Human colonic mononuclear cells: studies of cytotoxic function*

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SUMMARY We isolated lymphocytes from the lamina propria of colon from 19 patients with inflammatory bowel disease, colon cancer, and certain benign conditions to determine: (1) if these lymphocytes could mediate mitogen-induced (MICC) and spontaneous cell-mediated cytotoxicity (SCMC), and (2) if there were any differences in cytotoxic effectiveness which could relate to the underlying disease. We found that lamina propria lymphocytes functioned well in MICC reactions with phytohaemagglutinin, but not concanavalin A as the inducing mitogen (specific lysis $28 \pm 5\%$ vs $5 \pm 3\%$). Lamina propria lymphocytes did not mediate SCMC (specific lysis 0.3%). Neither the presence of inflammation nor the underlying disease of the patient influenced the cytotoxic activity. Peripheral blood lymphocytes from normal subjects and patients performed well in MICC assay with both phytohaemagglutinin and concanavalin A as the inducing mitogen and were equally effective in SCMC reactions.

Lamina propria lymphocytes are located just beneath the surface epithelium of the intestine. Their strategic location within the mucosa, as well as their increase in diseases such as ulcerative colitis¹ and gluten-sensitive enteropathy,² has prompted the hypothesis that lamina propria lymphocytes may be important, not only to local immune responses,³ but possibly also to disease-related intestinal damage. Pursuit of this hypothesis has been hampered by lack of sufficient quantities of lamina propria lymphocytes for study. Development of techniques for the isolation of lamina propria lymphocytes has allowed initial characterisation of this lymphocyte population.⁴⁻⁸

Inherent to the idea that lamina propria lymphocytes may cause tissue damage in disease is the concept that lamina propria lymphocytes can mediate various mechanisms of cytotoxicity. Such mechanisms as antibody-dependent (ADCC),⁹ mitogen-induced (MICC),¹⁰ and spontaneous cell-mediated cytotoxicity (SCMC)¹¹ are mediated by

peripheral blood lymphocytes. These activities may also be present in lamina propria lymphocytes. However, it is not clear that lamina propria lymphocytes can mediate all these reactions—for example, ADCC.^{8, 12-15}

The purpose of this study was to determine if isolated lamina propria lymphocyte populations of the colon mediate target cell killing by the mechanism of MICC, a T-cell function,¹⁰ and SCMC, a natural killer (NK) cell function.¹⁶ (The NK cell has an Fc receptor, but not surface immunoglobulin; there is controversy as to whether this cell may form rosettes with sheep red blood cells,¹⁷ as do T cells).

Methods

PATIENT GROUP

Nineteen specimens from patients undergoing colectomy for a variety of diseases were obtained at the time of surgery. Eight patients had colon cancer, five had inflammatory bowel disease (three with Crohn's disease and two with ulcerative colitis) and six patients had various colonic diseases, including three with diverticulosis, one with an adenomatous polyp, one with an intestinal lipoma, and one with a redundant sigmoid colon. Squares of tissue measuring 5-10 cm (2-4 in) per side were cut for study from areas of the bowel which were normal ('uninvolved').

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Eleven additional specimens were obtained from areas of the bowel which were grossly (and subsequently histologically) 'involved' with either cancer (six) or inflammatory bowel disease (three Crohn's disease, two ulcerative colitis). Peripheral blood lymphocytes were obtained from 10 healthy subjects, as well as from five of the 19 patients. All studies were approved by the Human Subjects Committee, Brigham and Women's Hospital, and informed consent was obtained from each participant.

PREPARATION OF EFFECTOR CELLS:

LAMINA PROPRIA LYMPHOCYTES

Lamina propria lymphocytes were isolated from colonic mucosa using the technique of Bull and Bookman.⁴ The procedure yielded mononuclear cells with 90% viability as estimated by trypan blue exclusion. The cell suspensions were maintained in RPMI-1640 medium (Grand Island Biological) containing 10% pooled AB, Rh-negative serum (American Red Cross Blood Services, Northeast Region, Boston, Mass.) and streptomycin, penicillin, amphotericin-B, and gentamicin. The preparation procedure does not alter the lymphocyte's ability to mediate ADCC,^{13 14 17} SCMC,^{13 14} or MICC.^{13 14} We confirmed this in our laboratory by putting peripheral blood lymphocytes through the same isolation procedure and showing no significant effect of the procedure on the cytotoxicity values.

PERIPHERAL BLOOD LYMPHOCYTES

Peripheral blood lymphocytes were obtained by standard techniques involving centrifugation of blood over Ficoll-Hypaque[®] gradients.¹⁸

TARGET CELLS

Mouse mastocytoma tumour cell line, P815, was maintained by passage in Eagle's minimal essential medium (MEM, Grand Island Biological) with added fetal calf serum (FCS), penicillin, streptomycin, and used in the study of MICC. Human chronic leukaemia cell line, K562, was maintained in RPMI-1640 medium with 10% FCS and was used to test SCMC. In studies of cytotoxicity, both target cell lines were labelled¹⁹ with sodium chromate⁵¹ (⁵¹Cr) (New England Nuclear, Boston, Mass.). 1×10^4 target cells generally had 20 000 counts per minute or more 'maximal releasable' counts (see below).

T CELL ENUMERATION

T cells from lamina propria lymphocytes were enumerated by the sheep red blood cell (E-rosette) method.²⁰ Sheep red blood cells were treated with 2-aminoethyisothiuronium bromide (AET) before being used. At least 200 cells per specimen were

counted and results expressed as percentage rosettes out of total lymphocytes.

CYTOTOXICITY TESTING

MICC¹⁰ was determined in V-bottom microtitre plates (Cooke Laboratories, Alexandria, Virginia) by adding mitogen, 1×10^4 ⁵¹Cr-P815 target cells, and effector lymphocytes (lamina propria lymphocytes or peripheral blood lymphocytes) to appropriate wells. All assays were done in triplicate. Maximal releasable ⁵¹Cr was determined by mixing labelled target cells with Triton X100. Spontaneous ⁵¹Cr release was determined by incubation of target cells with no effector cells. The plates were harvested after 18 hours of incubation at 37°C by centrifuging at $900 \times g$ for 10 minutes at 4°C. An aliquot of culture supernatant was counted in a liquid scintillation spectrometer.

The optimal concentrations of PHA and Con A for MICC assay were 10 µg/ml and 6.2 µg/ml (final concentration), respectively. In early experiments, various target:effector cell ratios were examined, ranging from 1:25 to 1:200. A plateau of activity was observed at a target-to-effector ratio of 1:50. This was used in the remainder of this study. Results were calculated according to the formula below and expressed as % specific lysis. Spontaneous ⁵¹Cr release from P815 cells averaged 28%. Specific ⁵¹Cr release from target cells incubated with PHA alone was 2–5%. Specific ⁵¹Cr release from target cells incubated with lamina propria lymphocytes alone was 1–2%.

SCMC¹¹ was determined using V-bottom microtitre plates. To the wells were added 1×10^4 ⁵¹Cr-K562 cells and effector lymphocytes (lamina propria lymphocytes or peripheral blood lymphocytes). Preliminary experiments showed that peripheral blood lymphocytes (PBL) had optimal killing at target:effector ratio of 1:25. Lamina propria lymphocytes (LPL) were used in ratios up to and including 1:100. The plates were incubated for 18 hours and harvested as above. Maximal ⁵¹Cr release was determined as above. Spontaneous ⁵¹Cr release was determined by incubation of K562 cells with media alone and averaged 10%. Cytotoxicity was expressed as percentage specific target cell lysis obtained with the following formula:

$$\% \text{ specific lysis} = \frac{\text{experimental } ^{51}\text{Cr release} - \text{spontaneous } ^{51}\text{Cr release}}{\text{maximal } ^{51}\text{Cr release} - \text{spontaneous } ^{51}\text{Cr release}} \times 100$$

for MICC: experimental ⁵¹Cr release = target + mitogen + effector cells, spontaneous ⁵¹Cr release = target + mitogens, maximal ⁵¹Cr release = target + triton $\times 100$ (same for SCMC).

Table 1 *T cell percentages and cell-mediated cytotoxicity of lamina propria lymphocytes*

	Colon cancer		IBD*		Miscellaneous†
	Involved	Uninvolved	Involved	Uninvolved	
% T cells	49%±3 (3)	43%±4 (3)	49%±3 (5)	52%±4 (5)	40%±3 (2)
MICC					
PHA	30±13‡	23±9	25±7	15±4	25±8
CON A	<1	7±4	8±5	<1	3±2
SCMC	<1	<1	<1	<1	<1
	(6)	(8)	(5)	(5)	(6)

*IBD: inflammatory bowel disease.

†See text for diagnoses.

‡% specific lysis (mean ± standard error).

Parentheses indicate number of individuals tested.

Target-to-effector ratio for MICC was 1:50 and for SCMC ranged from 1:25 to 1:100.

for SCMC: experimental ^{51}Cr release=target+media+effector cells, spontaneous ^{51}Cr release=target+media.

STATISTICAL METHODS

One-way analysis of variance and Student's *t* test were determined with a Hewlett-Packard HP-67 computer.

Results

LAMINA PROPRIA LYMPHOCYTE PREPARATION

Lamina propria lymphocyte preparations from involved and uninvolved colons yielded from 10×10^6 to 40×10^6 mononuclear cells per square cm of mucosa. The highest yields were from intestine involved with inflammation. Eighty to 95% of the cells were lymphocytes; macrophages and rare epithelial cells accounted for the remaining cells.

T CELL PERCENTAGE

Lamina propria lymphocytes forming rosettes with sheep red blood cells were determined in 18 specimens: three involved and three uninvolved with cancer, five involved and five uninvolved with inflammatory bowel disease, one with diverticulosis, and one with adenomatous polyp, respectively. Forty to 52% of the LPL formed E-rosettes. The percentage of E-rosettes was not significantly influenced by the underlying disease (>0.2) (Table 1).

CYTOTOXICITY—PERIPHERAL BLOOD LYMPHOCYTES

Peripheral blood lymphocytes from 10 normal individuals were examined for their capacity to mediate MICC and SCMC and compared with values obtained from five patients (three with colon carcinoma and one each with diverticulosis and polyposis) whose LPL were also studied. The PBL of the patients yielded results for MICC which,

Table 2 *Cell-mediated cytotoxicity of peripheral blood lymphocytes*

	Normals		Patients (5)
	Untreated (10)	Collagenase treated (5)	
MICC			
PHA	64±5	62±4	45±12
CON A	44±8	39±7	26±8
SCMC	50±4	46±5	52±12

*% specific lysis (mean ± standard error).

Parentheses indicate number of individuals tested.

Target-to-effector cell ratio for MICC was 1:50 and for SCMC was 1:25.

although lower than values from normal subjects, were not significantly different ($P>0.3$). SCMC assays also resulted in values for normal subjects and patients which were not significantly different from each other ($P>0.6$). Thus, in this group, PBL from normal subjects and patients mediated both MICC and SCMC without any effect from the underlying disease (Table 2).

CYTOTOXICITY—LAMINA PROPRIA LYMPHOCYTES

Mitogen-induced cytotoxicity (MICC)

LPL from all patient groups mediated MICC induced by PHA (Figure). Furthermore, no significant difference was detected between values of MICC of LPL from involved and uninvolved areas of the groups with cancer and inflammatory bowel disease ($P<0.2$) (Table 1). Therefore, neither the underlying disease among patient groups nor the presence or absence of disease involvement in individual specimens influenced the MICC cytotoxic activity of LPL induced by PHA.

MICC values for both PBL and LPL were strikingly lower when Con A was the inducing mitogen ($P<0.01$) (Figure, Tables 1 and 2). Thus, Con A-induced MICC was 31–42% less efficient than PHA-induced MICC when PBL were the effector cells (Table 2) and 84% less efficient when LPL were the effector cells (Figure). It appears from these data that the cells in LPL vs PBL which mediate Con A-induced MICC are either diminished markedly, less effective, or are more readily suppressed by Con A. Treatment of PBL with the same reagents used to prepare the LPL had no influence on the ability of the PBL to mediate Con A or PHA-induced MICC (Table 2).

Spontaneous cell-mediated cytotoxicity (SCMC)

In contrast with PBL, LPL were unable to mediate SCMC against K562 cells. The specific lysis for all specimens but one, regardless of disease diagnosis or activity, was less than 1%. A single specimen from

Figure 1 Percentage specific lysis for P815 or K562 target cells by colon lamina propria lymphocytes in assays of mitogen-induced (MICC: P815) and spontaneous cell-mediated cytotoxicity (SCMC: K562). The bars show the mean $\pm$ standard error of lamina propria lymphocytes from 11 specimens involved with inflammatory bowel disease or carcinoma (stippled), and 19 specimens either adjacent to inflamed or malignant areas or from colon specimens resected from patients with other diseases (hatched). Con A is much less efficient than PHA in inducing MICC ($P < 0.01$), and SCMC is not mediated by lamina propria lymphocytes at all.

a patient with cancer achieved a value of 12%. In four specimens, effector-to-target ratios of up to 300:1 still did not produce SCMC activity. The PBL of the patients, in contrast with their LPL, had a mean value of SCMC of 52%, not significantly different from normal controls (Tables 1 and 2). Incubation of PBL with the same reagents used to prepare the LPL did not affect the ability of the PBL to mediate SCMC (Table 2). Furthermore, incubation of LPL from three specimens of colon (one ulcerative colitis, two cancer) in medium for 40 hours to allow for possible regeneration of surface receptors did not result in these LPL gaining the ability to mediate SCMC (mean specific toxicity before incubation 1.2%; after incubation, 1.8%).

Discussion

We showed that LPL of human colon participate in the T cell mediated mitogen-induced cytotoxic reaction (MICC). Con A is less effective than PHA in inducing MICC when PBL and LPL are the effector cells. Also, LPL appear to be functionally different from PBL, as, although they perform well

in MICC when PHA is the mitogen, they perform more poorly than PBL when Con A is the inducing mitogen. There may be a relative paucity in the LPL population of the subset of T cells capable of interacting with Con A to result in MICC activity with this mitogen. It has been proposed that adherent cells and monocytes could be needed for complete expression of MICC.²¹ It is suggested that the adherent cell population is more important for the expression of Con A-induced than PHA-induced cellular cytotoxicity. Differences in accessory cells in PBL vs LPL could account, therefore, for the relative inability of the LPL to show effective MICC activity with Con A.

The finding in our study that LPL obtained from normal colon, colon uninvolved with cancer, and colon uninvolved with inflammation function equally in MICC without regard to the underlying disease leaves open the issue of relevance of the *in vitro* phenomenon of MICC to disease. We did not examine other target cells of potential interest, such as isolated colonic epithelial cells.

The cell which mediates SCMC is called the NK cell and may be identical with the K cell, which mediates antibody-dependent cellular cytotoxicity reactions.²² We found the function of SCMC lacking in the LPL population of human colon using K562 cells as the target. This target is especially susceptible to SCMC killing¹¹ and should show this function if it is present. It is noteworthy that the lack of SCMC activity occurs in 'normal' colon LPL as well as LPL from diseased colon. Inflammation and/or neoplasm *per se*, therefore, do not seem to attract NK cells or render the cells already present in the LPL functional in SCMC assays. Previous studies foreshadowed this finding: Clancy,¹² Fiocchi *et al.*,⁸ and Bookman and Bull¹⁵ showed that LPL from colon did not mediate ADCC, while Arnaud-Battandier *et al.*,¹⁷ showed that LPL from guinea-pig colon did not mediate ADCC or SCMC against the Chang cell line. It remains possible that lymphoid cells capable of mediating SCMC reactions are present in the LPL population, but their activity is suppressed by modulating or suppressor cells. Alternatively, the isolation procedure may remove a needed accessory cell. This is less likely, as PBL are not affected by the isolation procedure.

Chiba *et al.*¹³ and MacDermott *et al.*,¹⁴ in contrast with others,^{8, 12, 15, 17} showed that lamina propria lymphocytes are active in ADCC assays against chicken red blood cells (CRBC)^{13, 14} and Chang cells.¹³ However, the specific cytotoxic activity of lamina propria lymphocytes against Chang cells is quite low in Chiba's study (10–15%) and non-existent in MacDermott's study.¹⁴ Both results must, therefore, await confirmation before they can be

accepted as definitively showing K cell activity by lamina propria lymphocytes. As the K cell and the NK cell may, in fact, be the same cell,²² the lack of SCMC activity shown by us would go along with the lack of ADCC activity shown by others^{8 12 15 17} using cell lines as targets. It remains unclear whether 'ADCC' against chicken red blood cells is different from ADCC against other targets.

Additional study of the biological characteristics of the lamina propria lymphocytes will be crucial to our understanding of their role, if any, in inflammatory diseases.

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