Clin. exp. Immunol. (1984) **56,** 445–452

The mononuclear cells of human mesenteric blood, intestinal mucosa and mesenteric lymph nodes: compartmentalization of NK cells

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(Accepted for publication 6 January 1984)

SUMMARY

The proportions of T cell subsets and Leu 7^+ cells and the spontaneous cell-mediated cytotoxicity (SCMC) of isolated mononuclear cells have been determined across the mesenteric vascular bed and along the intestinal mucosal–mesenteric lymph node (MLN) axis in patients undergoing abdominal surgery. Whereas the proportion of T_4^+ and T_8^+ cells were similar in simultaneously taken PVB and mesenteric venous blood (MVB), the proportion of Leu 7^+ cells was higher in MVB in 16 of 17 studies ($15{\cdot}4 \pm 6{\cdot}8\%$, $10{\cdot}8 \pm 5{\cdot}1\%$). Additional studies showed that the proportions of lymphocyte subsets in peripheral arterial blood are the same as those in PVB. Thus, an enrichment of Leu 7^+ cells occurs across the mesenteric vascular bed. Isolated intestinal and MLN mononuclear cells contained similarly high proportions of T_4^+ and T_8^+ cells as in PVB but Leu 7^+ cells made up a minority subpopulation in intestinal ($1{\cdot}3 \pm 0{\cdot}8\%$) and MLN mononuclear cells ($1{\cdot}0 \pm 0{\cdot}9\%$). The SCMC of intestinal and MLN mononuclear cells was low and paralleled the proportion of Leu 7^+ cells. Despite the higher proportions of Leu 7^+ cells in MVB, the SCMC was less than that of PVB in eight patients with inflamed intestine and not significantly different from PVB in seven patients with normal intestines. These paradoxical findings were at least in part due to inhibitory factors in mesenteric plasma. In conclusion, NK cells appear to be largely confined within the vascular system and the enrichment of Leu 7^+ cells across the mesenteric vascular bed suggests that this compartmentalization may be due to differences in the traffic of lymphocyte subpopulations through the intestinal mucosa and MLN.

Keywords mononuclear cell subsets natural killer cells intestinal mucosa mesenteric lymph node mesenteric blood

INTRODUCTION

The development of monoclonal antibodies (MoAbs) which recognize subsets of human mononuclear cells with functional specificities has led to their wide application to the study of immune mechanisms at the tissue level as well as in peripheral blood. Recently, the mononuclear cell subpopulations of intestinal mucosa have been defined. In tissue sections, the proportion of helper

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to suppressor/cytotoxic T cells in the intestinal lamina propria is similar to that of peripheral blood (Selby & Jewell, 1983; Selby *et al.*, 1984). When mononuclear cells are isolated from the intestine, there is a small change in this ratio probably due to depletion of suppressor/cytotoxic cells (Selby *et al.*, 1984). Cells with the NK–K cell phenotype (Leu 7^+) are present in less than 3% of mononuclear cells isolated from intestinal mucosa whereas they comprise 10–20% of those from peripheral blood (Gibson *et al.*, 1984). Likewise, the spontaneous cell-mediated cytotoxicity (SCMC) of intestinal mononuclear cells is minimal when tested under similar assay conditions to peripheral blood MNC but is readily measurable when appropriate adjustments for the likely low proportion of NK cells is made (Gibson *et al.*, 1984).

The reason for the paucity of Leu 7^+ cells in the intestinal mucosa is not known but may relate to differences in the traffic patterns of various lymphocyte subpopulations through the gut. B and T cells are well documented to recirculate through gut associated lymphoid tissue and mesenteric lymph nodes (MLN) to the systemic circulation via thoracic duct at a rapid rate (Gowans, 1959; Gowans & Knight, 1964). As lymphocyte recirculation experiments are not possible in humans, the aim of this study was to assess indirectly the traffic of NK cells by measuring the proportion of Leu 7^+ cells and SCMC of mononuclear cells isolated from the intestinal mucosa, the mesenteric vascular bed, and the mesenteric lymph nodes.

MATERIALS AND METHODS

Patients and specimens. Different groups of patients were used for different parts of the study.

Experiment 1: lymphocyte subpopulations in peripheral and mesenteric venous blood. Five to ten millilitre specimens of blood were taken simultaneously from a peripheral vein (PVB) and from a tributary of the inferior or superior mesenteric vein (MVB) in 20 patients undergoing abdominal surgery. In 10 patients in whom a part of the intestine was inflamed due to Crohn's disease (seven) or ulcerative colitis (three), MVB was withdrawn from a vein draining a diseased segment. The other 10 patients were undergoing surgery for gastrointestinal malignancy (seven), peptic ulceration (two) and cholelithiasis (one).

Experiment 2: lymphocyte subpopulations in peripheral arterial and venous blood. In a further 13 patients, simultaneous 5–10 ml samples of peripheral arterial blood (PAB) and PVB were drawn from the same arm. Except in one volunteer, arterial lines were *in situ* as part of the management of the underlying disease. In eight patients, it followed major surgery, four had respiratory disease and others suffered from multiple injuries (1), self-poisoning (1) and diabetic complications (1).

Experiment 3: lymphocyte subpopulations of lamina propria and mesenteric lymph nodes. For mononuclear cells isolated from intestinal mucosa and MLN, T cell subsets were assessed in seven patients with Crohn's disease, four with ulcerative colitis, five with colorectal carcinoma and three patients who had non-malignant, non-inflammatory bowel disease. For NK phenotypic and functional studies, a subsequent group of patients was examined, comprising nine with Crohn's disease, four with ulcerative colitis, four with colorectal carcinoma and one with peptic ulceration of the colon.

In patients with colorectal carcinoma, the specimen of mucosa was taken distant from the tumour and was histologically normal whilst half of each MLN studied was histologically examined to ensure they were also free of tumour. Patients with Crohn's disease and ulcerative colitis in all of the above groups had active disease and were receiving high doses of prednisolone (20–64 mg) at the time of surgery.

Isolation of mononuclear cells. Blood specimens were immediately heparinized and processed within 2 h. Plasma was removed prior to mononuclear cells isolation in those specimens from which the plasma was also studied. Blood was diluted to two to three times its original volume with Hank's balanced salt solution free of calcium and magnesium (HBSS-CMF) and carefully layered onto a discontinuous Ficoll-Paque (Pharmacia) gradient (Böyum, 1968). Following centrifugation for 30 min at 400*g*, the mononuclear cells were harvested, washed twice with HBSS-CMF and resuspended in complete medium (RPMI-1640 with 10% fetal calf serum, 25 mM HEPES, L-glutamine, 100 μg/ml streptomycin, and 100 units/ml penicillin) until studied.

Intestinal mononuclear cells were isolated by a method modified from Bull & Bookman (1977). Within one hour of resection, the intestinal mucosa was dissected and minced into approximately 3 × 3 mm pieces. Six to eight grams of these were incubated with stirring in HBSS-CMF containing 0·75 M EDTA and 10 mM HEPES for 45 min at 37°C. This was repeated every 30 min until no more epithelial cells were seen in the supernatant. After washing two or three times with HBSS-CMF, the remaining tissue was incubated with 75–100 ml of complete medium containing 25 units/ml collagenase (CLSPA, Worthington) overnight at 37°C with stirring. After allowing most of the debris to settle, the supernatant was aspirated, centrifuged, and the resulting pellet of cells and debris then washed twice in HBSS-CMF. The mononuclear cells were isolated from this crude preparation by centrifugation on a Ficoll-Paque gradient as for blood.

Following the dissection of MLN from surrounding fat, they were cut into small pieces and gently pushed through a sterile stainless steel mesh to release the mononuclear cells. The resulting cell population was washed in HBSS-CMF and then resuspended in complete medium. No further purification step was used.

All cell preparations were counted using a haemocytometer (Neubauer chamber) and their viability checked with trypan blue (0·1%) exclusion. The viability of blood mononuclear cells was always greater than 95% and the intestinal cells greater than 85% whilst the viability of the MLN cells ranged between 80 and 90%.

Antibodies. For studies of intestinal and MLN T cell subsets, OKT4 and OKT8 (Ortho) were used whereas Leu 3a and Leu 2a (Becton Dickinson) were used for blood studies. As these two groups of antibodies appear to measure equivalent populations (Reinherz & Schlossman, 1980; Engelman *et al.*, 1981), they will be referred to in the remainder of the text as T4 and T8. The IgM MoAb, anti-Leu 7 (Becton Dickinson), was used to determine proportions of cells with the NK–K phenotype (Abo & Balch, 1981). As a control, antibodies to thymocytes (IgG) and to granulocytes (IgM) were used in place of the above first layer antibodies. Second layer antbodies were goat anti-mouse IgG conjugated with fluorescein isothiocyanate and goat anti-mouse IgM conjugated with tetramethyl rhodamine isothiocyanate (Nordic).

Immunofluorescence. Indirect immunofluorescence techniques were used as previously described (Selby & Jewell, 1983). Briefly, 1×10^6 mononuclear cells were washed in phosphate-buffered saline containing 0·1% sodium azide (PBS/A) then resuspended in 20 μl of PBS/A and incubated for 10 min at room temperature with 2 μl of the first layer antibody. Following washing twice with PBS/A, the cells were incubated with the second layer antibody similarly to the first layer at a concentration of 1 in 10. Following washing twice, the cells were fixed in 10% formalin and placed on glass slides in 90% glycerol 10% PBS. A coverslip was placed over the cells and sealed with nail varnish. The cell suspensions were examined by phase and fluorescent microscopy. At least 200 cells were counted except when a minority population was being examined at which time more than 400 cells were counted. The proportion of positive cells was expressed as a percentage of the total mononuclear cell count.

Cytotoxicity assay. K-562 cells were used as target cells; $0{\cdot}5–1 \times 10^6$ K-562 cells were incubated in 100 μl of fetal calf serum and 100 μCi of sodium ^{51}chromate for 1 h at 37°C. Following washing three times in complete medium, the cells were resuspended at a concentration of 1×10^5 cells/ml. To U bottomed wells of 96 well microtitre plates, 50 μl of labelled target cells were added to varying numbers of effector cells according to the effector:target (E:T) ratio being tested. Generally, blood mononuclear cells were tested at an E:T ratio of 50:1 and intestinal and MLN cells at 50:1 and 500:1. Each well was made up to a final volume of 200 μl with complete medium. Maximal release was assessed by lysing target cells with Triton X-100 in complete medium and spontaneous release determined by incubating labelled K-562 cells in 200 μl of complete medium alone. After 4 h incubation at 37°C and 5% CO_2, 100 μl of cell free supernatant was carefully aspirated and counted in a gamma counter. The average of triplicate or quadruplicate wells was taken and the cytotoxicity determined by the following formula: $\%\text{lysis} = E - S/M - S \times 100$, where E = experimental release, S = spontaneous release, and M = maximal release. Spontaneous release was 5–12% of maximal release.

Statistics. Paired and unpaired data were statistically compared using Student's *t*-test. Data have been expressed as mean ± standard deviation.

RESULTS

Distribution of lymphocyte subsets

Data on Leu 7^+ cells were obtained from 17 of the 20 patients (in the remaining three patients, methodological difficulties prevented adequate cell enumeration). Of these 17 patients, 16 showed a higher proportion of Leu 7^+ cells in MVB compared with PVB (Table 1 & Fig. 1). This difference was independent of the underlying disease process. Sufficient cells were available from 11 of the 20 patients to quantitate the $T4^+$ and $T8^+$ cells which were found in similar proportions in MVB and PVB (Table 1).

To determine whether lymphocyte proportions in PVB reflect those of mesenteric arterial blood (MAB), simultaneous PVB and PAB were taken from patients in whom an arterial line was *in situ* as part of the management of their underlying disease. No difference in $T4^+$ ($33{\cdot}7 \pm 6{\cdot}5\%$, $33{\cdot}6 \pm 7{\cdot}2\%$, $T8^+$ ($21{\cdot}3 \pm 4{\cdot}7\%$, $22{\cdot}0 \pm 5{\cdot}8\%$), or Leu 7^+ cell proportions ($7{\cdot}9 \pm 5{\cdot}5\%$, $7{\cdot}6 \pm 5{\cdot}6\%$) were found in PVB and PAB, respectively.

High proportions of $T4^+$ and $T8^+$ cells were found in isolated intestinal mononuclear cells ($44{\cdot}2 \pm 8{\cdot}3\%$, $17{\cdot}8 \pm 5{\cdot}3\%$) and in autologous MLN cells ($57{\cdot}5 \pm 6{\cdot}1\%$, $13{\cdot}1 \pm 5\%$) (Table 2). In contrast, Leu 7^+ cells comprised a minority subpopulation of intestinal ($1{\cdot}3 \pm 0{\cdot}8\%$) and MLN mononuclear cells ($1{\cdot}0 \pm 0{\cdot}9\%$). Again, no disease related differences were apparent (data not shown).

Table 1. The proportions of Leu $3a^+$, Leu $2a^+$ and Leu 7^+ cells in mononuclear cells isolated from simultaneously taken peripheral venous and mesenteric venous bloods

	%LEU $3a^+$ ($n=11$)	%LEU $2a^+$ ($n=11$)	%LEU 7^+ ($n=17$)
Peripheral venous blood	$31{\cdot}5 \pm 7{\cdot}5$*	$24{\cdot}1 \pm 9{\cdot}3$	$10{\cdot}8 \pm 5{\cdot}1$
Mesenteric venous blood	$30{\cdot}1 \pm 8{\cdot}2$	$22{\cdot}0 \pm 11{\cdot}5$	$15{\cdot}4 \pm 6{\cdot}8$

* Mean ± standard deviation.

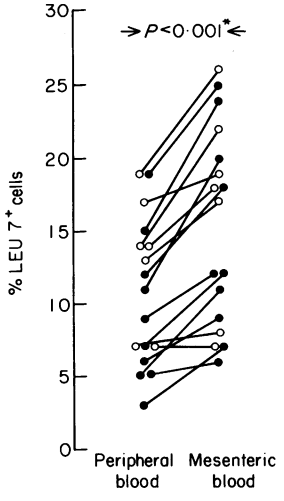

Fig. 1. Comparison of Leu 7^+ cell proportions in mononuclear cells from simultaneously obtained peripheral and mesenteric venous bloods from patients with inflamed intestine (●——●) and non-inflamed intestine (○——○). * Paired *t*-test.

Table 2. The proportion of OKT4$^+$, OKT8$^+$ and Leu 7$^+$ cells in mononuclear cells isolated from intestinal mucosa and mesenteric lymph nodes

	%OKT4$^+$ ($n=19$)	%OKT8$^+$ ($n=19$)	%LEU 7$^+$ ($n=18$)
Intestinal mucosa	44·2±8·3*	17·8±5·3	1·3±0·8
Mesenteric lymph node	57·5±6·1	13·1±5·0	1·0±0·9

* Mean ± standard deviation.

Distribution of SCMC

The SCMC of mononuclear cells from MVB was less than from PVB in eight patients with active inflammatory bowel disease (21±17%, 26±18%, $P<0·01$) whereas no difference was found in five patients with non-inflammatory diseases (30±20%, 32±20%, $P>0·1$) (Fig. 2). The SCMC of mononuclear cells isolated from PAB (13.6+12·9%) was the same as that from PVB (12·7±12·0%) in six studies. Thus the PVB SCMC reflects that of the mesenteric artery.

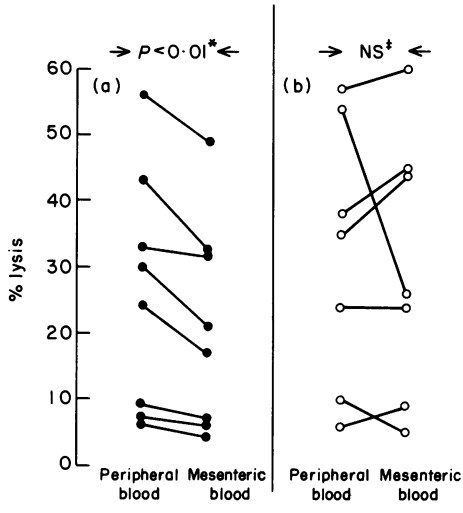

Fig. 2. Comparison of SCMC of mononuclear cells isolated from simultaneously taken peripheral and mesenteric venous blood from patients with (a) inflamed and (b) non-inflamed intestine. * Paired *t*-test; † NS = not significant.

The SCMC of intestinal and MLN mononuclear cells was minimal when tested at an E:T ratio similar to that for peripheral blood. When the E:T ratio was increased to 500:1, so that the Leu 7$^+$:T ratios were roughly similar to peripheral blood, significant SCMC of intestinal and MLN mononuclear cells was detected in the majority of populations tested and approached the levels of SCMC found in autologous peripheral blood mononuclear cells (Fig. 3). The underlying disease had no obvious effect on the measured SCMC.

Effect of mesenteric venous plasma on SCMC

The paradoxical finding of similar or lower SCMC in MVB compared to PVB despite the higher proportions of cells of the NK phenotype suggested that inhibitory factors may be released from

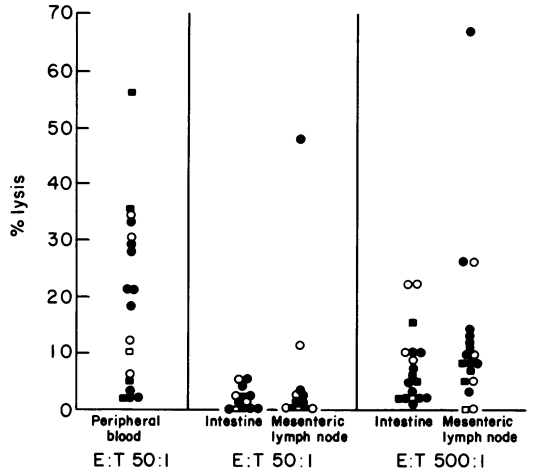

Fig. 3. Comparison of SCMC of mononuclear cells isolated from autologous peripheral blood, intestinal mucosa and mesenteric lymph nodes in patients with colorectal carcinoma (○), redundant sigmoid colon (□), Crohn's disease (●) and ulcerative colitis (■). Intestinal and mesenteric lymph node mononuclear cells were tested at E:T ratios of 50:1 as for peripheral blood, and 500:1 when the Leu 7^+:T ratio was similar to that of peripheral blood.

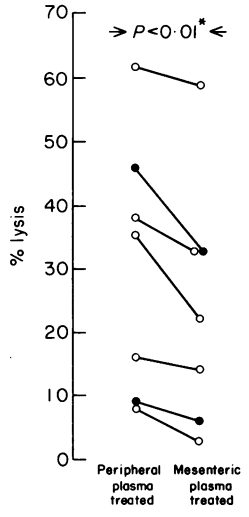

Fig. 4. Effect of 2 h pre-incubation of PBMNC in autologous peripheral and mesenteric plasma on SCMC. Bloods were simultaneously obtained from patients with inflamed intestine (●——●) and non-inflamed intestine (○——○). * Paired *t*-test.

intestinal mucosa into the MVB. PVB mononuclear cells were incubated in autologous MVB and PVB plasma for 2 h at 37°C and their resulting SCMC tested. In all seven studies, the mesenteric plasma suppressed SCMC by $27 \pm 20\%$ (Fig. 4).

DISCUSSION

A consistent increase in the proportions of Leu 7^+ cells was observed in the mononuclear cells of MVB compared to those of simultaneously taken PVB in patients undergoing abdominal surgery.

Since the mononuclear cell subpopulations of PAB and PVB were similar, it is reasonable to suppose that the MAB subpopulations are also similar to PVB. Thus a selective increase in the proportion of Leu 7^+ cells in MVB must occur as blood traverses the mesenteric vascular bed.

The study of cell phenotype of mononuclear cell populations isolated from intestinal mucosa may not accurately reflect exact *in situ* proportions due to the effects of the long isolation procedure (Selby *et al.*, 1984) but it does give a rough guide to relative numbers of various mononuclear cell subsets present. Although direct quantitative comparison of cell subpopulations in intestinal and MLN mononuclear cells is therefore not possible, this study has shown that roughly similar proportions of the subsets assessed are present in both cell populations apart from an increased $T4^+$:$T8^+$ ratio in MLN. The Leu 7^+ cell comprises a minority subpopulation in both the lamina propria and MLN in contrast to its high proportions in PVB.

The enrichment of Leu 7^+ cells across the mesenteric vascular bed and their paucity in intestinal mucosa and MLN compared to the uniform distribution of T cell subsets suggest that $T4^+$ and $T8^+$ cells may be leaving the intravascular space of the mesenteric bed at a greater rate than Leu 7^+ cells. Animal studies have demonstrated the continuous traffic of lymphocytes through gastrointestinal and MLN tissue and their recirculation via the thoracic duct (Gowans, 1959; Gowans & Knight, 1964). Factors controlling this migration are poorly understood. The migration of lymphocytes into lymph nodes, Peyer's Patches (Gowans & Knight, 1964; McConnel, 1983) and lymphoid aggregates in inflamed tissue (Smith, McIntosh & Morris, 1970; Freemont, 1983) appears to be largely via venules with specialized endothelium, the high endothelial venules (HEV). A recent report has suggested the presence of receptors on the surface of lymphocytes which determine the pattern of migration through HEV (Gallatin, Weissman & Butcher, 1983). It is conceivable that Leu 7^+ cells lack the surface receptors necessary for their migration from the mesenteric vascular bed into the intestinal mucosa. As recently suggested for mice (Zoller *et al.*, 1982), human NK cells (defined by anti-Leu 7) do not apear to form part of the recirculating lymphocyte pool.

The SCMC of most intestinal and MLN mononuclear cell populations was minimal when asayed at an E:T ratio of 50:1 at which the activity of PVB is optimally tested. This probably reflects, at least in part, the paucity of effector cells at the tissue level. A previous study (Gibson *et al.*, 1984) indicated that the SCMC of intestinal and autologous PVB mononuclear cells parallels the proportions of Leu 7^+ cells in that cell population but direct demonstration of the phenotype of the effector cell has only been reported for peripheral blood (Abo & Balch, 1981). SCMC of MLN mononuclear cells appears also to parallel Leu 7^+ proportions. When assessed at approximately equivalent Leu 7^+ ratios, intestinal SCMC is generally similar to that of MLN but is usually less than that of autologous PVB (Fig. 3).

Despite the higher proportions of Leu 7^+ cells in MVB, the SCMC of MVB was the same as or less than that of PVB. The mesenteric plasma appears to contain factors which suppress SCMC and these presumably are being released from the intestinal mucosa as they are not present in peripheral plasma. The nature of these factors has not yet been detemined but prostaglandins and plasminogen activator are potential candidates. Inflamed mucosa would potentially release more soluble factors associated with inflammation into the circulation than normal mucosa and this probably explains the higher PVB:MVB ratio of SCMC in patients with active inflammatory bowel disease. However, the *in vivo* significance of such observations is probably of little importance as the cells are in contact with MVB plasma for only a very brief period of time before being diluted in the portal vein and subsequently in the inferior vena cava.

The conclusions reached in this study are: (1) Leu 7^+ cells appear compartmentalized to the vascular system and spleen and the enrichment of Leu 7^+ cells across the mesenteric vascular bed suggests that this compartmentalization may be due to the relative inability of Leu 7^+ cells to leave the mesenteric bed; (2) SCMC parallels that of Leu 7^+ cell proportions in peripheral blood, intestinal mucosa, and MLN and (3) and paradoxical finding of similar or lowered SCMC of mononuclear cells from MVB compared to those obtained simultaneously from PVB is probably due at least in part to the release of soluble modulating factors into the mesenteric circulation from the intestinal mucosa.

Leu 7, Leu 2a and Leu 3a antibodies were generously donated by Dr N. Warner of the Becton Dickinson Monoclonal Antibody Centre, Mountain View, California, USA. PRG was in receipt of a grant from the Oxfordshire Regional Health Authority. WSS was supported by the Pharmacia Fellowship. The authors wish to thank Mr M.W.G. Kettlewell, Mr E.G. Lee and their colleagues for obtaining intestinal and mesenteric blood specimens.

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