Isolation and purification of human large bowel mucosal lymphoid cells: effect of separation technique on functional characteristics

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SUMMARY Human large bowel lamina propria lymphoid cells have been isolated using both mechanical and enzymatic techniques. Their separation from other cell types after isolation was effected with greater efficiency by sedimentation on isokinetic gradients than by filtration through glass bead columns. After being purified, the capacity of the lamina propria lymphocytes to function *in vitro* as effector cells in antibody-dependent cellular cytotoxicity was determined. Mechanical disruption of the mucosa gave low yields of lymphoid cells, which lacked the capacity for cytotoxicity. Enzymatic digestion of mucosal tissue, by comparison, yielded large numbers of viable lymphoid cells which retained a significant level of cytotoxic activity. Investigation revealed that mechanical homogenisation stimulated the synthesis of prostaglandin E_2 , and inhibitor studies showed that this mediator was responsible for the lack of cytotoxic activity in mechanically-liberated lymphocytes.

The assessment of peripheral immunological parameters in intestinal diseases may reveal little in relation to events occurring at the site of the mucosal lesion and, consequently, little in relation to possible immune aetiology of the disease. It is possible, for example, that the contradictory reports concerning anergy in cell-mediated immunity in Crohn's disease (Verrier-Jones *et al.*, 1969; Richens *et al.*, 1974; Meuwissen *et al.*, 1975; Meyers *et al.*, 1976; MacPherson *et al.*, 1976; Auer *et al.*, 1978a,b) result from an ignorance of simultaneous mucosal immunological status. Ideally, mucosal and peripheral immune competence should be assayed simultaneously. The development of techniques separating high yields of viable lymphocytes from human intestinal mucosa is, therefore, of great importance.

Such procedures have been described, using either mechanical methods (Breucha *et al.*, 1974a,b; Mavligit *et al.*, 1974; Rudzik and Bienenstock, 1974; Clancy, 1976; Goodacre *et al.*, 1979) or enzymatic methods (Bull and Bookman, 1977; Arnaud-Battandier *et al.*, 1978; Crofton *et al.*, 1978) to disrupt the mucosal tissue. It is important that these two categories of technique should be com-

pared, not only in terms of efficiency—that is, numbers of lymphoid cells recovered—but also in terms of lymphoid cell function after separation.

This report describes the use of a mechanical method and an enzymatic method for the separation of human large bowel lamina propria lymphoid cells, and also two techniques for their subsequent purification. The ability of the recovered lymphoid cells to function in *in vitro* cytotoxicity reactions has also been assessed.

Methods

MEDIA

Eagle's Minimum Essential Medium (EMEM) and calcium- and magnesium-free Eagle's Minimum Essential Medium (MEMS) were obtained as 10× concentrates from Flow Laboratories, Irvine, Scotland, and were routinely supplemented at single strength with the following: 10% foetal calf serum (FCS, Flow Laboratories), previously heat-inactivated for 30 minutes at 56°C (except Chang cell culture, see below); 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 used without additives and calcium- and

magnesium-free PBS (PBSA, Dulbecco 'A', Oxoid) was supplemented as shown in the text.

TISSUES

Specimens of colon or rectum were obtained at operation from patients undergoing surgical resection for Crohn's disease (CD) or adenocarcinoma of the large intestine (AC). Only macroscopically normal tissue at least 40 mm from the edge of any inflammatory or neoplastic lesion was used. Tissues were placed in ice-cold MEMS immediately after resection and separation procedures were initiated within 30 minutes.

LIBERATION OF LAMINA PROPRIA

LYMPHOID CELLS

Plastic petri dishes and tubes (Sterilin) or siliconised glassware were used throughout all cell-handling procedures.

Mechanical method

Approximately 5 g of mucosa was carefully dissected free of all underlying submucosal and muscle tissue and was washed extensively in MEMS to remove surface debris. The mucosal tissue was then finely minced with scissors into pieces measuring approximately 1–2 mm² and homogenised in a glass/Teflon tissue homogeniser (tolerance approximately 0.030 in). The homogenate was then forced through a stainless steel grid (mesh size, 0.5 mm) using a rubber policeman and through 19 G, then 21 G hypodermic needles to further disrupt tissue masses. Mucus was removed from the resulting suspension using the method of Clancy (1976); the suspension was shaken on a vortex mixer (Griffin), centrifuged at 400 g for five minutes, and the supernatant containing disrupted mucus discarded; the cell pellet was resuspended in MEMS and the process was repeated six times. At this stage the supernatant appeared to be clear of mucus, although microscopically the cells, when resuspended, still showed clumping in mucus remnants to a variable degree. These cell clumps were further disaggregated by filtration of the suspension through two successive columns constructed from 10 ml plastic syringe barrels, each loosely packed with 5 ml nylon wool, which were subsequently flushed with 2 × 10 ml MEMS. The resultant suspension of lymphoid, epithelial, and other cell types was then purified using either columns of siliconised glass beads or isokinetic gradients of Ficoll in MEMS, as described below.

Enzymatic method

The technique of Bull and Bookman (1977) was used as follows. Approximately 5 g mucosa was dissected and washed, as described above for the

mechanical method. It was then cut into 20 mm² sections in PBSA (supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin, and 40 µg/ml gentamicin) and incubated, with stirring, in 50 ml 1 mM DL-dithiothreitol (DL-DTT, Sigma) in supplemented PBSA for 15 minutes at room temperature to remove surface mucus. The tissue was then removed, washed in supplemented PBSA, and incubated with continuous stirring for 90 minutes in 50 ml 0.75 mM disodium EDTA (Sigma) in supplemented PBSA at 37°C to remove the surface epithelium. The tissue was then washed, preincubated in EMEM and stirred overnight at 37°C in EMEM (further supplemented with 100 units/ml penicillin and 100 µg/ml streptomycin) containing 20 units/ml purified collagenase (type VI, Sigma). All incubations were carried out in sealed, sterile 100 ml polyethylene specimen containers. The resultant heterogeneous cell suspension was filtered through two nylon wool columns (see Mechanical method section) to disaggregate cell clumps, washed × 3 in MEMS, and subjected to isokinetic gradient centrifugation as described below.

PURIFICATION OF LAMINA PROPRIA

LYMPHOID CELLS

Siliconised glass bead columns

Siliconised glass bead columns, constructed as described by Rudzik and Bienenstock (1974), were used to remove epithelial cell debris from the mechanically-separated lymphoid cell suspensions in some experiments. Each column comprised a 50 ml plastic syringe barrel containing 2 cm (vertical height) of 1 mm diameter glass beads sandwiched between 1 cm (below) and 5 cm (above) of 3 mm diameter glass beads. A 16-mesh stainless steel grid at the base held the beads in place. All glass beads (Ballotini, Jencons Scientific) were siliconised and the columns were autoclaved before use. The flow rate through the columns was regulated to 1 ml/min using a 23 G 1 in hypodermic needle. Before use, each column was equilibrated with 25 ml MEMS, which was then discarded. The cell suspension, reduced to 25 ml in MEMS, was then run through the column, which was finally flushed with a further 25 ml MEMS. Total, differential, and viability counts were carried out on the eluted cells.

Isokinetic gradient centrifugation

Isokinetic gradients of Ficoll (Ficoll 400, Pharmacia) in MEMS were generated in siliconised Pyrex tubes measuring 150 mm × 20 mm diameter using a Buchler Multistaltic Pump. Each 40 ml gradient varied linearly from 2.7% w/v Ficoll at the sample-gradient interface 90 mm from the centre of revolution, to 5.5% w/v Ficoll at the gradient-cushion

interface 205 mm from the centre of revolution, and was formed over a cushion of 30% w/v Ficoll.

Three millilitres of MEMS containing $10\text{--}20 \times 10^6$ total cells was layered over each gradient. Gradients were centrifuged at 38 *g* for 14 minutes at 4°C in a MSE Coolspin centrifuge with an eight-place Universal swing-out head. Tubes were held in 50 ml metal buckets fitted with 110 mm $\times$ 25 mm diameter polypropylene sleeves. Acceleration was controlled so that maximum revolutions were reached after one minute and deceleration was at a similar rate.

After centrifugation, the 3 ml starting sample was removed from the top of the gradient, followed by successive fractions measuring 5 ml each. Total, differential, and viability counts were carried out on the cells recovered in all fractions and the fractions were then pooled as required. Recovered lymphoid cells were washed $\times 3$ in EMEM before inclusion in *in vitro* cytotoxicity assays.

PROSTAGLANDIN EXTRACTION AND BIOASSAY

The presence of prostaglandin (PG)-like activity in the cell suspension supernatants after mechanical homogenisation of mucosal tissue was determined using acidic lipid extracts of the supernatants. Two 1 ml samples were removed from the 50 ml supernatant medium. After the pH had been adjusted to 4.0, each sample was extracted twice with two volumes of diethyl ether, mixing thoroughly at each stage. Each ether extract was then evaporated to dryness under nitrogen, reconstituted with 1 ml Krebs's buffered saline (KBS), and stored at -20°C until assayed. Bioassay of PG-like activity was carried out using two gut baths in series, the first containing rat fundus strip and the second containing rat colon strip. The superfusate consisted of KBS containing 1.13 μM methysergide, 0.36 μM phentolamine, 7.7 μM propranolol, 0.35 μM atropine, and 0.50 μM mepyramine as antagonists. The PG-like activity shown by each extract was quantified against calibration curves for the activity of the rat gut preparations to known amounts of purified PGE_2 and $\text{PGF}_{2\alpha}$ (Upjohn).

CYTOTOXIC ASSAYS

The antibody-dependent cellular cytotoxic (ADCC) potential of peripheral blood lymphocytes (PBL) and of isolated lamina propria lymphocytes was assayed in a ^{51}Cr -release assay using Chang liver cells (CLC) as targets. CLC were grown in continuous culture as monolayers in 100 ml glass bottles in EMEM (containing 10% FCS, which had not been heat-inactivated), and were harvested from confluent cultures by incubation with 0.6 mM disodium EDTA in PBSA for 20 minutes at 37°C. Harvested

CLC were washed $\times 3$ in EMEM and labelled with 100 μCi sodium chromate (^{51}Cr , Radiochemical Centre, Amersham) for one hour at 37°C, washed $\times 3$ in EMEM, and adjusted to 10^5 cells/ml. Anti-CLC antiserum was raised in rabbits by repeated intramuscular injections of 10^7 CLC in Freund's complete adjuvant. Antiserum from a single rabbit was used in all the tests and was heat-inactivated at 56°C for 30 minutes before storage at -70°C .

PBL were separated from human heparinised whole venous blood obtained from healthy volunteer donors by sedimentation over Ficoll-Triosil.

ADCC assays were carried out in triplicate in round-bottomed 164 mm $\times$ 10 mm diameter plastic tubes (Luckhams). To each experimental tube was added 100 μl EMEM, 100 μl labelled CLC (10^4 cells), 100 μl lymphocyte suspension (2.5×10^6 cells), and 100 μl anti-CLC antiserum (10^{-8} and 10^{-4} dilutions). Spontaneous cell-mediated cytotoxicity (SCMC) was assessed from tubes containing target and effector cells but without antiserum, and spontaneous isotope release (SR) from the target cells was determined in tubes containing target cells and EMEM only. Maximum release, assessed by detergent lysis of the target cells, was always 100%. Tubes were capped and incubated at 37°C for 18 hours. After incubation, 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 ICN Gamma Set 500 gamma counter. Percentage specific ^{51}Cr release from the target cells was calculated as follows:

$$\begin{aligned} \%^{51}\text{Cr release} &= \frac{\text{Supernatant cpm} \times 2}{\text{Supernatant cpm} + \text{Residual cpm}} \\ \% \text{ Specific } ^{51}\text{Cr release} &= \frac{(\text{Experimental } ^{51}\text{Cr release} - \text{SR})}{(\text{SCMC } \% ^{51}\text{Cr release} - \text{SR})} \end{aligned}$$

Results

MECHANICAL METHOD

The efficiency of the mechanical method, in terms of lamina propria lymphoid cell numbers recovered, is depicted in Table 1. The mean number of lymphoid cells released per g of mucosal tissue was $3.8 \times 10^6/\text{g}$ (range, $1.7\text{--}7.3 \times 10^6$). Subsequent treatment of the cell suspension to disrupt and remove mucus reduced this mean value to $1.9 \times 10^6/\text{g}$ (range, $1.0\text{--}3.9 \times 10^6$), a loss of lymphoid cells equivalent to 50% of the original number recovered. In addition, Table 1 shows that subsequent filtration of the suspension through nylon wool did not reduce the cell recovery. Indeed, in most experiments recovery was apparently improved using this procedure. This

Table 1 *Lymphoid cell yield after mechanical homogenisation of gut mucosal tissue*

Patient	Lymphoid cells $\times 10^6$ /g mucosal tissue			
	After homogenisation	After removal of mucus	After nylon column filtration	After glass bead column purification
CD/1	3.1	—	2.0	—
CD/2	4.0	1.7	1.7	—
CD/3	2.5	1.6	2.2	—
CD/4	7.3	—	5.9	—
AC/1	2.6	1.6	1.6	—
AC/2	2.2	2.1	2.1	—
AC/3	1.7	1.3	2.1	—
AC/4	3.6	1.0	1.5	—
AC/5	5.6	3.0	3.4	—
AC/6	3.5	1.2	—	0.7
AC/7	5.7	3.9	—	1.6
Mean $\pm$ SD	3.8 $\pm$ 1.7	1.9 $\pm$ 0.9	2.5 $\pm$ 1.4	1.2 $\pm$ 0.6

CD: Crohn's disease.

AC: adenocarcinoma of the large bowel.

may be attributed to the disaggregation of cell clumps by the nylon wool, releasing further cells into single cell suspension.

At this stage, the lymphoid cell suspension was heavily contaminated with epithelial cells and cell debris and required purification before further investigation. Contamination with residual mucus frequently caused aggregation of cells within the suspension, causing problems with the subsequent purification procedures. Lymphoid cells were 85–95% viable at this stage as assessed by Trypan blue exclusion.

GLASS BEAD COLUMN PURIFICATION

Table 1 shows the results of two experiments in which the siliconised glass bead column method of purification was attempted. Prior removal of mucus from the suspension again caused a substantial loss (45%) of originally-separated cells. In both experiments, passage through the glass bead columns resulted in further lymphoid cell loss. Mean cell numbers were reduced to 25% of original. Although the majority of cells eluted from the columns were of lymphocyte and plasma cell lineage (Table 2), there was a variable degree of monocyte/macrophage and

polymorphonuclear cell contamination, and removal of epithelial cells was not complete. Lymphocyte Trypan blue viability was slightly improved after column treatment.

ISOKINETIC GRADIENT PURIFICATION

Because of the substantial loss of lymphoid cells experienced using the glass bead columns, all further gut lymphoid cell suspensions were purified using isokinetic gradients of Ficoll in MEMS. Figure 1 shows a typical distribution of gut lymphoid cells recovered in 5 ml fractions from such a gradient. Preliminary experiments indicated that the optimum number of cells loaded on gradients of the dimensions used was between 10×10^6 and 20×10^6 total cells. Application of higher cell numbers overloaded the gradient and the separation became less efficient with more spread of cells from their modal populations into adjoining fractions. Differential counts of the types of cells recovered from each portion of the gradient are given in Table 2. For clarity, the fractions have been pooled into three groups labelled A, B, and C. The 3 ml starting sample contained only platelets after centrifugation and was discarded. The modal population of small lymphocytes was contained in the three 5 ml fractions at the top of the gradient (A, Fig. 1). Table 2 shows that the lymphocytes recovered from these fractions were highly purified. Small lymphocytes and plasma cells were inevitably recovered in the same fractions but contamination with other cell types was minimal. Recovered lymphocytes showed 95–100% Trypan blue viability. The mid-portion of the gradient (B, Fig. 1, Table 2) contained very few cells, the majority of which were medium-sized lymphocytes with an increased content of cells of the monocyte/macrophage type. The fractions recovered from the gradient-cushion interface (C, Fig. 1, Table 2) contained a large number of non-viable and disrupted cells of variable morphology, aggregates of epithelial cells, cell debris, and a number of large lymphocytes.

A frequent observation when using the isokinetic gradients was that high yield of lymphocytes and

Table 2 *Differential counts of gut mucosal cells purified either by glass bead column treatment or isokinetic gradient sedimentation*

	% Total cells				
	Lymphocytes	Plasma cells	Monocytes/ macrophages	Polymorphs	Disrupted/ unidentifiable
Glass bead column	68–82	4–10	5–11	1–3	5–7
Isokinetic gradient					
A	50–97	3–50	< 1	0	0
B	95–97	0	3–5	< 1	0
C	50	0	2–5	< 1	45–50

Fig. 1 Typical separation profile of large bowel lamina propria lymphoid cells after isokinetic gradient sedimentation. S: sample volume (3 ml). All successive fractions measured 5 ml. See Table 2 for differential cell counts within A, B, and C.

good separation from other cell types was dependent upon prior efficient removal of mucus from the sample suspension. Even small amounts of remnant mucus induced the formation of cell aggregates, streaming of cells within the gradient, and a consequent loss of separation efficiency. These problems were commonly encountered using suspensions derived from mechanically-separated samples but rarely so in the case of enzymatically-separated samples.

The high yield of lymphocytes minimally contaminated with other cell types prompted the continued use of this method. In all the ADCC assays reported below, therefore, the lamina propria lymphocytes used were those recovered from the top three 5 ml fractions (A, Fig. 1) from isokinetic gradients.

ADCC OF SEPARATED LAMINA PROPRIA LYMPHOCYTES

Mechanically-separated cells

When the ADCC activity of mechanically-separated lamina propria cells was assessed, values consistently approaching zero were encountered. This prompted an investigation into the presence and nature of possible inhibitors of ADCC released during the separation procedure. Subsequently, it was found that when indomethacin (15 µg/ml) was added to the culture medium throughout the homogenisation procedure, the liberated lymphocytes showed significantly raised ADCC activity (Table 3).

Table 3 Effect of separation technique on subsequent ADCC of liberated mucosal lymphocytes

Patient	% Specific ^{51}Cr release		
	Mechanical		Enzymatic
	Medium alone	Medium and indomethacin	
CD/1	1.1	8.6	—
CD/3	1.4	—	—
CD/4	0.7	10.1	8.7
AC/1	2.2	—	—
AC/2	1.8	11.7	13.0
AC/3	1.6	—	—
AC/5	1.6	11.6	—
Mean $\pm$ SD	1.5 $\pm$ 0.5	10.5 $\pm$ 1.5	10.9 $\pm$ 3.0

Addition of indomethacin to the separation medium also gave an appreciable increase in cell recovery (Table 4). Indomethacin, at the concentrations used, was not responsible *per se* for the observed increase in ADCC activity. This was demonstrated by the assessment of ADCC in PBL which had previously been incubated for two hours at room temperature in indomethacin of varying concentration (Table 5). It can be seen that concentrations in excess of those used during tissue homogenisation have little effect on ADCC capacity.

These results suggest that the addition of indomethacin suppressed the formation of an inhibitory factor released during tissue homogenisation. Subsequent extraction of acidic lipids from the supernatants of tissue homogenisations carried out in MEMS or MEMS with indomethacin, followed by

Table 4 Effect of separation technique on yield of mucosal lamina propria lymphoid cells

Patients	Lymphoid cells $\times 10^6$ /g mucosal tissue		
	Mechanical method		Enzymatic methods
	Medium alone	Medium and indomethacin*	
CD/1	3.1	7.5	—
CD/4	7.3	8.9	32.8
AC/1	2.6	4.2	—
AC/2	2.2	3.7	6.5

*Indomethacin concentration = 15 µg/ml.

Table 5 Effect of indomethacin on ADCC of peripheral blood lymphocytes

Indomethacin concentration (µg/ml)	% Specific ^{51}Cr release ($\pm$ SD)
0	29.4 $\pm$ 5.1
10	30.3 $\pm$ 1.3
15	32.1 $\pm$ 4.7
20	35.6 $\pm$ 5.7
30	25.8 $\pm$ 2.9

Results shown are the mean values from two experiments carried out in triplicate, using PBL from the same donor.

Fig. 2 Bioassay of prostaglandin-like activity in supernatant extracts from mechanically homogenised large bowel mucosa. A: extract of supernatant from mucosa (patient AC/2) homogenised in MEMS alone. A_{Ind}: extract of supernatant from mucosa (patient AC/2) homogenised in MEMS+indomethacin (15 µg/ml). B: extract of supernatant from mucosa (patient CD/1) homogenised in MEMS alone. B_{Ind}: extract of supernatant from mucosa (patient CD/1) homogenised in MEMS+indomethacin (15 µg/ml).

bioassay of these extracts on rat fundus and colonic strip preparations revealed the possible nature of this inhibitory factor. Figure 2 demonstrates that the rat fundus preparation was more sensitive to PGE₂ than to PGF₂α, and that the rat colon was preferentially sensitive to PGF₂α. Assay of the biological activity of extracts from the homogenate supernatants of two mechanical separation experiments clearly showed substantial amounts of PG-like activity, attributable to PGE₂ alone. The activity in the extract from supernatant A (patient AC/2) was equivalent to 31 ng PGE₂/ml of homogenate supernatant. An adjacent section of mucosa homogenised in MEMS containing indomethacin yielded only 6.5 ng PGE₂/ml of supernatant, an inhibition of PG synthesis equivalent to 71% when adjusted for tissue weight. In the second experiment depicted in Fig. 2, homogenisation of mucosa in MEMS yielded 6.3 ng PGE₂/ml of supernatant B (patient CD/1) and a barely detectable 0.75 ng PGE₂/ml of supernatant was stimulated when adjacent mucosa was homogenised in MEMS with indomethacin, a weight-corrected inhibition of 90%.

Table 6 Effect of prostaglandin E₂ on ADCC of peripheral blood lymphocytes

PGE ₂ concentration (ng/ml)	% Specific ⁵¹ Cr release (±SD)
0	30.9±0.4
10	24.0±4.7
20	4.8±6.2

Results shown are the mean values from two experiments carried out in triplicate using PBL from the same donor.

To investigate the possibility of PGE₂ acting as an inhibitor of ADCC, PBL were incubated in various concentrations of purified PGE₂ for two hours at room temperature before assaying their ADCC potential. The results (Table 6) clearly show that PGE₂ can, indeed, exert a marked effect on lymphocyte ADCC activity.

Enzymatically-separated cells

Because of the PG-stimulating effect of mechanical homogenisation of mucosal tissue, described above, an alternative technique employing enzymes to release the lamina propria lymphoid cells was attempted. Column chromatography-purified collagenase (type VI, Sigma) described as 'essentially protease-free' was used in the method described by Bull and Bookman (1977) to minimise possible effects on lymphocyte surface membrane receptors.

In two experiments, the efficiencies of mechanical and enzymatic methods were determined using identical tissue samples for each method (Table 4). Treatment with collagenase yielded significantly more lymphoid cells than tissue homogenisation in medium alone, or even in medium containing indomethacin. Moreover, the cell suspensions obtained from collagenase-digested mucosal tissue contained no detectable mucus, indicating the efficient mucolytic action of DTT. Liberated lymphoid cells exhibited 95–100% Trypan blue viability.

After isokinetic gradient purification, the enzymatically liberated lymphocytes retained ADCC activity (Table 3) comparable with that measured in lymphocytes liberated mechanically in medium containing indomethacin.

Discussion

Several attempts to separate and characterise gut mucosal lymphoid cells have been described, both in animals (Rudzik and Bienenstock, 1974; Arnaud-Battandier *et al.*, 1978) and in man (Breucha *et al.*, 1974a,b; Mavligit *et al.*, 1974; Clancy, 1976; Bull and Bookman, 1977; Crofton *et al.*, 1978; Goodacre *et al.*, 1979). In the only reported study comparable with the present investigation (homogenisation of large bowel mucosa), Goodacre *et al.* (1979) demonstrated a mean recovery of 12.7×10^6 lymphoid cells per gram of mucosa. This is considerably higher than the recovery experienced by ourselves (3.8×10^6 lymphoid cells per gram of mucosa) using a similar method. As in the present study, however, Goodacre *et al.* (1979) report a considerable range of recoveries ($2.8\text{--}38 \times 10^6/\text{g}$; cf. present study, $1.7\text{--}7.3 \times 10^6/\text{g}$). There are three possible reasons for this wide range of cell recoveries using the mechanical method. Firstly, the extent of lymphoid cell infiltration is variable between mucosal samples. Secondly, although the characteristics of the homogenisation procedure were standardised as far as possible, the effectiveness of homogenisation remained variable—indeed, such variability is inherent in the method. Finally, and most importantly, the mucus content differed between tissues, causing cell aggregation and trapping to a variable extent. The technique described by Clancy (1976) for removal of mucus from cell suspensions, in our hands accounted for a loss of 50% of the original lymphoid cells separated. It is likely that such cell loss would be greater from separations using large intestine—for example, this study—with a high mucus content, than from separations using small intestine—for example, Clancy, 1976—having a relatively low mucus content.

Glass bead column purification of the liberated cells in the present study accounted for a further 30% loss of cells. It is interesting that, in those experiments of Goodacre *et al.* (1979) where cell suspensions were subsequently treated using glass bead columns, the final cell recovery was, on average, 20% below the recovery from experiments in which columns were not used. Thus, although siliconised glass bead columns are relatively efficient at removing epithelial cell debris, they also remove considerable numbers of lymphoid cells. Subsequent experiments (unpublished) using PBL on glass bead columns indicated that most of this cell loss may be attributed to trapping by cell aggregates rather than to cell adherence to the column.

In comparison, isokinetic gradient purification was highly efficient, both in terms of cell recovery and of lymphoid cell purity. Thus, it was possible to

recover 95–100% of the mononuclear cells layered onto each gradient. Approximately 70% of these cells were recovered as the modal population of small lymphocytes and plasma cells. The gradients were centrifuged at a centrifugal force (38 g) much less than that (97 g) routinely used by Pretlow and his co-workers in their numerous reports of cell separation by isokinetic gradient sedimentation (for example, Pretlow *et al.*, 1971, 1973, 1977). We have found that, although the use of higher centrifugal forces applied to the gradients gives improved separation of cell types, when applied to mucosal cell preparations, decreased yields are experienced. This may be explained by the presence, in mucosal cell preparations, of cell doublets and triplets which, when applied to an isokinetic gradient, act as cells of correspondingly increased diameter and are forced towards the gradient-cushion interface. However, it has been shown that separations of cell suspensions with a greater heterogeneity of cell types—for example, from tumours—certainly require the application of higher centrifugal forces to produce lymphocyte populations of satisfactory purity (Pretlow *et al.*, 1977). Other workers (Bull and Bookman, 1977; Arnaud-Battandier *et al.*, 1978) have purified the gut lymphoid cells by sedimentation over discontinuous Ficoll-Hypaque gradients. In our experience, lymphocyte recovery and purity were always less after discontinuous gradient sedimentation than when isokinetic gradients were used to purify the cell suspension.

The dramatic effects on subsequent cell recovery and lymphocyte cytotoxic capacity of adding indomethacin to the culture medium throughout mechanical separation experiments suggested that prostaglandins were implicated in the suppression of lymphocyte functional capacity. Subsequent bioassay of homogenate supernatants clearly showed that substantial PGE_2 synthesis was taking place during the homogenisation procedure. The addition of indomethacin, a potent PG synthetase inhibitor (Vane, 1971), greatly inhibited this synthesis. It appears that the homogenisation procedure causes disruption of cell membranes, releasing the arachidonic acid precursors of PG synthesis. E-series PGs have been shown to inhibit many human lymphocyte functions *in vitro*. Thus, Cook *et al.* (1978) have shown inhibition of the lymphocyte anamnestic antibody response; Lomnitzer *et al.* (1976) demonstrated PGE inhibition of leucocyte inhibitory factor production by PHA-stimulated lymphocytes; Smith *et al.* (1971a,b) report that the raised levels of lymphocyte cyclic AMP induced by PGE have marked inhibitory effects on $[^3\text{H}]$ thymidine incorporation into PHA-stimulated lymphocytes; Goodwin *et al.* (1977 a,b) have shown inhibition of

mitogen stimulation by low concentrations of PGE₁ and PGE₂ and have recently postulated a specific receptor for PGE on human lymphocytes (Goodwin *et al.*, 1979), which may account for these potent and wide-ranging effects. With relevance to the present study, Droller *et al.* (1978a,b) have shown that PGE₂, in concentrations as low as 10^{-8} M, have, in their system, an inhibitory effect on lymphocyte ADCC which they attribute to PG-induced increases in lymphocyte intracellular cyclic AMP. We have demonstrated the presence in mucosal tissue homogenate supernatants of PGE₂ concentrations of 6.3 ng/ml and 31 ng/ml in two experiments, and have shown that, in our system, significant suppression of ADCC in PBL could be achieved by preincubation of the effector cells in PGE₂ concentrations at least as low as 10 ng/ml (2.8×10^{-8} M).

The stimulation of PGE synthesis induced by membrane disruption in mechanical separation procedures will, presumably, vary, depending upon the degree of cellular breakdown. However, it is unlikely that all PG synthesis can be avoided during tissue homogenisation as Piper and Vane (1971) have shown that even mild mechanical stimuli can induce PG synthesis. Subsequent assessment of lymphocyte responses must, therefore, take account of this. Goodacre *et al.* (1979) report a delayed and reduced mitogenic response of gut lamina propria lymphocytes to PHA, when compared with PBL. The lymphocytes in their study were released by a mechanical method and it is possible that consequent PG synthesis and raised lymphocyte cyclic AMP levels caused suppression of the lymphocyte mitogenic response, a mechanism which is well documented (Smith *et al.*, 1971a,b; Goodwin *et al.*, 1977a,b). The weak stimulatory effect of low concentrations of cyclic AMP on the spontaneous rate of DNA synthesis in mitogen unstimulated lymphocytes reported by Smith *et al.* (1971b) may explain the variability in this parameter found by Clancy (1976) in mechanically-separated mucosal lymphocytes. The effects of indomethacin on cell recovery after mucosal homogenisation observed in the present study are difficult to explain. Subjectively, cell suspensions derived from tissue homogenisation in medium with indomethacin contained less mucus. Raised cell recovery may, therefore, be due to a possible mucolytic action of indomethacin resulting in less cell aggregation and trapping.

The present study provides the first direct evidence for the existence of cytotoxic effector cells in the human large bowel lamina propria. This conflicts with previous reports revealing no evidence for the existence of K cells in the colonic mucosa (Bookman *et al.*, 1977; Bull and Bookman, 1977), but substantiates the work of Strickland *et al.* (1975) who

reported histological evidence for the presence of Fc-receptor bearing cells in the colonic lamina propria of normal individuals and of patients with Crohn's disease. The importance of such cells as possible mediators of cytotoxic reactions to colonic epithelial cells in Crohn's disease has been reported by Stobo *et al.* (1976).

The lymphoid cell yield per gram of mucosal tissue was greatly increased by the use of an enzymatic method of tissue disaggregation. In the two experiments reported, cell recovery was 32.8×10^6 /g and 6.5×10^6 /g, which compares with 15.0×10^6 /g described by Bull and Bookman (1977) who used an identical method (if we assume that 1 cm² of mucosa weighs approximately 0.5 g). The enzymatically-liberated lymphocytes, after purification on isokinetic gradients, showed similar ADCC activity to lymphocytes liberated mechanically in medium containing indomethacin. This further confirms the inhibitory effect of PGE₂ synthesised during mechanical homogenisation.

All techniques designed to liberate lymphoid cells from a complex tissue such as the gut must represent a compromise. However, we have shown that, as a method liberating high yields of lymphocytes retaining intact membrane receptors and the capacity for ADCC, enzymatic digestion of the gut mucosa, as described, demonstrates distinct advantages over methods employing mechanical homogenisation of the tissue. Further characterisation of the cytotoxic capacity of lamina propria lymphocytes is therefore in progress using this technique, in conjunction with isokinetic gradient sedimentation to purify the liberated cells.

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