further support for this idea comes from recent studies which indicate phenotypic differences between intestinal and splenic NK cells (Tagliabue *et al.*, 1982). It is unclear whether these discrepancies represent the existence of two fully mature populations of NK cells and an alternative explanation for our results is that the IEL are a precursor population

requiring prolonged target cell contact for differentiation and function. Clearly further studies are required to elucidate the relationship between intestinal and peripheral NK cells.

In mice (Parrott *et al.*, 1982) and humans (Selby *et al.*, 1981) the majority of IEL carry the suppressor T lymphocyte marker and an outstanding feature of our studies was the consistent suppression which IEL exerted on spleen NK activity. Thus, we considered that this suppression could account for the lack of 4 h activity in IEL. However, the IEL were still actively suppressive in 18 h assays, despite their own high NK activity in these tests, so it is unlikely that suppression alone can account for the low 4 h activity we have observed. The suppressor effect was most marked at high cell concentrations but we do not consider that crowding effects are responsible. In support of this, similar numbers of lymph node cells did not suppress the NK activity of spleen cells, while thymocytes did so inconsistently and to a lesser extent than IEL. Although suppression of NK activity by T cells (Osband & Parkman, 1978) and macrophages (Cudkowicz & Hochman, 1979) has been described, we have not been able to attribute suppression to these mechanisms. Thus, removal of macrophages using Sephadex did not eliminate the suppressor activity of the IEL. In addition, suppressor cells in the IEL were not sensitive to anti-Thy $1.2+C^1$. Suppression was still present in gluteraldehyde treated preparations of IEL and this would indicate that the IEL were not acting by competing with spleen cells for recognition sites on the target cells. Furthermore, we could not detect lysis of labelled IEL by syngeneic spleen cells, indicating that IEL were not competing with the YAC-1 cells as targets. Similar findings have recently been reported for a suppressor subpopulation of mouse thymocytes (Nair *et al.*, 1981). These workers also found that this suppression was not H-2 restricted, a finding which we have confirmed for the IEL (Mowat & Parrott, unpublished observations). The nature of the thymic suppressor cell has not been elucidated. Finally, the suppressor activity of the IEL could be related to the granules which a proportion of IEL contain. It has been proposed that granulated IEL are related to mucosal mast cells (Guy-Grand, Griscelli & Vassalli, 1978) and histamine released by mast cells is a known inhibitor of cellular immune functions (Rocklin, 1978). It is therefore conceivable that intestinal NK activity may be locally modulated by secretory products released by IEL. Future experiments are designed to investigate this possibility.

This study lends support to the concept that the IEL are a heterogenous population of cells, with a range of morphology (Ferguson, 1977) and surface phenotype (Parrott *et al.*, 1982). Since the intestinal epithelium is in intimate contact with a large amount of antigen, it is not surprising that several types of lymphoid effector cell are present in this site. We have now identified both cytotoxic T cell (Davies & Parrott, 1981a) and NK cell activity among the IEL and would postulate that these cells may play an important role in mucosal defence against intracellular organisms such as viruses or parasites. In addition, our results suggest that immune regulatory mechanisms may also exist concurrently within the epithelial layer.

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