Preparation of lymphoid cells from small specimens of human gastrointestinal mucosa

R. W. CROFTON[1], CHARLOTTE COCHRANE, AND D. B. L. McCLELLAND[2]

From the University Department of Therapeutics, Royal Infirmary, Edinburgh

SUMMARY Several methods for the preparation of cell suspensions from human gastrointestinal mucosa were investigated. Satisfactory suspensions were obtained by incubating tissue fragments in a solution of collagenase and hyaluronidase overnight at 4°C followed by 30 minutes at 37°C. The resulting suspension contained large numbers of intact lymphoid cells; in addition, variable amounts of epithelial cells and cell debris were present. A high proportion of the lymphoid cells were shown by immunofluorescence to contain immunoglobulin (mainly IgA). Viability of these cells was demonstrated by dye exclusion, their ability to survive in short-term culture, and their ability to incorporate radio-labelled amino acid into immunoglobulin *in vitro*.

There have been many investigations of the function of peripheral blood lymphocytes in patients with disease affecting the gastrointestinal mucosa. However, peripheral blood lymphocytes are not necessarily representative of the lymphocytes in the gut. For example, the relative anergy in Crohn's disease (Meuwissen *et al.*, 1975; Strickland *et al.*, 1975; Meyers *et al.*, 1976) has been attributed to the influx of T-lymphocytes into the bowel wall where they have been identified in large numbers (Meuwissen *et al.*, 1976).

In view of these differences we attempted to isolate lymphoid cells from the gastrointestinal mucosa in order to study their characteristics and function *in vitro*. There have been several attempts in the past to do this (Nind *et al.*, 1973; Breucha *et al.*, 1974a,b; Mavligit *et al.*, 1974; Clancy, 1976) but in no case have full details been published of the characteristics and properties of the cells isolated. The aim of this study was to develop a method of isolating the lymphoid cells of the human gut mucosa and to permit study of their characteristics and properties.

Methods

TISSUES
Specimens of stomach, small bowel, or colon were

obtained from patients undergoing operation for peptic ulcer and gastric and colonic carcinoma. Specimens of jejunum were obtained by Crosby capsule from patients undergoing investigation for possible coeliac disease. Rectal tissue was obtained by rectal biopsy at sigmoidoscopy. Specimens from patients with carcinoma were obtained as far from the tumour as possible and were macroscopically normal in appearance.

The mucosa and submucosa were dissected free of the muscular layer, washed thoroughly to remove blood and debris, and chopped into fragments about 1 mm in diameter. Aliquots of 100 mg of tissue were then weighed out.

ENZYMES
The enzymes used and their source and concentrations are shown in Table 1.

Table 1 *Enzymes used in preparing suspensions of mucosal cells*

Enzyme	Source and description	Concentration (mg/ml)
Collagenase	Sigma: type 1 from *Clostridium histolyticum* ammonium sulphate fraction (cat. no. C 0130)	0·5
Hyaluronidase	Sigma: type 1 from bovine testes (cat. no. H 2001)	1·0
Pronase	BDH: broad spectrum protease from *Streptomyces griseus* (cat. no. 39052 2P)	2·0
DNAase	BDH: electrophoretically purified from bovine pancreas (cat. no. 39101 2C)	0·025

[1]Present address: Aberdeen Teaching Hospitals, Aberdeen.
[2]Address for reprints: Dr D. B. L. McClelland, University Department of Therapeutics, Royal Infirmary, Edinburgh EH3 9YW.

CELL ISOLATION

All incubations were performed in Hank's balanced salt solution (HBSS) with the pH adjusted to 7·0-7·2. Each aliquot of 100 mg tissue was added to 10 ml HBSS containing the appropriate concentration of enzyme in a 20 ml sterile glass universal container. Where indicated, the container was left at 4°C overnight. The incubation was performed in a 37°C waterbath. A Teflon coated stirring bar was added to the container and the mixture stirred gently on a magnetic stirrer.

At the end of the incubation, the crude cell suspension was filtered through a metal sieve (40 mesh standard) to remove debris. The cells were washed three times in phosphate buffered saline supplemented with a 2·5% heat-inactivated fetal calf serum, gentamicin 100 μg/ml, and nystatin 100 units/ml. The cells were finally suspended in Ham's F10 medium containing 20% heat-inactivated pooled normal human serum, gentamicin 100 μg/ml, and nystatin 100 units/ml. All glassware was sterilised and siliconised before use.

The total cell count was determined in a Neubauer chamber and the viability by exclusion of 0·1% Trypan blue. Differential counts were carried out on Giemsa-stained preparations made in a Shandon cytocentrifuge using 0·2 ml of cells suspended at a concentration of 10^5 cells/ml spun for five minutes at 500 r.p.m.

METHODS OF ISOLATION

The methods assessed are listed in Table 2. Other methods were also tried but are not listed in this or the following tables because there is only a limited amount of data on them.

Table 2 *Methods assessed for preparation of mucosal cell suspensions*

1. Stirring in HBSS for 30 min at 37°C*
2. Stirring in hyaluronidase 12·5 mg/ml for 15 min followed by stirring in pronase 2 mg/ml for 30 min at 37°C
3. Stirring in pronase 2 mg/ml for 30 min at 37°C
4. Soaking in pronase 2 mg/ml overnight at 4°C followed by stirring in pronase 2 mg/ml and DNAase 0·025 mg/ml for 30 min at 37°C
5. Soaking in collagenase 0·5 mg/ml and hyaluronidase 1 mg/ml overnight at 4°C followed by stirring for 30 min at 37°C

All enzymes were dissolved in HBSS.
*Stirring in 10 ml/100 mg tissue in a 20 ml glass universal container with magnetic stirrer.

CELL CULTURE

For cell culture, the cells were suspended in Ham's F10 medium supplemented with human serum and antibiotics as described above. The cell concentration was adjusted to 10^6 viable cells/ml. Cultures were carried out in rigid polystyrene round-bottom microtitre plates (Flow Laboratories: cat. no.

76-211-05), each well of which contained 0·1 ml of the cell suspension (approximately 10^5 viable cells). The plates were sealed and then incubated at 37°C. At the end of the culture period, the cells were harvested from the wells by aspirating the suspension and washing the wells twice with 0·1 ml PBS, counted and their viability determined. Differential counts were made on cytocentrifuge preparations.

IMMUNOFLUORESCENCE

Alcohol-fixed cytocentrifuge preparations were stained with fluorescein isothiocyanate-conjugated rabbit anti-human IgG, IgA, and IgM antisera (all obtained from Behringwerke). The specificity of the fluorescence was checked by absorbing the antisera with the appropriate immunoglobulin and by blocking the fluorescence with unlabelled antisera. The microscope was a Zeiss Photomicroscope II with incident illumination by an HBO 200 W/4 lamp.

In vitro IMMUNOGLOBULIN SYNTHESIS

This was assessed by the method originally described by Hochwald *et al.* (1961). Cell suspensions were incubated in 1 ml modified Eagle's medium containing 1 μCi/ml each of ^{14}C-lysine and ^{14}C-isoleucine (both with specific activity greater than 270 m Ci/mmol: Radiochemical Centre), gentamicin 100 μg and nystatin 100 units. After 48 hours incubation, the cultures were frozen and thawed, dialysed, lyophilised, and then reconstituted and subjected to micro-immunoelectrophoresis. Carrier proteins as described by Lai A Fat *et al.* (1976) were used because of the small amount of protein present in the concentrated culture fluid. The antisera used were also as described by Lai A Fat *et al.* (1976). Autoradiographs were made of the washed and dried immunoelectrophoresis slides.

Results

CELL PREPARATION METHOD

After an incubation of 30 minutes, fragments of undigested tissue and mucus remained, regardless of which enzyme or enzyme combination was used. Clumping in the cell preparations obscured the cell morphology and made it difficult to obtain accurate cell counts. To try to reduce clumping, EDTA at a concentration of 1 mM/l was added to the washing medium. However, it only partially succeeded in reducing the clumping and also reduced the total cell yield compared with the normal washing medium.

Incubation periods longer than 30 minutes resulted in lower cell yields. This effect was shown by the action of pronase on peripheral blood lymphocytes. After 30 minutes' incubation, more

than 95% were recovered, while after 90 minutes only 22% were still viable.

After 30 minutes' incubation at 37°C, considerable numbers of cells remained in the lamina propria when fixed sections of the remaining tissues were examined. It was therefore decided to assess the effect of leaving the tissue in the enzyme solution at 4°C overnight in the hope that this would allow better penetration of enzyme into the fragments. This modification gave much better cell preparations and yields with much greater tissue breakdown and little apparent loss of viability in the recovered cells.

The cell yields and the percentage of 'lymphoid' cells obtained are shown in Tables 3 and 4. In the differential counts, the cells have been classified into epithelial, 'lymphoid', and other cells. Cells termed 'lymphoid' were typical lymphocytes and plasma cells; the 'other' cell category included macrophages,

Fig. 1   *Cytocentrifuge preparation of cell suspension prepared from human small intestinal mucosa, showing mononuclear cells with the appearance of plasma cells, and damaged epithelial cells. Giemsa × 750.*

Table 3   *Total yield of cells from gastric mucosa with percentage of 'lymphoid' cells and viability*

Method*	No. of samples	Yield/100 mg ± SEM	Per cent 'lymphoid' cells	Per cent viability
Hyal.† + Pro.	11	$1.03 \pm 0.25 \times 10^6$	35.9	83
Pro. for 30 min	6	$1.19 \pm 0.66 \times 10^6$	27.7	67
Collag. + Hyal. overnight	7	$11.44 \pm 2.20 \times 10^6$	47.4	86

*Details of method listed in Table 2.
†Hyal.: hyaluronidase; Pro.: pronase; Collag.: collagenase.

Table 4   *Total yield of cells from small intestinal mucosa with percentage of lymphoid cells and viability*

Method*	No. of samples	Yield/100 mg ± SEM	Per cent lymphoid cells	Per cent viability
Stirring	12	$6.61 \pm 1.37 \times 10^6$	32.4	30
Hyal.† + Pro.	12	$4.30 \pm 1.09 \times 10^6$	25.6	87
Pro-overnight + DNAase	4	$8.58 \pm 3.07 \times 10^6$	32.4	92
Collag. + Hyal.	8	$6.32 \pm 1.06 \times 10^6$	34.5	87

*Details of method listed in Table 2.
†Key to abbreviations: see Table 3.

Fig. 2   *Cell suspension prepared from small intestinal mucosa. Phase contrast illumination. Trypan blue stained. Many epithelial cells which have taken up dye are present; most mononuclear cells exclude the dye.*

eosinophils, mast cells, and cells that could not be identified. Figure 1 illustrates a cytocentrifuge preparation of the cell suspension obtained from a specimen of gastric mucosa, in which cells with the appearance of plasma cells are recognisable, together with damaged epithelial cells and some cell debris. Figure 2 illustrates a suspension of cells from jejunal mucosa viewed by phase contrast after trypan blue staining. The epithelial cells have taken up the dye, but the mononuclear cells are unstained.
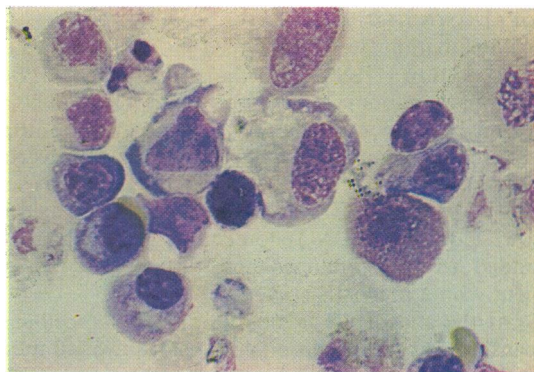
With gastric mucosa (Table 3), the yield of 11.4×10^6 cells/100 mg was approximately 10 times higher with incubation overnight in the cold followed by 30 minutes at 37°C than with other methods. Incubation with collagenase and hyaluronidase for 30 minutes at 37°C without overnight incubation in one experiment gave a yield of 4.3×10^6 cells/100 mg while collagenase alone for 30 minutes at 37°C resulted in 2.7×10^6 cells/100 mg. Other enzymes and enzyme combinations including neuraminidase and trypsin, gave similar yields of $3 - 4 \times 10^6$ cells/100 mg. In view of the results with collagenase and hyaluronidase using overnight incubation plus 30 minutes at 37°C, these other methods were not investigated further.

The results obtained with small intestinal mucosa are listed in Table 4. Regardless of the method used, the total yield of cells was of the range $4.3 - 8.6 \times$

10^6 cells/100 mg tissue. Other methods which were used on one or two occasions, such as pronase and trypsin, for 30 minutes at 37°C or pronase alone with overnight incubation at 4°C followed by 30 minutes at 37°C gave similar yields but with lower viability or a lower percentage of 'lymphoid' cells. Incubation with collagenase and hyaluronidase in the cold followed by 30 minutes at 37°C resulted in the best preservation of cell morphology compared with the other methods along with the highest percentage of lymphoid cells.

A variety of methods of isolating cells from the colon were attempted but none has been satisfactory, giving yields of less than 10^6 cells/100 mg tissue. These cell yields are not presented in detail because the large amount of mucus still present at the end of the incubation period causes cell clumping which makes the counts unreliable.

CELL CULTURE
The cell suspensions could be satisfactorily maintained in short-term culture. Ten technically satisfactory cultures were set up in microplates. In eight of these cultures, the yield of 'lymphoid' cells after 24 hours' incubation was 50% or more of the original number of 'lymphoid' cells in the culture. Cells recovered after incubation periods of 24 and 40 hours are illustrated in Fig. 3. The result of a typical experiment is shown in Table 5. This shows that there was a considerable fall in cell numbers in the first 24 hours, after which the cell counts declined slowly.

In vitro IMMUNOGLOBULIN SYNTHESIS
Nine cultures of stomach and small bowel cell suspensions were set up, each containing $0.5 - 5.5 \times 10^6$ 'lymphoid' cells. The amount of immunoglobulin synthesised was graded on a semi-quantitative scale as described by van Furth *et al.* (1966). IgA synthesis was detected in seven cultures. The

Table 5 *Recovery of 'lymphoid' cells after culture in vitro*

Time (h)	Cell number	Per cent 'lymphoid' cells	Number 'lymphoid' cells	Per cent recovery
0	15.0×10^5	87	13.1×10^5	100
24	9.2×10^5	87	8.0×10^5	61
48	7.6×10^5	91	6.9×10^5	53

intensity of labelling of the IgA line tended to increase with the number of 'lymphoid' cells in the culture (Table 6). IgG production was found in six of the cultures and IgM in one. In all these preparations, the intensity of the labelling was much less than that observed for IgA.

Table 6 In vitro *synthesis of immunoglobulins and secretory component by cultures of gut lymphoid cells*

Source of cells	Culture number	Number of lymphoid cells in culture	Intensity of labelling autoradiograph		
			IgA	IgG	IgM
Stomach	530	1.1×10^6	±	±	−
	531	1.1×10^6	±	±	−
	532	0.8×10^6	±	−	−
	554	5.4×10^6	+++	±	−
Small intestine	464	0.4×10^6	−	−	−
	473	1.5×10^6	++++	±	+
	481	1.2×10^6	±	±	−
	551	1.5×10^6	±	±	−

IMMUNOFLUORESCENCE
When ethanol-fixed cytocentrifuge preparations were stained with anti-immunoglobulin conjugates, large numbers of IgA containing cells and small numbers of IgM and IgG containing cells were seen (Fig. 4). With seven specimens, three separate cytocentrifuge preparations were made, one of which was stained with each anti-immunoglobulin antiserum. The total number of fluorescent cells in five high-power fields was counted for each prepara-
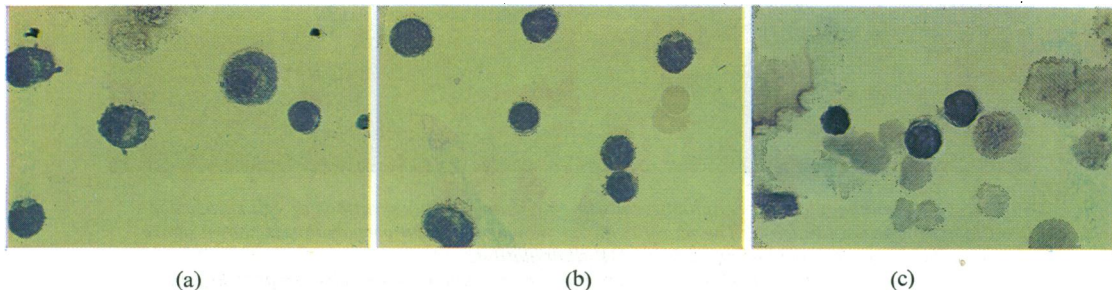

(a)   (b)   (c)

Fig. 3 *Cytocentrifuge preparation of cells from gastric mucosa recovered after culture in microplates.* (a and b) *Cells removed from culture after 24 hours.* (c) *After 40 hours in culture the cytoplasmic staining is darker, but both cell types are still present. Giemsa × 600.*
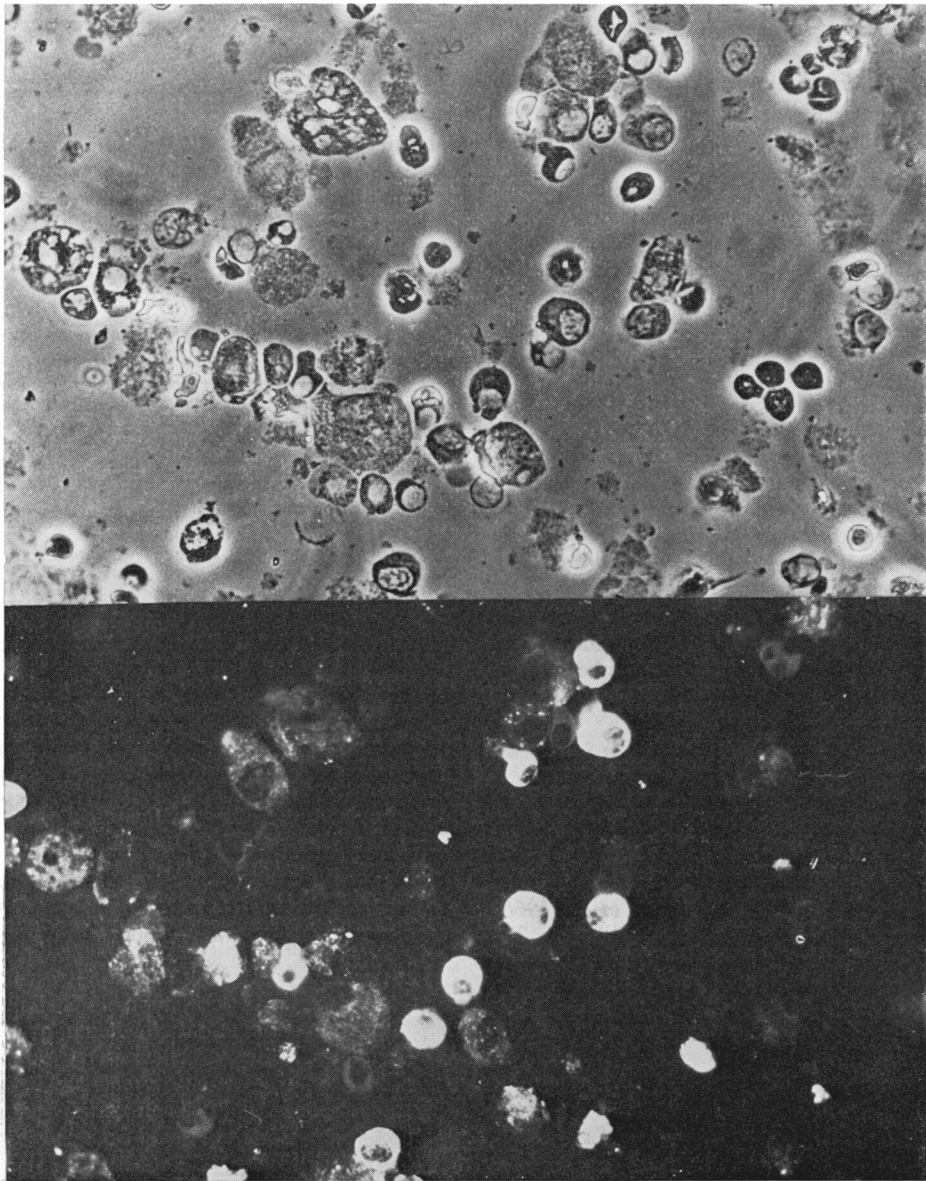
Fig. 4   (a-c) *Immunoglobulin containing cells in cytocentrifuge preparations of cell suspensions from human intestinal mucosa. The phase contrast view of the fixed preparation is shown above the same field photographed using incident UV illumination.*
(a) *Colonic mucosal cells (anti IgA). Intensely fluorescent mononuclear cells are present, and weaker granular fluorescence is seen in some large cells (probably damaged epithelial cells).*
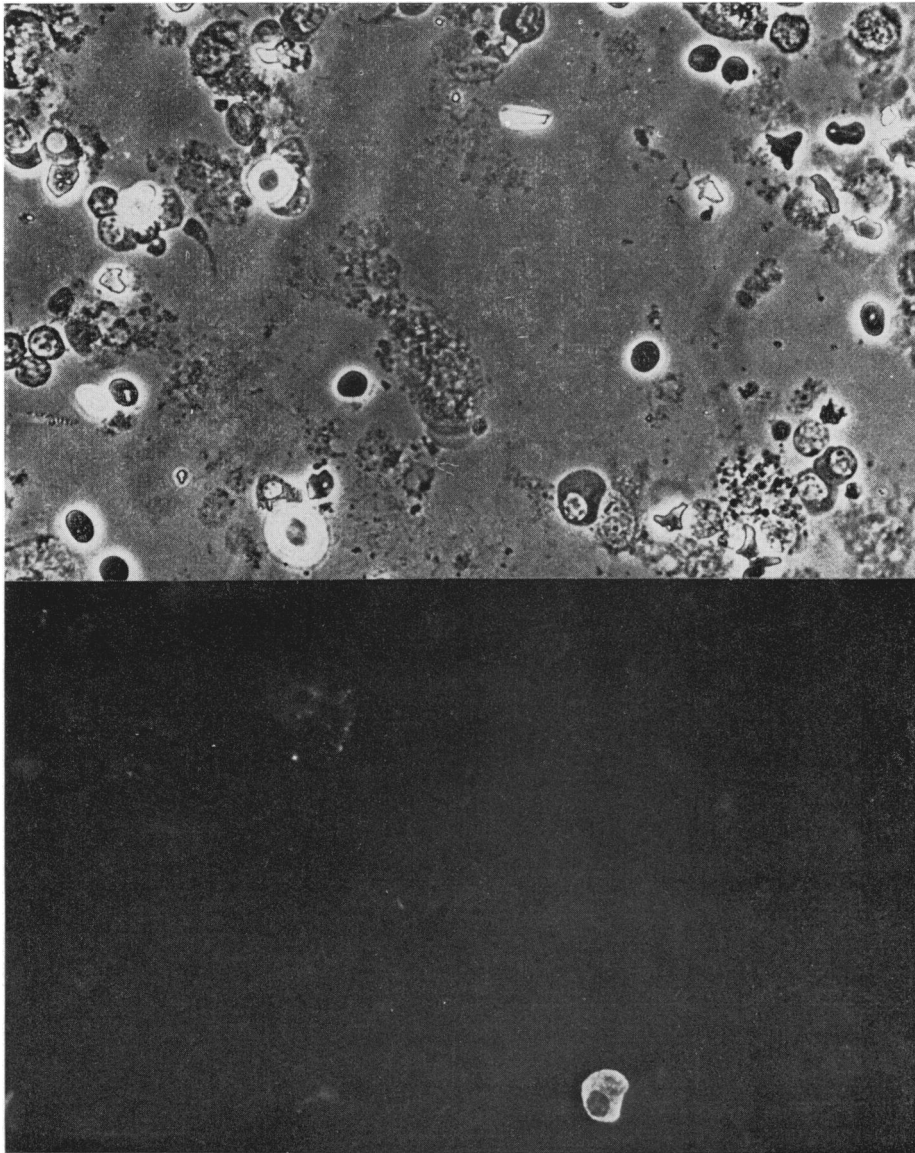
Fig. 4b   *Colonic mucosal cells (anti IgM). A single intensely fluorescing cell is seen in a field containing both mononuclear and epithelial cells.*
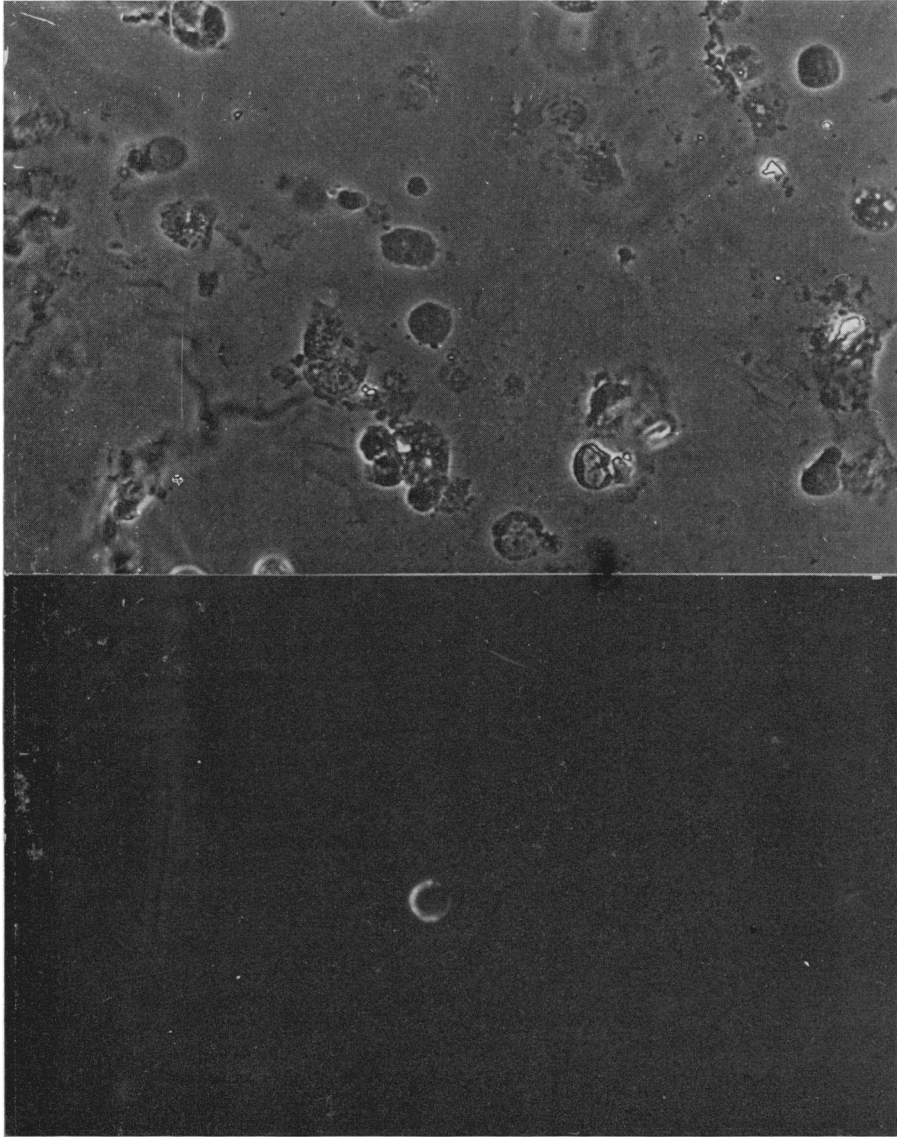
Fig. 4c   *Small intestinal mucosal cells (anti IgG). A single cell showing cytoplasmic fluorescence is present in this field.*

tion and the ratio of cell numbers for each Ig class determined. In these seven specimens, the ratio of IgA to IgG containing cells ranged from 2·6 to 1 up to 15 to 1 and the IgM to IgG ratios ranged from 0·5 to 1 up to 3·8 to 1.

An approximation of the percentage of lymphoid cells containing intracellular IgA was obtained by counting the number of fluorescent cells and the total number of cells in at least five high-power fields. The number of IgA cells was then expressed as a percentage of the total number of cells. From the differential counts performed on Giemsa-stained preparations, the percentage of lymphoid cells was known and from these two percentages, the percentage of lymphoid cells which contain IgA was calculated. This came to a mean of 40·1 % (SEM ± 8·7 %) in 14 preparations.

Discussion

CELL ISOLATION
Of all the methods tested, overnight incubation with collagenase and hyaluronidase in the cold followed by stirring for 30 minutes at 37°C gave the best results. Other methods described here, or variations of them, failed to give as good total yields or as many viable cells.

Further work is needed to develop a satisfactory method for making cell suspensions from normal colonic mucosa. The two main problems appear to be the very thick layer of mucus and the relatively small numbers of lymphoid cells compared with the stomach and small intestine.

Several other methods of isolating human lymphoid cells from the normal gut have been described but they have failed to give good results in our hands. Nind *et al.* (1973) worked with cell suspensions prepared from colonic carcinomas, which therefore cannot be assumed to be similar to the lymphocytes present in the normal gut. These workers gave no indication of the cell yield in relation to the weight of the original tissue, nor of the cell type isolated.

Breucha and his colleagues (Breucha *et al.*, 1974a,b) have published two abstracts of methods of isolating gut lymphocytes, one using filtration through a nylon wool column and the other combining that with collagenase treatment. The results have not yet been published in detail. Mavligit *et al.* (1974) isolated gut lymphocytes by scraping the mucosa and passing the resulting suspensions through a wire mesh. Again, no details of cell yields are given.

Clancy (1976) obtained yields of 10 − 30 × 10⁶ cells 'per square inch' of mucosa by homogenising the mucosa and filtering the crude suspension through two columns of siliconised glass beads. The final cell preparations contained 97 % lymphocytes

but, as no information was provided about cell yields during the separation procedure, the results cannot be compared directly with ours.

In summary, no method of isolation of lymphoid cells of the human gastrointestinal tract has been adequately documented and illustrated. All previous reports omit important information, particularly with respect to cell yields at each point in multistage isolation procedures. Furthermore, in attempts to prepare enriched suspensions of lymphoid cells from mucosa, the possibility has not been excluded that there may be selective enrichment of certain cell types, yielding a final preparation which is not representative of the lymphoid cells present in the mucosa itself. Pretlow *et al.* (1975) have emphasised the importance of documenting such points in applying any cell separation techniques.

IMMUNOFLUORESCENCE
The data on immunoglobulin-containing cells have been expressed as ratios of IgA: IgG and IgM: IgG. These ratios give only an approximate indication of the relative proportions of the various immunoglobulin-containing cells. To obtain accurate data on the relative proportions of immunoglobulin-containing cells, labelling with two fluorochromes is required as described by Brandtzaeg *et al.* (1974). However, some points can be made from this preliminary study.

In all cell suspensions, approximately 40 % of the 'lymphoid cells' contained immunoglobulin and the majority of these stained with anti IgA. This is in agreement with the observations of many other workers—for example, Crabbé *et al.* (1965)—who have shown in sections of normal gut mucosa that the vast majority of immunoglobulin-containing cells stain for IgA. There is no comparable description of suspensions of human gut cells. Rudzik and Bienenstock (1974) studying rabbit mucosa found that only 1 % of a suspension of gut lymphoid cells showed cytoplasmic immunofluorescence (usually IgA). This figure is very low in comparison with observations on tissue sections, and suggests that an important selection of cells occurred in the initial separation or subsequent fractionation procedures. In suspensions of mouse lamina propria cells, there were about 20 % IgA plasma cells (Cebra *et al.*, 1977) which is comparable with our findings (40·1 %).

IMMUNOGLOBULIN SYNTHESIS
The smallest numbers (0·5 − 1 × 10⁶) of lymphoid cells used in the cultures synthesised amounts of immunoglobulin which were just detectable by labelling of the autoradiographs. This may be compared with the findings of van Furth (1966) who found that cultures of 1 − 1·5 × 10⁶ spleen cells synthesised amounts of immunoglobulins which

were just detectable using a similar technique. Allowing for differences in the lymphoid cell populations in gut and spleen, the amount of IgA synthesised by 10^6 gut lymphoid cells appears roughly comparable with the amount of IgG produced by a similar number of spleen cells.

In some experiments, the total number of cells isolated from 100 mg fragments were incubated in the culture system. Radioautographs of the supernatant fluids of these cultures showed a similar intensity of labelling to that seen when whole tissue fragments of similar weight were studied in the same system.

CELL CULTURE

It has proved possible to maintain these crude cell preparations in short-term culture. Over the first few hours, there is usually a heavy loss for which three possible explanations have been considered. The first is that the number of viable cells is less than that indicated by Trypan blue exclusion. The second reason is that the cell suspensions contain many dead and dying cells and cell debris which may be cytotoxic and so reduce the number of cells recovered.

It is also likely that a proportion of the cells is not recovered because they have adhered to the microplate and are therefore not harvested at the end of the incubation.

Conclusion

We have described a satisfactory method for the initial preparation of suspensions of cells from small fragments of human gastrointestinal mucosa. These cells contain and synthesise immunoglobulins and can be maintained in short-term culture. Further work is needed to obtain satisfactory cell yields from the colon. The next stage in this work is to develop methods which will remove epithelial cells from the suspension without selectively depleting subpopulations of lymphoid cells.

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