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The nature of the natural killer (NK) cell of human intestinal mucosa and mesenteric lymph node

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SUMMARY

The relationship of the mononuclear cell (MNC) from human intestinal mucosa and mesenteric lymph node mediating anti-K-562 activity with that of peripheral blood has been assessed. Depletion of macrophages did not alter the measured cytotoxicity confirming that the effector cells were lymphocytes. Complement lysis of Leu 7 and Leu 11b coated cells reduced intestinal natural killer (NK) activity by a similar degree to that of peripheral blood but mesenteric lymph node NK activity was affected to a lesser extent. The response in NK activity of mucosal and nodal MNC to short incubation with lymphoblastoid interferon was similar to that for peripheral blood MNC. Twenty-four hours incubation of MNC with low concentrations of purified interleukin-2 (IL-2) consistently augmented intestinal and nodal NK activity but failed to augment that of peripheral blood MNC. No differences between the inhibitory effects of cAMP and prostaglandin E_2 on NK activity from the three sites were seen. In addition, inhibition of cyclo-oxygenase activity with indomethacin had no effect on NK activity of intestinal and peripheral blood MNC while the lipoxygenase inhibitor, nordihydroguaiaretic acid, suppressed intestinal and peripheral blood NK activity similarly. In conclusion, anti-K-562 activity by intestinal MNC is mediated by NK cells with similar phenotypic and functional properties to those of peripheral blood. However, the increased sensitivity of mucosal NK cells to IL-2 suggests that higher proportions of NK cell precursors may be present in intestinal MNC populations.

Keywords natural killer cells intestinal mucosa mesenteric lymph node interferon interleukin-2

INTRODUCTION

The pheotypic and functional characteristics of NK cells of human intestinal mucosa are largely unknown whereas those from peripheral blood have been extensively studied. Evidence has been recently presented that the subpopulation of murine intraepithelial lymphocytes exhibiting natural cytotoxicity demonstrates different phenotypic (Tagliabue *et al.*, 1982) and functional (Mowat *et al.*, 1983) properties to those of autologous splenic natural killer (NK) cells. On the other hand, rat intraepithelial NK cells appear to exhibit the same target specificity as do splenic NK cells (Flexman, Shellam & Mayrhofer, 1983; Nauss *et al.*, 1984) although the responses to interferon stimulation differ (Flexman *et al.*, 1983). Similar studies of mucosal MNC devoid of intraepithelial lymphocytes have not been reported.

The aim of this study was to define the relationship of mucosal and mesenteric lymph node NK cells to those of peripheral blood in man and this was performed by: (a) investigating the phenotype

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of the mucosal and mesenteric lymph node NK cell with respect to its lymphocyte nature and surface antigenic expression; (b) determining the response of the mucosal and lymph node NK cells to soluble modulating factors and (c) comparing the above findings with those of peripheral blood NK cells.

MATERIALS AND METHODS

Patients and specimens. Intestinal mucosa and mesenteric lymph nodes were obtained from patients undergoing surgical resection of intestine for inflammatory bowel disease, colorectal carcinoma or miscellaneous intestinal diseases. In patients with carcinoma, the specimen was taken at least 10 cm from tumour. Adjacent tissue was examined histologically as was half of each lymph node studied from patients with carcinoma.

Prior to or immediately following induction of general anaesthesia, 10–40 ml of peripheral blood was obtained in every patient. Peripheral blood was also obtained from healthy subjects.

Isolation of MNC populations. Peripheral blood MNC were isolated by the method of Böyum (1968) as previously described (Gibson *et al.*, 1985).

The intestinal specimens were opened immediately following their resection and washed with tap water to remove faecal and other contents. A segment of full thickness intestinal wall was dissected and placed immediately in sterile Hank's balanced salt solution free of calcium and magnesium containing penicillin 100 units/ml and streptomycin 100 μg/ml (HBSS-CMF). Intestinal MNC were then isolated by a method modified from that of Bull & Bookman (1977) as previously described (Gibson *et al.*, 1985). Briefly, intestinal mucosa was dissected from the muscularis and treated with EDTA to remove epithelial cells and mucus and then digested overnight in complete medium (RPMI 1640 with 10% fetal calf serum, 25 mM HEPES, 2 mM L-glutamine, and antibiotics as above) containing 25 units/ml collagenase (CLSPA, Worthington). The resulting cell population was then purified over a Ficoll-Paque gradient (Pharmacia).

Lymph nodes were dissected from the mesentery of the resection specimens where possible and placed into sterile HBSS-CMF. Following dissection of the lymph nodes from surrounding fat, they were cut into pieces and gently pushed through a sterile stainless steel mesh to release the MNC. The resulting cell population was washed once with HBSS-CMF. No further purification step was used.

All cell populations were suspended in complete medium and counted using a haemocytometer (Neubauer chamber). Viability was assessed by 0·1% trypan blue exclusion. Half to two million cells were isolated per 10 ml of peripheral blood and viability was always greater than 95%. Two to thirty-five million MNC were isolated per gram of intestinal mucosa. The viability of intestinal MNC was 80–95% while that of mesenteric lymph node MNC was 70–90%.

NK assay. Intestinal MNC were cultured for 24 h in complete medium to reverse the inhibitory effects of the isolation process (Gibson, Hermanowicz & Jewell, 1984b) prior to functional assay. Peripheral blood and mesenteric lymph node MNC were tested either fresh or following 24 h incubation in complete medium. In previous studies, 24 h incubation of peripheral blood MNC had little influence overall on NK activity (Gibson *et al.*, 1984b).

NK activity was assessed as previously described (Gibson *et al.*, 1985) using ^{51}Cr-labelled K-562 cells as target. The effector to target (E:T) ratio for peripheral blood MNC was 50:1 and for mesenteric lymph node and intestinal MNC 500:1 (Gibson *et al.*, 1984a). Incubation time was 4 h at 37°C and 5% CO_2. The average of triplicate experimental release values (E) and sextuplicate spontaneous (S) and maximal (M) values were calculated and the cytotoxicity determined by the following formula:

$$\% \text{ lysis} = (E - S)/(M - S) \times 100.$$

Spontaneous release was 5–12% of maximal release. Cytotoxicity was considered to be significant if it were greater than 2% (Gibson *et al.*, 1984a).

Macrophage depletion. Intestinal macrophages are reported to be adherent to plastic (Golder & Doe, 1983; Beeken, St Andre-Ukena & Gundel, 1983) and to glass (Bull & Bookman, 1977). In initial experiments, very few intestinal MNC were found to stick to plastic. Thus, adherent cells

were removed by incubating intestinal and lymph node MNC in complete medium in 16 mm diameter tissue culture treated plastic wells containing 15 mm round glass coverslips. After 2 h culture at 37°C 5% CO_2, the medium was removed following vigorous pipetting and the wells washed twice with HBSS-CMF. The non-adherent population was then washed and resuspended at the desired concentration for morphological and functional assessment. Air dried smears were stained with May–Grünwald–Giemsa or for non-specific esterase using α-naphthyl acetate as substrate (Li, Yam & Crosby, 1972). In addition, cell suspensions were fixed in 4% glutaraldehyde and pellets were formed by centrifugation. Thin (0·75 μm) sections were stained with azure A in 1% borax. A reduction of intestinal macrophages from 8–15% to 3–4% and of lymph node macrophages from 8–12% to less than 2% was found.

To remove cells capable of phagocytosis, intestinal and lymph node MNC were suspended in 15 ml of complete medium to which was added carbonyl iron (Koch-Light). The cells were incubated at 37°C for 30 min with intermittent mixing. They were then passed over a magnet, the cells washed, and the magnet treatment repeated. The resultant cell population contained $\leqslant 1\%$ macrophages.

Complement lysis of antibody coated cells. Three to five million peripheral blood MNC or $8–15 \times 10^6$ intestinal or mesenteric lymph node MNC were incubated for 30 min at 4°C in 100 μl of complete medium containing 1 in 10 anti-Leu 7, anti-Leu 11b (Becton-Dickinson), or no antibody. The cells were then incubated in complete medium containing 1 in 2 concentration of guinea pig serum at 37°C. Freshly prepared guinea pig serum was immediately frozen and stored in small aliquots at −70°C and thawed shortly before use. In prior experiments the serum was minimally toxic to MNC populations *per se*. Following 60 min incubation, the cells were washed twice in HBSS-CMF, counted, resuspended in complete medium. The control population was diluted such that the E:T ratio in the NK assay was 50:1 for peripheral blood and 500:1 for intestinal and lymph node MNC. The antibody treated cell populations were resuspended in the same volume as the control cells prior to testing their NK activity.

Soluble modulating factors. Lymphoblastoid inerferon was kindly donated by the Wellcome Research Laboratories. It was diluted in complete medium and stored in small aliquots at −70°C. Repeated freezing and thawing was avoided. Highly purified human interleukin-2 (IL-2) was purchased from Genzyme (Koch-Light) and contained no interferon activity. It was stored at −20°C at which it is stable for 6 months (Genzyme information). cAMP (adenosine 3-5-cyclic monophosphate) and cGMP (guanosine 3-5-cyclic monophosphate) were purchased from Sigma Chemicals, suspended in complete medium at a concentration of 10^{-4} M and stored in small aliquots at −20°C. PGE_2 1 mg/ml in ethanol (Upjohn) was diluted to 10^{-4} M in complete medium and stored in small aliquots at −20°C. Nordihydroguaiaretic acid (NDGA, Sigma Chemicals) and indomethacin (Merck, Sharpe & Dohme) were dissolved in complete medium and stored at −20°C in aliquots at a concentration of 500 μM and 100 μM, respectively.

Assay of the effects of modulating factors. Interferon was added to MNC at a concentration of 1,000 units/ml in triplicate or quadruplicate microtitre wells (total volume 150 μl) and incubated at 37°C 5% CO_2 for 1·5 h. Target cells were then added to assess NK activity.

MNC were incubated in 100 units/ml of IL-2 in complete medium at a concentration of 4×10^6 MNC/ml in 16 mm flat bottomed tissue culture wells for 24 h. The cells were then washed, counted, and resuspended in complete medium and tested for NK activity.

cAMP, cGMP, NDGA, and indomethacin were added to triplicate or quadruplicate wells of microtitre plates containing MNC just prior to the addition of labelled target cells.

RESULTS

Effect of macrophage depletion on NK activity

Depletion of glass adherent cells from intestinal and lymph node MNC populations did not change NK activity (Table 1). Removal of phagocytosing cells with carbonyl iron and a magnet reduced the proportion of macrophages to 1% or less. This likewise did not change the NK activity of intestinal or lymph node MNC whether unstimulated or interferon stimulated (Table 1).

Table 1. The effect of depleting glass adherent and iron phagocytosing cells from intestinal MNC on NK activity

Diagnosis	Control	Non-adherent	Non-phagocytic
Intestinal MNC			
Carcinoma	6	6	—
Carcinoma	9	13	—
UC	2	2	—
CD	3	3	—
Carcinoma	8 (24)*	—	8 (24)
UC	1 (3)	—	0 (4)
CD	2 (14)	—	3 (14)
Carcinoma	1 (14)	—	6 (14)
Carcinoma	3 (9)	—	3 (8)
Mesenteric lymph node MNC			
CD	18	33	—
RSL	1 (8)	0 (7)	—
Caecal volvulus	13	—	17
UC	35 (44)	—	44 (52)

* Interferon stimulated NK activity in parentheses.
RSL = redundant sigmoid loop; UC = ulcerative colitis; CD = Crohn's disease.

Effect of complement lysis of antibody coated cells

Complement lysis of Leu 7^+ and Leu $11b^+$ cells reduced the NK activity of peripheral blood MNC by $66 \pm 10\%$ and $70 \pm 12\%$ respectively in six studies. Similar treatment of intestinal MNC reduced NK activity by $70 \pm 5\%$ for both antibodies in three studies in patients with colorectal carcinoma, Crohn's disease, or ulcerative colitis. In contrast, depletion of Leu 7^+ cells from lymph node MNC decreased NK activity in two patients with colonic carcinoma and Crohn's disease by 50% and 58%

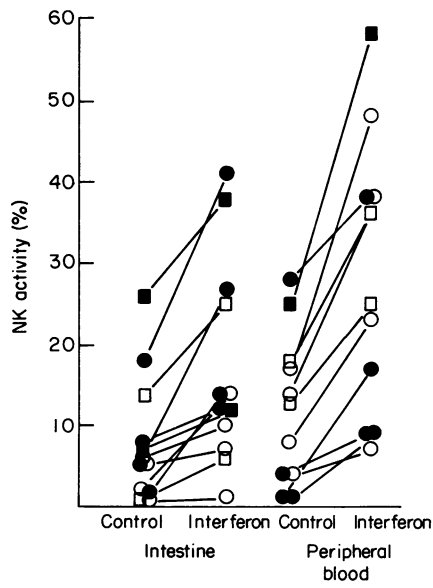

Fig. 1. The response of NK activity of intestinal and autologous peripheral blood MNC to 1·5 h pre-incubation in 1,000 units/ml interferon. Cells were isolated from patients with colorectal carcinoma (○), miscellaneous intestinal diseases (□), Crohn's disease (●) or ulcerative colitis (■).

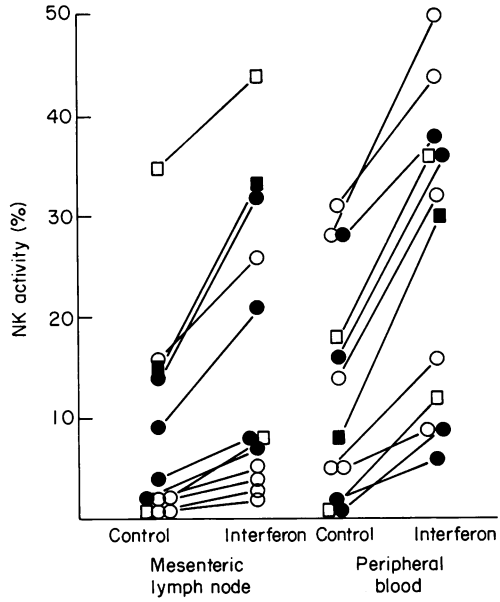

Fig. 2. The response of NK activity of mesenteric lymph node and autologous peripheral blood MNC to 1·5 h pre-incubation in 1,000 units/ml interferon. Cells were isolated from patients with colorectal carcinoma (○), miscellaneous intestinal diseases (□), Crohn's disease (●) or ulcerative colitis (■).

and the depletion of Leu 11b$^+$ cells by 40% and 43% whereas, in a further patient with cancer, NK activity was reduced by less than 10% for both antibodies.

Effect of soluble factors on NK activity

One and a half hours of pre-incubation of peripheral blood, intestinal, and mesenteric lymph node MNC in interferon (1,000 units/ml) increased NK activity in most cell populations tested. The results for intestinal and autologous peripheral blood MNC are presented in Fig. 1 and for

Table 2. The effect of 24 h pre-incubation with IL-2 (100 units/ml) on NK activity of MNC isolated from intestinal mucosa, mesenteric lymph nodes and peripheral blood

	Peripheral blood MNC		Intestinal MNC		Mesenteric lymph node MNC	
Diagnosis	control	IL-2	control	IL-2	control	IL-2
Crohn's disease	5	5	5	10	3	9
Megacolon	—	—	1	10	—	—
Caecal volvulus	38	35	11	31	47	55
Redundant sigmoid loop	—	—	0	3	0	0
Carcinoma	53	60	21	29	2	5
Ulcerative colitis	4	4	—	—	16	27
Normal	22	22	—	—	—	—
Normal	19	25	—	—	—	—
Normal	35	34	—	—	—	—

Table 3. The effect of PGE_2 and cAMP on unstimulated and interferon stimulated NK activity of MNC isolated from intestinal mucosa, mesenteric lymph nodes and peripheral blood

	Unstimulated			Interferon stimulated		
Diagnosis	Control	PGE_2	cAMP	Control	PGE_2	cAMP
Intestinal MNC						
Ulcerative colitis	7	4	1	12	10	7
Ulcerative colitis	26	13	2	38	25	5
Carcinoma	6	1	0	10	5	0
Mesenteric lymph node MNC						
Carcinoma	1	1	0	6	3	0
Ulcerative colitis	15	13	0	33	26	3
Peripheral blood MNC						
Normal	32	17	2	54	44	4
Normal	23	12	0	43	25	4
Normal	28	14	1	51	46	3

mesenteric lymph node and autologous peripheral blood MNC in Fig. 2. The degree of enhancement of NK activity was similar for MNC from the three sites.

Twenty-four hours of pre-incubation of intestinal and lymph node cells in IL-2 also increased NK activity in all but one population tested (Table 2). However, autologous peripheral blood MNC and normal peripheral blood MNC preincubated under the same conditions did not demonstrate enhanced activity except in two experiments in which the relative increase was far lower in magnitude than that for intestinal MNC.

cAMP and PGE_2 have been well documented to inhibit NK activity of peripheral blood MNC and cGMP to have no or a slight stimulating effect (Roder & Klein, 1979; Brunda, Herberman & Holden, 1980; Lang *et al.*, 1982; Hall *et al.*, 1983). Concentrations of these agents known to inhibit the NK activity of peripheral blood MNC were assessed for their effect on NK activity of intestinal and mesenteric lymph node MNC (Table 3). The effect of cGMP (10^{-3}M) on intestinal MNC was assessed in two studies and little influence was observed on unstimulated (7% *vs* 6%; 26% *vs* 29%) or interferon stimulated NK activity (12% *vs* 10%, 38% *vs* 36%). cAMP (10^{-3}M) had a potent inhibitory effect on both stimulated and unstimulated NK activity of peripheral blood, intestinal, and nodal NK activity. PGE_2 also inhibited NK activity but, at the concentration used (10^{-6}M), not as strongly as cAMP especially for interferon pre-treated cells. As PGE_2 was dissolved in ethanol, MNC were also tested against 0·03% ethanol in complete medium and no effect on NK activity was seen for any cell population.

Indomethacin, at concentrations capable of inhibiting cyclo-oxygenase activity (1–10 μM) did not affect the NK activity of intestinal MNC ($n=2$). At a concentration of 40 μM, the lipoxygenase inhibitor, NDGA, suppressed intestinal NK activity by 38 and 69% in two studies while NK lysis by peripheral blood MNC was suppressed by $52 \pm 10\%$ in four studies.

DISCUSSION

Human intestinal mucosal and mesenteric lymph node MNC are capable of exerting cell-mediated cytotoxicity of K-562 target cells. The spontaneous cytotoxicity is not exerted by adherent or by phagocytic cells and is almost certainly lymphocyte-mediated. Methods to remove macrophages from MNC populations are not absolute in their effectiveness particularly with adherence of intestinal MNC. Nevertheless, in no studies was cytotoxicity decreased but, in some, was increased

as might be expected by the relative enrichment of NK cells with the removal of mononuclear phagocytes (Koren *et al.*, 1981). Thus, the cytotoxicity observed by intestinal and mesenteric lymph node MNC is truly 'NK' activity and the effector cells truly 'NK' cells.

In studies of MNC isolated from murine intestinal lamina propria and epithelium, Tagliabue *et al.* (1981, 1982) found a different spectrum of surface antigenic expression of NK cells in the epithelium compared with splenic NK cells. In this study, the effect of complement lysis of cells coated with antibodies recognizing the subpopulations of peripheral blood MNC in which most NK activity resides was assessed. NK activity of intestinal MNC was decreased to a similar degree as for peripheral blood MNC but, for MNC from lymph nodes, lysis of Leu 7^+ and Leu $11b^+$ cells reduced NK activity to a lesser degree. Thus, these preliminary experiments suggest that mucosal NK cells to be phenotypically similar to those of peripheral blood whereas those from mesenteric lymph nodes may have slightly different surface antigenic expression. This is in accord with other studies in which differences between the phenotype of peripheral blood and peripheral lymph node NK cells were reported (Eremin, Ashby & Stephens, 1978a; Eremin *et al.*, 1978b).

Peripheral blood NK cells are subject to a large number of modulating influences (Roder, Karre & Kiessling, 1981). Two major factors which stimulate NK activity are interferon (Ortaldo, Herberman & Pestka, 1981) and IL-2 (Domzig, Stadler & Herberman, 1983). Most MNC populations from intestinal mucosa and mesenteric lymph nodes exhibited enhanced NK activity following treatment of the cells with interferon and the magnitude of this increase was similar to that of peripheral blood MNC. Interferon was also recently reported by other workers to stimulate mucosal NK activity (Targan *et al.*, 1983) but Flexman *et al.* (1983) found poor response of rat intraepithelial NK cells to interferon.

Recently, other groups have found that IL-2 enhances intestinal NK activity (Hogan *et al.*, 1983; Fiocchi & Youngman, 1984). In this study, MNC from intestinal mucosa and mesenteric lymph nodes also demonstrated increased NK activity when pre-incubated with low concentrations of IL-2 for 24 h. In contrast, the same concentration of IL-2 had a variable effect on NK activity of peripheral blood MNC populations and, when enhanced activity was observed, it was much less than the relative increase seen for intestinal MNC. It has been suggested that IL-2 activates 'pre-NK' cells as well as augmenting the activity of NK cells (Domzig *et al.*, 1983). Miyasaka *et al.* (1984) have shown that IL-2 increases the proportion of Leu 7^+ cells in peripheral blood MNC and that these, at least in part, generate from the Leu 7^- cell population. The enhanced sensitivity of intestinal MNC to IL-2 compared with peripheral blood MNC may relate to a higher relative proportion of NK cell precursors in the mucosal cell population. Alternatively, intestinal NK cells may have much less *in vivo* exposure to IL-2 than do peripheral blood NK cells and, thus, respond more vigorously to *in vitro* stimulation. However, intestinal MNC are capable of producing IL-2 *in vitro* although this may be depressed in inflammatory bowel disease (Fiocchi *et al.*, 1984) and in colon cancer (Hogan *et al.*, 1983). Most of the patients whose intestinal MNC were studied had neither of these diseases.

Of the many factors which may inhibit NK activity of peripheral blood MNC, cAMP and PGE_2 are probably of major physiological *in vivo* importance in the minute-to-minute modulation of NK cell function—they are present in most cells and tissues, their effects are potent and of rapid onset and offset, and their synthesis, secretion and tissue or intracellular concentrations are under fine control. Intestinal and mesenteric lymph node cells exhibit similar inhibitory responses to cAMP and PGE_2 as previously reported for peripheral blood MNC (Roder & Klein, 1979; Braunda *et al.*, 1980; Lang *et al.*, 1982; Hall *et al.*, 1983). In addition, the resistance of MNC to PGE_2 inhibition conferred by interferon stimulation reported for peripheral blood MNC (Leung & Koren, 1982) also occurs for mucosal NK cells.

The recent demonstration that lipoxygenase inhibitors potently suppress peripheral blood NK cell activity (Seaman, 1983; Suthanthiran *et al.*, 1984) has suggested that arachidonic acid metabolism via the lipoxygenase pathway may also play an important part of the lytic process of the NK cells, perhaps via the supply of hydroxyl radicals (Suthanthiran *et al.*, 1984). Similar potent inhibition of intestinal and mesenteric lymph node NK activity was observed when a lipoxygenase inhibitor (NDGA) was added to the NK assay. As for peripheral blood NK cells (Roder & Klein, 1979; Hall *et al.*, 1983; Suthanthiran *et al.*, 1984), the cyclooxygenase pathway does not appear to be

important for NK cell cytotoxicity as indomethacin had no effect on NK activity when used at concentrations known to inhibit cyclo-oxygenase activity.

The conclusions reached in this study are as follows. (1) Spontaneous cell-mediated cytotoxicity of K-562 cells by intestinal and mesenteric lymph node MNC populations is mediated by lymphocytes. Thus, the effector cells are, by definition, NK cells. (2) Intestinal NK cells have a similar phenotype to those of peripheral blood whereas mesenteric lymph node NK cells exhibit some differences in surface antigen expression. (3) The pattern of response to stimulating and inhibiting soluble factors of intestinal and mesenteric lymph node NK cells is similar to those of peripheral blood except for an enhanced sensitivity to IL-2 of intestinal and nodal MNC suggesting the presence of higher proportions of NK cell precursors in those populations.

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