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# Peripheral, mucosal, and tumour-infiltrating components of cellular immunity in cancer of the large bowel

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SUMMARY A reliable technique has been devised for the preparation of colorectal tumour-infiltrating lymphocytes (TIL). The immune capacity of these lymphocytes has been assessed *in vitro* and compared with that of lymphocytes infiltrating the lamina propria of adjacent normal mucosa (LPL) and with autologous peripheral blood lymphocytes (PBL). Assay of natural killer (NK) cell function revealed the absence of such activity in TIL and LPL despite the presence of normal levels in PBL. Antibody-dependent (K cell) cytotoxic activity was also absent in TIL and LPL. Both TIL and LPL showed significant mitogen-induced cytotoxic responses, although higher levels were detected in PBL. Tumour-infiltrating lymphocytes revealed depressed levels of spontaneous DNA synthesis, but mitogen stimulation of TIL was equivalent to that of LPL. T-cell proportions in TIL preparations were equivalent to those in PBL, but LPL comprised significantly fewer T cells.

The emergence in recent years of techniques for the isolation of lymphoid components from intestinal mucosa[1–5] has resulted in the partial characterisation of these cells in terms of surface receptors and functional status determined in *in vitro* assays.

Because of the apparent inability of host defence mechanisms effectively to recognise and destroy the neoplastic cells of established solid tumours, despite infiltration of the tumours by host immune components, the functional capacity of these tumour-infiltrating lymphocytes (TIL) is of great interest. Almost invariably, however, TIL function has been assessed in relation to functional parameters assayed in autologous peripheral blood lymphocytes (PBL) or in lymph nodes draining the tumour. Investigations of lymphoid cell ecotaxis between blood, lymph node, and intestinal lamina propria[6] suggest that these compartments differ in their transient lymphocyte populations. In the case of large bowel adenocarcinoma, therefore, it is more relevant to compare TIL function with that of lymphocytes infiltrating the tissue of tumour origin—that is, lymphocytes infiltrating normal large bowel lamina propria (LPL).

We have isolated TIL from large bowel adenocarcinomata and LPL from adjacent histologically normal lamina propria using identical techniques. *In vitro* cytotoxic and blastogenic assays performed on these two populations and on autologous PBL revealed marked differences between peripheral and intestinal lymphocyte populations but smaller distinctions between LPL and TIL, suggesting that tumour modulation of TIL may be more subtle than previous reports suggest.

## Methods

PATIENTS

A series of 24 patients (15 male, nine female), with ages ranging from 47 years to 87 years (mean 72 years), undergoing surgical resection of large bowel adenocarcinomata (five caecum, one ascend-

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ing colon, one descending colon, 10 sigmoid colon, seven rectum) was studied. None of the patients was receiving radiation or cytotoxic chemotherapy.

MEDIA

Eagle's Minimum Essential Medium (EMEM), Eagle's Minimum Essential Medium modified for suspension cultures (MEMS), and RPMI 1640 medium (RPMI) were obtained as ×10 concentrates from Flow Laboratories, Irvine, Scotland, and were routinely supplemented at single strength with the following: 10% heat-inactivated fetal calf serum (FCS, Flow Laboratories) or 10% heat-inactivated autologous plasma (AP); 20 mM HEPES; 4·5 mM sodium bicarbonate; 2 mM L-glutamine; 40 μg/ml gentamicin, and 0·125 μg/ml amphotericin B (Fungizone, Squibb). Phosphate buffered saline (PBS) was free of calcium and magnesium ions.

TISSUES

Twenty to 30 millilitres of heparinised blood was obtained from patients two to 24 hours before surgery and PBL were isolated by density gradient centrifugation over Ficoll-Triosil. These lymphocytes were thrice washed in RPMI before inclusion in assays.

Representative segments of tumour and normal mucosa were transferred to ice-cold EMEM immediately after resection and were processed within 30 minutes. LPL were isolated as previously described.[7] Briefly, 1–5 g mucosa was dissected free of submucosa, washed extensively, cut into small sections, and incubated sequentially in 50 ml PBS containing 1 mM DL-dithiothreitol (DTT, Sigma) for 15 minutes at room temperature to remove mucus; 50 ml PBS containing 0·75 mM disodium EDTA for 90 minutes at 37°C to dissociate epithelial cells; and 50 ml EMEM containing 20 units/ml purified collagenase (type VI, Sigma or CLSPA, Worthington), and 25 μg/ml (approximately 50–65 units/ml) deoxyribonuclease 1 (Worthington) overnight at 37°C to dissociate lamina propria components. The resultant cell suspension was filtered twice through loosely-packed nylon wool columns to remove remaining cell aggregates and thrice washed in MEMS. Tumour tissue was simultaneously treated in an identical fashion to release TIL.

After assessment of mononuclear cell viability (0·1% Trypan blue), LPL and TIL were separated from other cell types on isokinetic gradients of 2·7% to 5·4% Ficoll (Pharmacia) in MEMS constructed exactly as described.[7] Ten to 20 million total cells in 3 ml MEMS were layered over each gradient. Gradients were centrifuged at 98 *g*, measured at the sample gradient interface, for 14 minutes at 4°C. After centrifugation, the LPL or TIL enriched fraction was removed (see Results), thrice washed in RPMI, and included in the assays as described below.

ASSAYS

*E-rosettes*

Proportions of lymphocytes in PBL, LPL, and TIL populations forming E-rosettes with 2-aminoethylisothiouronium-coated sheep red blood cells (AET-SRBC) were determined using the method of Kaplan and Clark.[8]

CYTOTOXIC ASSAYS

Spontaneous cell-mediated cytotoxicity (SCMC), antibody-dependent cell-mediated cytotoxicity (ADCC), and mitogen-induced cell-mediated cytotoxicity (MICC) of PBL, LPL, and TIL populations were assayed in $^{51}$Cr-release assays using Chang liver cells (CLC) as targets. In four patients SCMC and ADCC were additionally assayed using respectively K562 (an erythroblastoid cell line) and chicken red blood cells (CRBC) as targets. Target cells ($10^6$) were labelled with 100 μCi sodium chromate-$^{51}$Cr (250–500 μCi/μg, Radiochemical Centre, Amersham) for one hour at 37°C, washed three times, and adjusted to $10^5$ cells/ml in RPMI. Assays were carried out in triplicate in plastic tubes. To each experimental tube was added 100 μl labelled target cells ($10^4$ cells); 100 μl lymphocyte suspension ($2·5\times10^5$ cells, or, in the case of K562 targets, $5·0\times10^6$ cells); and 100 μl rabbit anti-target cell antiserum (ADCC assays: $10^{-3}$ and $10^{-4}$ dilutions of anti-CLC or $10^{-2}$ and $10^{-3}$ dilutions of anti-CRBC), or 100 μl RPMI containing purified phytohaemagglutinin (PHA, Wellcome: MICC assays, final concentration 1 μg/ml), or RPMI alone in SCMC assays to give a final incubation volume in all assays of 400 μl. Tubes were capped and incubated at 37°C for 18 hours (except SCMC assays using K562 targets which were incubated for four hours. Spontaneous $^{51}$Cr release (SR) was estimated in tubes containing target cells and RPMI only. Maximum release (MR) was estimated by detergent lysis of the target cells. After incubation the tubes were centrifuged at 150 *g* for five minutes; 200 μl of each culture supernatant was withdrawn and counted with the residual supernatant plus cell pellet in a gamma counter. Percentage specific $^{51}$Cr release was calculated as follows:

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$$\text{Percentage } ^{51}\text{Cr release} = \frac{\text{Supernatant cpm} \times 2}{\text{Supernatant cpm} + \text{Residual cpm}} \times 100$$

$$\text{Percentage Specific } ^{51}\text{Cr release} = \frac{\text{Test release} - \text{SR}}{\text{MR–SR}} \times 100$$

*Blastogenic assay*

All manipulations and incubations were carried out in RPMI+10% AP. One hundred microlitre aliquots containing $10^5$ lymphocytes were pipetted into round-bottom wells of a microtitre plate (Sterilin). Purified PHA was added to the wells in 100 $\mu$l volumes to give final concentrations of 0, 1, 5 and 10 $\mu$g/ml. The plate was covered and incubated at 37°C for 72 hours in an atmosphere of 95% air, 5% $CO_2$. All cultures were then pulsed for four hours with 1 $\mu$Ci $^3$H-thymidine (specific activity 5 Ci/mmol, Radiochemical Centre, Amersham). Cultures were harvested onto glass fibre discs (Whatman, GF/C) using an Ilacon cell harvester. After drying, the discs were counted for activity in a toluene-based scintillant.

*Statistical analysis*

Because of variations in the number of each type of lymphocyte population measured, it was not possible to perform all assays with autologous PBL, LPL, and TIL. Results from all patients in each group were pooled and significance was determined by application of the Mann-Whitney U test.

## Results

ISOKINETIC GRADIENTS

Because of the heterogeneity of cell types released after tumour digestion, it was necessary to subject isokinetic gradients to a higher centrifugal force (98 *g*) than previously reported[7] for the preparation of LPL from normal mucosa. The efficiency and reproducibility of lymphocyte purification on these gradients was assessed in two separate experiments using PBL. A total of $16\times10^6$ cells ($15\times10^6$ mononuclear+$10^6$ erythrocytes) in 3 ml MEMS was layered over each of two identical isokinetic gradients. After centrifugation at 98 *g* for 14 minutes at 4°C, the sample volume of 3 ml was removed, followed by successive 4 ml fractions. Total and differential cell counts were performed on each gradient fraction. Mononuclear cell recoveries from the gradient fractions are depicted in Fig. 1. Of the $15\times10^6$ mononuclear cells layered onto gradient 1, 77% ($11\cdot5\times10^6$) were recovered, 89% ($10\cdot2\times10^6$) of these sedimenting into gradient fractions 3,4,5 and 6. Recovery from gradient 2 was very similar: 79% ($11\cdot8\times10^6$) total recovery with 89% ($10\cdot5\times10^6$) recovered from the modal population. The combined differential count for fractions 3,4,5, and 6 was 94% lymphocytes, 6% monocytes, ≤1% polymorphs. Receptors for SRBC, and spontaneous and antibody-dependent cytotoxic responses were unaffected by isokinetic gradient treatment. Responses to PHA were reduced by approximately 10% after isokinetic gradient treatment, but spontaneous DNA incorporation remained unaffected.

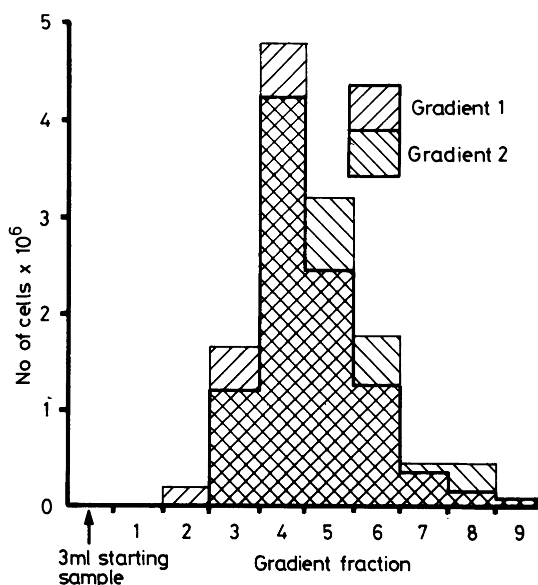


Fig. 1 *The distribution of mononuclear cells recovered from two identical isokinetic gradients. Fifteen million peripheral blood cells were applied to each gradient and the gradients were centrifuged at 98 g for 14 minutes at 4°C. Each gradient fraction measured 4 ml. Accumulated recoveries and differential counts are given in the text.* ▨: *gradient 1* ▧: *gradient 2.*

The portion of the gradient represented by fractions 3, 4, 5, and 6 in the experiments described above was therefore recovered after LPL and TIL preparation and treated as the modal population of these lymphocytes. After such treatment of tumour digests, tumour cells sedimented to the gradient-cushion interface and contamination of TIL by tumour cells was always less than 1%.

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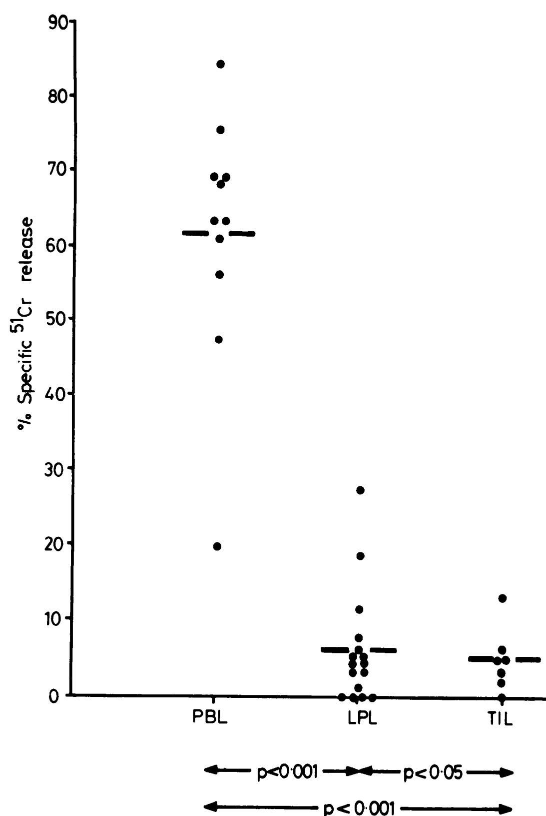


Fig. 2 *Antibody-dependent cell-mediated cytotoxicity (ADCC) of PBL, LPL, and TIL to $^{51}$Cr-labelled Chang liver cells. Effector: target cell ratio, 25:1; incubation time, 18 hours, anti-target cell antiserum dilutions, 1:1000 and 1:10 000. Each point represents the mean specific $^{51}$Cr release from triplicate assays performed with effector cells from a single patient. Horizontal bars represent the mean for each group.*

CYTOTOXIC RESPONSES

The ADCC activity of PBL, LPL, and TIL populations to CLC targets is shown in Fig. 2. It is clear that, of the three lymphocyte populations, only PBL were capable of mediating ADCC. Similarly, in four individuals tested for ADCC with CRBC targets, only PBL were reactive (mean 56·9%, range 47·0% to 62·8%). LPL were unreactive (mean 2·7%, range 0% to 9·8%) and TIL were not tested in this assay. Figure 3 shows that LPL and TIL were similarly unreactive in the NK cell-mediated SCMC assay with CLC targets, whereas PBL induced a mean specific $^{51}$Cr release of 31·7% from the target cells. The low SCMC activity of LPL was confirmed in assays using K562 target cells with lymphocytes from three patients (PBL, mean 39·6%, range 17·4%–57·5%; LPL, mean 9·1%, range 1·5%–18·9%).

Contrasting with their low levels of reactivity in SCMC and ADCC assays, both LPL and TIL showed significant levels of responsiveness in the MICC assay (Fig. 4). Their responses did not differ in this assay but both populations showed significantly less activity than PBL.

The extent of variability in all three cytotoxic parameters assessed in PBL was considerably greater than that normally experienced in PBL from healthy controls (not shown), although no direct correlation with any recorded disease parameters could be found to account for this variability.

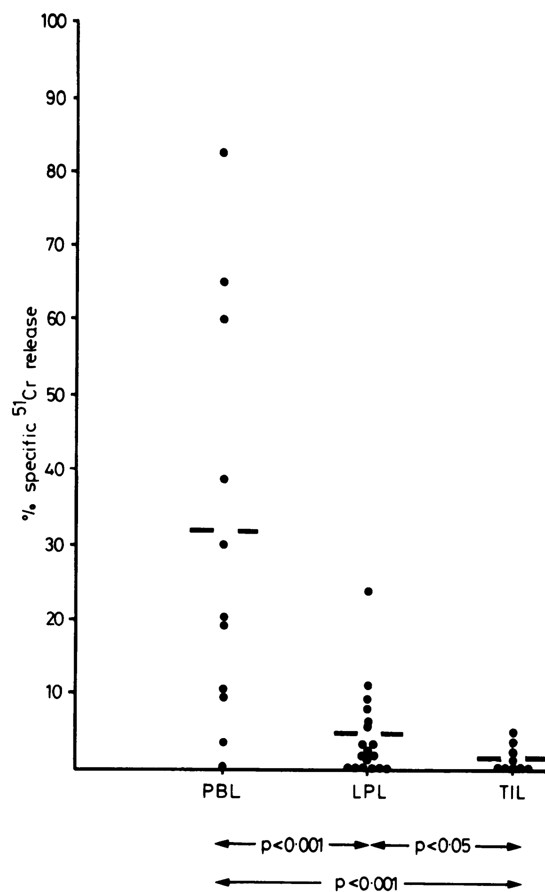


Fig. 3 *Spontaneous cell-mediated cytotoxicity (SCMC) of PBL, LPL, and TIL to $^{51}$Cr-labelled Chang liver cells. Effector: target cell ratio, 25:1; incubation time, 18 hours. Each point represents the mean specific $^{51}$Cr release from triplicate assays performed with effector cells from a single patient. Horizontal bars represent the mean for each group.*

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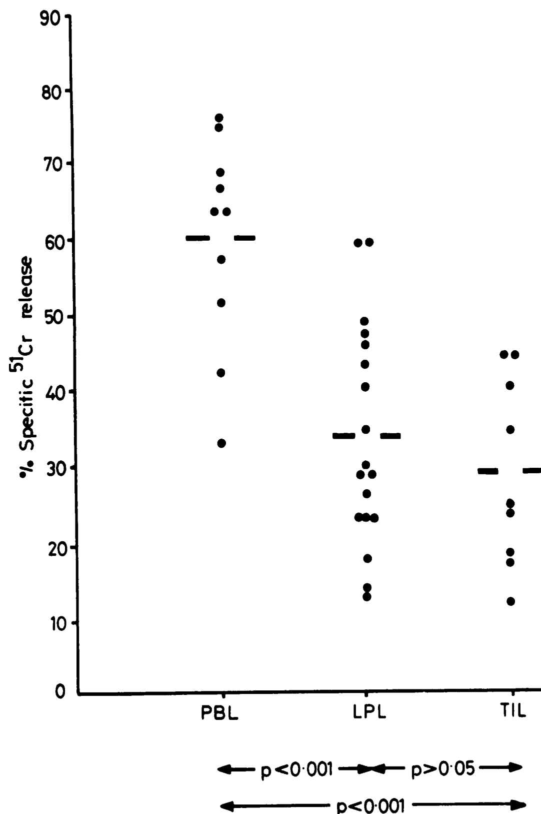


Fig 4 *Mitogen-induced cell-mediated cytotoxicity (MICC) of PBL, LPL, and TIL to $^{51}$Cr-labelled Chang liver cells. Effector: target cell ratio, 25:1; incubation time 18 hours; PHA concentration 1 μg/ml. Each point represents the mean specific $^{51}$Cr release from triplicate assays performed with effector cells from a single patient. Horizontal bars represent the mean for each group.*

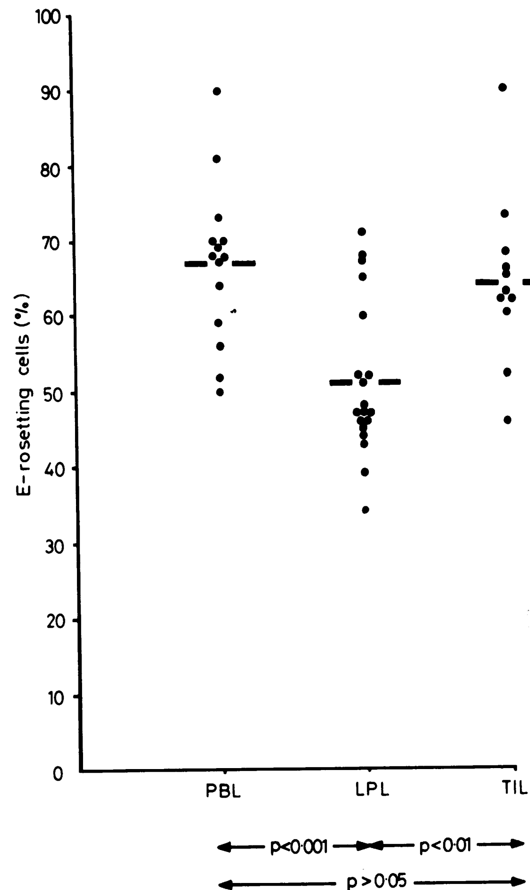


Fig. 5 *Percentages of PBL, LPL, and TIL forming rosettes with AET-coated SRBC. Each point represents the mean of triplicate assays performed with cells from a single patient. Horizontal bars represent the mean for each group.*

### E-ROSETTES

Percentages of E-rosetting cells (Fig. 5) in PBL and TIL were not significantly different (means: PBL=67%; TIL=64%), but LPL comprised significantly fewer T cells (mean, 51%).

### BLASTOGENIC RESPONSES

Spontaneous rates of DNA synthesis in the three lymphocyte populations, measured by incorporation of $^{3}$H-thymidine, are shown in Fig. 6. Incorporation into TIL was significantly less than into either PBL or LPL. Incorporation into these latter two populations did not differ significantly, although greater variation was evident in the values of LPL.

Blast transformation by PBL in response to PHA was found to be greater at all three concentrations of the mitogen than that induced in LPL or TIL (Fig. 7). This difference between peripheral and local lymphocyte responsiveness is significant even when the effect of preparative procedure is taken into account. In addition, PBL responded maximally at 5 μg PHA/ml, whereas both LPL and TIL showed declining responses at concentrations of PHA greater than 1 μg/ml. There was no significant difference between LPL and TIL responses at any of the PHA concentrations used.

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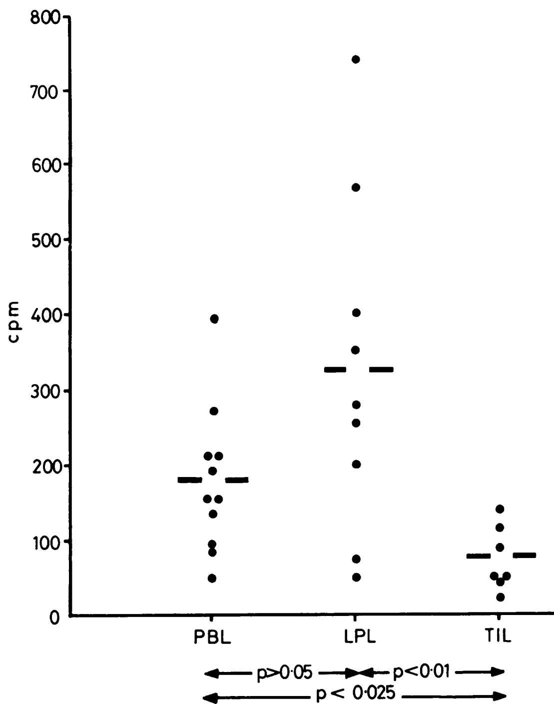


Fig. 6 *Spontaneous incorporation of ³H-thymidine into PBL, LPL, and TIL after 72 hours' incubation at 37°C. Each point represents the mean of three or more assays performed with cells from a single patient. Horizontal bars represent the mean for each group.*

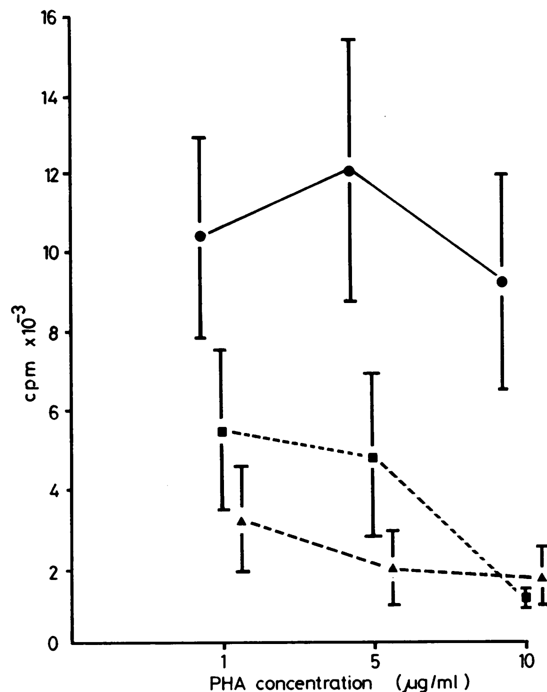


Fig. 7 *The blastogenic response of PBL (●), LPL (■), and TIL (▲) to three concentrations of PHA in 72 hour incubations at 37°C. Cultures were pulsed with 1 µCi ³H-thymidine four hours before termination. Each point represents the mean±SE of three or more assays performed with cells from at least five patients. PHA 1 µg/ml: PBL vs LPL: P>0·05; LPL vs TIL: P=0·245; PBL vs TIL: P<0·025. PHA 5 µg/ml: PBL vs LPL; P<0·05: LPL vs TIL; P=0·183: PBL vs TIL; P<0·025. PHA 10 µg/ml: PBL vs LPL; P<0·001: LPL vs TIL; P=0·357: PBL vs TIL; P=0·067.*

## Discussion

There are numerous reports in recent literature of attempts to demonstrate K-cell activity in the gut mucosa, stimulated largely by histological evidence[9] of Fc receptor-bearing cells in the colonic lamina propria and by the proposal[10] of an antibody-dependent cytotoxic mechanism directed against colonic epithelial cells as the pathogenetic factor in inflammatory bowel disease. Isolated LPL, however, have been shown by several investigators to possess low ADCC activity.[11-14] More recently it has been proposed[15 16] that the K-cell activity of isolated LPL depends upon the target cell used in the assay, erythrocyte targets showing greater susceptibility to lysis than cell lines. Fiocchi *et al.*[14] on the other hand, found no evidence of LPL K-cell induced lysis of CRBC in plaque or ⁵¹Cr-release assays. Our results, presented here, substantiate this latter view, that LPL do not mediate ADCC against cell line or erythrocyte targets. Fc receptor-bearing LPL have been reported as either comparable in number with those in PBL[15] or completely absent.[13] It has been suggested that ADCC may be dependent upon other unknown factors in addition to possession by the effector cell of Fc receptors.[15] Indeed, we have occasionally encountered significant ADCC activity in large bowel LPL isolated from resected specimens in various disease states, suggesting the need for a carefully controlled study correlating lytic ability with possible modulating factors—for example, blocking of receptors by locally-produced antibody or antigen/antibody complexes, and suppressor cell activity.

The lack of LPL responsiveness in NK-mediated SCMC paralleled the low antibody-dependent cytotoxic ability of these cells and agrees with previous

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reports.[4,15-17] This also suggests a deficit in Fc receptor availability in the lamina propria or the modulation of effector cells by unknown factors in the mucosal environment.

Assay of natural and antibody-dependent killing by TIL revealed that these mechanisms are either absent or suppressed within large bowel tumours. Other workers[18,19] have shown that K562 target cells are similarly resistant to lysis by TIL isolated from lung and breast tumours despite the presence of Fc receptor-bearing cells in isolates. Further, inhibition of NK activity by co-culture of effector cells with adherent cell populations was demonstrated[19] and the possibility was raised of suppression of NK activity by prostaglandins secreted within the tumour milieu by macrophages and monocytes. If this suppressive mechanism exists *in vivo* in tumours, it may also be an important factor in K and NK cell suppression within the normal bowel lamina propria. We have already shown[7] that LPL K cell activity can be boosted by pre-treatment of the cells with a prostaglandin synthetase inhibitor. This could be an important mode of suppression in the gut lamina propria, particularly in inflammatory bowel disease where increased monocyte/ macrophage populations have been reported.[20]

The finding of significant MICC activity in LPL confirms previous reports[4,15-17,21] Although initially devised to assess polyclonal T-cell cytotoxicity, this assay is now thought to reflect both polyclonal and Fc receptor-bearing T-cell components.[22] It is interesting that, in the present study, MICC levels in TIL were not significantly different from levels in LPL despite the presence of a greater proportion of T-cells in TIL. This may indicate either that differences exist in the $T\mu$ and $T\gamma$ subpopulations[23] infiltrating normal and malignant mucosa, or that TIL T-cell activity is depressed by some factor within the tumour milieu.

Spontaneous DNA synthesis by lymphocytes from normal mucosa did not differ from PBL values. In contrast, TIL showed little spontaneous incorporation of $^{3}$H-thymidine over 72 hours. The similarity of PBL and LPL values agrees with a previous report[3] by workers who isolated LPL using an enzymatic technique, but contrasts with a study[5] in which LPL were isolated by mechanical techniques and which showed reduced spontaneous activity in LPL compared with PBL. The possibility that mechanical isolation procedures can release metabolic inhibitors has already been discussed.[7] Our results suggest that lymphocytes in peripheral blood and in the gut lamina propria exist, not unnaturally, in a state of activation induced by the readily accessible antigenic environment. The low rate of spontaneous DNA synthesis by TIL suggests either that these lymphocytes are sequestered from lumenal antigens, or that their ability to become activated is impaired by the tumour environment.

The impaired ability of LPL and TIL to respond to PHA, compared with the responsiveness of PBL, is a common finding.[2,3,5,24] That TIL transformation was equivalent in amplitude to LPL blast formation may indicate that, in spite of their low levels of spontaneous DNA synthesis, TIL are able to respond to antigenic stimulus, although not necessarily to tumour antigens. Indeed, it has been shown[18] that although TIL from lung and breast tumours are reactive to PHA, they do not respond to autologous tumour antigens, despite the fact that PBL from the same patients showed responsiveness to both PHA and autologous tumour. The peripheral blood and gut-associated lymphocyte populations responded maximally to different concentrations of PHA. This may reflect differences in density and availability of cell membrane receptors for the mitogen between the two lymphocyte compartments.

In summary, the present work suggests that reports of depressed immune competence existing within tumours ought to be reassessed in terms of lymphocyte reactivity within the normal tissue of tumour origin, particularly in relation to tumours of mucosal surfaces. In the case of large bowel neoplasia, the availability of tissues representing the normal-adenoma-carcinoma transition enables observation of possible changes in lymphocyte character and function taking place during the development of malignancy. Such investigations are currently in progress using the methodology described above.

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