Human colonic intraepithelial lymphocytes suppress in vitro immunoglobulin synthesis by autologous peripheral blood lymphocytes and lamina propria lymphocytes

G K Sachdev, H R Dalton, P Hoang, M C DiPaolo, B Crotty, D P Jewell

Abstract

Human colonic intraepithelial lymphocytes have been shown to suppress the proliferation of autologous lamina propria lymphocytes and allogeneic peripheral blood mononuclear cells. This study has shown that, in vitro, intraepithelial lymphocytes suppress IgA and total immunoglobulin synthesis (but not IgG or IgM production) by autologous peripheral blood and lamina propria lymphocytes. This down regulation of IgA production is mediated by a soluble factor secreted by the intraepithelial lymphocytes. There is no difference in immunoglobulin down regulation by intraepithelial lymphocytes of control subjects and patients with inflammatory bowel disease.

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Human intraepithelial lymphocytes (IEL) are found on the basement membrane between the enterocytes and have a close spatial relationship with luminal antigens, including many potentially pathogenic organisms. Although phenotypically most IEL are CD3⁺ CD8⁺,¹⁻⁵ the function of these cells, especially in humans, is not well defined. This is mainly because of the difficulty in isolating IEL in samples adequate in size and purity. A method of isolating IEL from resected human colon has recently been developed,⁶ the isolated IEL preparation having a similar distribution of phenotypes as determined by tissue section. The human colonic IEL thus isolated were shown to suppress the lectin driven proliferation of autologous lamina propria lymphocytes.⁶ In another recent study, IEL isolated from human small intestine suppressed the proliferative response of allogeneic peripheral blood lymphocytes (PBL).⁷ The effect of human IEL on immunoglobulin (Ig) production has also been studied, but with conflicting results. Greenwood *et al*¹ showed an immunoregulatory effect of IEL on pokeweed mitogen stimulated Ig synthesis by peripheral blood PBL. IEL helped Ig synthesis by PBL when added in low numbers, but increasing the proportion of IEL in the cocultures suppressed the secretion of IgA and IgM classes of Ig. However, Smart *et al*⁸ failed to confirm this effect of IEL using a similar system.

This study aimed to evaluate further the suppressor function of human colonic IEL and, in particular, to assess the effect of IEL on Ig production by PBL and lamina propria lymphocytes (LPL) in controls and patients with inflammatory bowel disease.

Methods

PATIENTS

For the PBL studies, colonic resection specimens were collected from nine control patients (carcinoma (7), diverticular disease (2); 4 men and five women, with a mean (SD) age of 61 (10) years) and from nine patients with inflammatory bowel disease (ulcerative colitis (7), and Crohn's disease (2), four men and five women with a mean age of 39 (14) years).

For the LPL studies, colonic tissue was used from eight control patients (carcinoma (5), idiopathic constipation (1), appendicular abscess (normal caecum) (1), and 'cap polyposis' (1); five men and three women with a mean age of 55 (17) years. Six patients with inflammatory bowel disease were studied (Crohn's disease (4) and with ulcerative colitis (2); mean age 36 (18) years.

Specimens from the control patients were taken at least 7 cm away from any macroscopic lesion and were subsequently shown to be histologically normal. In the patients with inflammatory bowel disease, uninfamed or the least inflamed parts of the specimen were collected.

ISOLATION OF IEL

The method used was the same as that described by Hoang *et al*.⁶ In brief, the specimen was washed three times with Hanks balanced salt solution (HBSS) without calcium and magnesium (Flow, Irvine, Scotland) containing penicillin 100 U/ml and gentamicin 50 µg/ml. The mucosa was carefully dissected and washed several times in the same buffer. The mucosal strips were incubated for 15-20 minutes at 37°C in 1 mM dithiothreitol (DTT, Sigma) in RPMI 1640 with L-glutamine and 25 mM Hepes buffer supplemented with 10% fetal calf serum (FCS) and antibiotics, as above. After washing the mucosal strips with HBSS a further three times, they were incubated with 0.75 mM disodium EDTA (prepared in HBSS and supplemented with 10% FCS, antibiotics, and 10 mM Hepes buffer) in a shaking water bath at 37°C at 160 oscillations per minute for 35 minutes. The supernatant was collected and the mucosal strips were incubated twice more with 0.75 mM EDTA for 30 minutes (the supernatant was collected between each incubation). The supernatants were pooled and centrifuged at 500 g for 8 minutes and the cell pellet was washed three times with RPMI 1640 with FCS (10%). The cells were finally resuspended in RPMI and stored overnight at 4°C. IEL were separated from

Department of
Gastroenterology, The
Radcliffe Infirmary,
Oxford

G K Sachdev
H R Dalton
P Hoang
M C DiPaolo
B Crotty
D P Jewell

Correspondence to:
Dr D P Jewell, The Radcliffe
Infirmary, Oxford OX2 6HE.

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the epithelial cells by passing the suspension through a glass-wool column (Sigma). The filtrate was centrifuged and the IEL containing cell pellet was further purified by performing a two step Percoll gradient of 44% and 67.5% at 800 g, as previously described.⁹ IEL were recovered from the interface, washed, and suspended in RPMI with 10% FCS to a final concentration of 1×10^6 cells/ml.

ISOLATION OF LPL

In some experiments LPL were isolated from the same mucosal strips, using a modification of the method described by Bull and Bookman.¹⁰ Briefly, mucosal strips from the above experiment were incubated twice more with 5 mM EDTA in a shaking water bath at 37°C, washed with HBSS, and the supernatant discarded. The strips were subsequently minced into fine pieces and incubated overnight in a shaking water bath at 37°C with 100 ml of RPMI containing 10% FCS, antibiotics, and 30 mg *Clostridium histolyticum* collagenase (Boehringer Mannheim GmbH, Germany). At the end of the incubation, the digestate was mixed vigorously, filtered through a 100 μ m nylon mesh (Lockertex, Warrington, UK), and washed a further three times in HBSS. LPL were recovered after centrifugation over a Ficoll-Paque (Pharmacia, LKB, Uppsala, Sweden) gradient at 500 g, washed three times in RPMI with 10% FCS, and resuspended to a final concentration of 1×10^6 cells/ml.

ISOLATION OF PBL

Heparinised venous blood was obtained from the same patients. It was diluted with an equal volume of HBSS with Ca and Mg, and the PBL were isolated over a Ficoll-Paque gradient. The cells were washed three times with HBSS and

once with RPMI with 10% FCS before suspending them in RPMI with 10% FCS to a concentration of 1×10^6 cells/ml.

PHENOTYPIC ANALYSIS

IEL were stained with monoclonal antibodies (Simultest helper/suppressor – CD4/CD8, Becton-Dickinson) and were analysed by flow cytometry (FACScan, Becton Dickinson, Sunnyvale CA, USA) as previously described.⁶ The viability of the cells were assessed using 0.1% Trypan blue.

CELL CULTURE AND COCULTURE

0.5×10^6 PBL were cultured in triplicate at 37°C with 5% CO₂ for 9 days, in the presence or absence of pokeweed mitogen (Gibco) at a final concentration of 1 in 100. In initial experiments (n=4), 0.1×10^6 or 0.2×10^6 IEL were added to PBL. In all subsequent experiments 0.5×10^6 IEL were added to 0.5×10^6 PBL to a final volume of 1 ml.

In a further series of experiments, 0.5×10^6 LPL were used as responder cells (instead of PBL) and similar cocultures with 0.5×10^6 IEL were performed as described above. At the end of the incubation period, the supernatant was collected from all the cultures, centrifuged, and stored at -20°C for subsequent Ig analysis by ELISA.

In 10 of the above patients (five control and five inflammatory bowel disease) proliferation suppression studies were performed simultaneously, as previously described.⁶ Briefly, 5×10^4 LPL with 10 μ g/ml of phytohaemagglutinin were cultured with or without 1×10^5 IEL in 96-well plates for 6 days. Sixteen hours before the end of the culture, 1 μ Ci [³H] methyl thymidine (Amersham Laboratories, Amersham, UK) was added to each well. The cells were then harvested onto filter paper discs and suspended in 10 ml of scintillation fluid (Optiphase Safe, LKB, Poole, Dorset, UK) and counted in a scintillation counter (1215 Rackbeta scintillation counter, LKB).

In two experiments, 1×10^6 /ml IEL were cultured alone for 5 days. The supernatant was then collected, centrifuged, and filtered through a 22 μ m filter. 0.5×10^6 PBL (isolated from normal subjects) were cultured with or without 0.5 ml of IEL supernatant. After 9 days of incubation at 37°C with 5% CO₂, the supernatant was collected and stored at -20°C for subsequent Ig assay.

ASSAY OF Ig BY ELISA

A standard ELISA method as described by Wu *et al*¹¹ was used for measuring total Ig, IgG, IgA, and IgM in the culture supernatant. Briefly, polyvalent goat anti-human Ig (Sigma) and monoclonal rabbit anti-human IgG, IgA, and IgM (Dakopatts) were diluted in 0.01 M bicarbonate buffer (pH 9.6) to a concentration of 4 μ g/ml. 200 μ l of this solution were added to each well in separate 96-well plates, which were then incubated overnight at 4°C. The next day the plates were washed five times with PBS containing 0.1% Tween 20 (PBS-T), and 150 μ l

Figure 1: Secretion of total immunoglobulin by normal peripheral blood lymphocytes from control subjects (n=9) and patients with inflammatory bowel disease (n=9). 0.5×10^6 peripheral blood lymphocytes were cultured for 9 days with or without 0.5×10^6 intraepithelial lymphocytes, both in the absence or presence of pokeweed mitogen. The results are expressed as ng/ml of culture supernatant (5×10^5 cells/ml).

Figure 2: Secretion of IgG by normal control subjects ($n=9$) and patients with inflammatory bowel disease ($n=9$). (See legend to Figure 1.)

of RPMI 1640 with 10% FCS were added to each well. After a further overnight incubation at 4°C and washing the plates five times with PBS-T, 100 μ l of suitably diluted supernatant or standard solution of human Ig (Binding Site, Birmingham, UK) were added. The incubation was performed overnight at 4°C. The plates were washed again with PBS-T. Peroxidase conjugated goat polyvalent anti-human Ig (Sigma) or monovalent rabbit anti-human IgG, IgA, and IgM (Dakopatts) were diluted 1 in 1000 in PBS. 100 μ l aliquots of these antibodies were added to each well and the plates were incubated for 1 hour at room temperature. A fresh substrate was prepared by dissolving 8 mg of OPD (Dakopatts)

Figure 3: Secretion of IgA by normal peripheral blood lymphocytes from control subjects ($n=9$) and patients with inflammatory bowel disease ($n=9$). (See legend to Figure 1.)

in 12 ml of 0.1 M citrate buffer (pH 5.6) containing 5 μ l of H_2O_2 (30% v/v). After washing the plates, 100 μ l of substrate solution were added to each well and the reaction was stopped after 2 minutes by adding 150 μ l of 1M H_2SO_4 . The plates were read within 5 minutes at 492 nm on a Titertek Multiscan reader. A standard curve was constructed and the values of the various immunoglobulins were calculated and expressed as ng/ml of supernatant.

CALCULATION OF THE SUPPRESSOR INDEX (SI)

The suppressor index was calculated for both Ig secretion and proliferation using the following formulae:

$$\text{Immunoglobulin SI} = \left[\frac{1 - \text{PBL} + \text{IEL}}{\text{PBL}} \right] - 100$$

where PBL=immunoglobulin secreted by PBL alone (ng/ml) and PBL+IEL=immunoglobulin secreted by PBL in the presence of IEL (ng/ml)

$$\text{Proliferation SI} = \left[\frac{1 - \text{LPL} + \text{PHA} + \text{IEL}}{\text{LPL} + \text{PHA}} \right] - 100$$

where LPL+PHA=proliferation of 5×10^4 autologous LPL plus 10 μ g/ml PHA (dpm) and LPL+PHA+IEL=proliferation of 5×10^4 autologous LPL plus 10 μ g/ml PHA plus 1×10^5 IEL (dpm)

Positive SI values indicated suppression, and negative values help.

STATISTICS

The results were analysed using the Mann-Whitney U test; $p < 0.05$ was taken as significant.

ETHICS

Approval for the study was given by Central Oxford Research and Ethics Committee.

Results

CELL POPULATION CHARACTERISTICS

The yields of IEL were mean (SD), 0.49 (0.08) and 0.50 (0.07) $\times 10^6$ /g of tissue from the control subjects and inflammatory bowel disease patients respectively. The viability of IEL >90% in all cases, and the mean (SD) CD4/CD8 ratios were 0.20 (0.14) for control and 0.19 (0.086) for inflammatory bowel disease subjects. Populations of IEL with a CD4/CD8 ratio >0.45 were discarded. The yield of LPL was 2.1 (0.4) $\times 10^6$ /g tissue and the viability was >90% in all subjects.

IG SYNTHESIS BY PBL

Compared with control PBL, unstimulated PBL from patients with inflammatory bowel disease produced significantly more total Ig, IgA, and IgM ($p < 0.05$). In control subjects pokeweed mitogen stimulated secretion of total Ig, IgG, and IgM. In contrast, there was a tendency for IgA production to be suppressed. In inflammatory bowel disease patients, however, pokeweed

Figure 4: Secretion of IgA by normal peripheral blood lymphocytes from control subjects ($n=9$) and patients with inflammatory bowel disease ($n=9$). (See legend to Figure 1.)

mitogen did not significantly effect the secretion of immunoglobulins by PBL (Figs 1–4).

Ig SECRETION BY PBL/IEL COCULTURES

In initial experiments, the effect of varying IEL/PBL ratios was examined. Although some suppression occurred at a 1:5 ratio, maximal effects were seen at a ratio of 1:1. An IEL/PBL ratio of 1:1 was therefore used in all subsequent experiments.

In control subjects, IEL significantly ($p<0.05$) suppressed secretion of total Ig from a median of 1066 ng/ml (range 707–1325) to 771 ng/ml (range 356–1093), and IgA from a median of 629 ng/ml (range 213–960) to 315 ng/ml (range 18–451). IEL had no effect on the secretion of these immunoglobulins by PBL incubated with pokeweed mitogen (Figs 1–4).

Figure 5: Suppression of secretion of IgA by peripheral blood lymphocytes from control subjects when intraepithelial are added in a ratio of 1:1 ($p<0.05$). The results are expressed as ng/ml of culture supernatant (peripheral blood lymphocytes 5×10^5 cells/ml $\pm$ intra-epithelial lymphocytes 5×10^5 cells/ml).

Unstimulated PBL from inflammatory bowel disease patients tended to produce less total Ig when cultured with IEL, although this difference was not significant. Secretion of IgA by PBL from these patients was suppressed by IEL both in the absence (711 ng/ml (range 487–993) to 486 ng/ml (range 0–693); $p<0.05$) and presence (551 ng/ml (range 99–871) to 387 ng/ml (range 0–602); $p<0.05$) of pokeweed mitogen. The production of IgG and IgM by PBL was not altered by IEL (Figs 2 and 3).

Although there was considerable variability in the amount of Ig produced by PBL from both control and inflammatory bowel disease patients, IgA production was suppressed in 17 of 18 assays (Figs 5 and 6).

Ig SECRETION BY LPL/IEL COCULTURES

Taking the patients as a whole ($n=14$), IEL significantly suppressed secretion of IgA (from a median (range) of 1369 ng/ml (1216–1742) to 1051 ng/ml (174–1428); $p<0.005$) and total Ig (from 3130 ng/ml (2014–3956) to 2604 ng/ml (1739–3566); $p<0.05$) by unstimulated LPL. In addition, IEL significantly suppressed secretion of IgA (1270 (970–1598) to 911 (94–1319) ng/ml, $p<0.05$) and total Ig (2559 (1928–3890) to 2073 (1846–2906) ng/ml, $p<0.05$) by LPL cultured with pokeweed mitogen, as shown in Figures 7 and 8. However, there were no statistical differences in the amount of suppression of IgA or total Ig by IEL in the control group and the patients with inflammatory bowel disease. As with PBL, IEL had no significant effect on the secretion of IgG or IgM by LPL (data not shown).

SUPPRESSION INDICES (SI)

LPL proliferation experiments

In 10 experiments, IEL mediated suppression of LPL proliferative responses was compared simultaneously with IEL mediated suppression of IgA and total Ig synthesis by PBL.

In all 10 subjects the proliferative response of LPL to 10 μ g/ml of phytohaemagglutinin was

Figure 6: Suppression of secretion of IgA by peripheral blood lymphocytes from patients with inflammatory bowel disease when intraepithelial lymphocytes are added in a ratio of 1:1. (See legend to Figure 5.)

suppressed by IEL from a median of 62376 dpm (24681–132785) to 30411 dpm (59710–8632), $p < 0.05$. The median SI in these proliferative studies was +53.5% (range +30.9 to +98.4).

In the same subjects, simultaneous experiments showed that IEL also suppressed IgA and total Ig secretion by PBL (in all experiments), the median SIs being +40.0% (range +21.6 to 86.8) and +33.1% (range +7.9 to +71.4) respectively. Despite the magnitude of the suppression being similar, there was no linear relationship between the suppressive activity of IEL on the proliferative responses of LPL and IgA or total Ig production.

Supernatant experiments

Supernatant from 5 days' culture of IEL when added to PBL from healthy volunteers ($n=5$) suppressed the secretion of Ig as shown in Figures 9 and 10. There was, however, no effect on the secretion of IgG or IgM (data not shown).

Discussion

IEL constitute a discrete compartment of the immune system. Phenotypically, most IEL are CD8⁺ (suppressor/cytotoxic) T lymphocytes. The CD4/CD8 ratio of isolated IEL in the present study showed a similar distribution to that seen in tissue section. A recent study⁷ failed

to show any cytotoxic activity in a population of human IEL, a finding confirmed by Hoang, Dalton, and Jewell (unpublished observations). Suppressor activity of human colonic IEL to autologous LPL has recently been shown.⁶ The present study shows that IEL are also able to suppress immunoglobulin synthesis by autologous PBL as well as LPL.

There are only two previous studies^{1,8} that have investigated the immunomodulatory role of human colonic IEL on immunoglobulin production. Greenwood *et al*¹ showed that IEL added to PBL in a ratio of 1:20 (IEL:PBL) stimulated IgA and IgM secretion, but increasing the ratio of IEL to PBL resulted in an increasing suppression of the immunoglobulin secretion. In contrast, Smart *et al*⁸ failed to show any effect. One possible reason for these discordant results is the difference in the purity of cell populations isolated in the two studies. The CD4/CD8 ratios of these isolated IEL populations were 0.06–0.14 and 1.21 respectively, suggesting that the latter population was probably significantly contaminated with LPL. The data presented here (using an IEL preparation with a CD4/CD8 ratio similar to the Greenwood study) show that when IEL are used in a ratio of 1:1 with PBL or LPL,

Figure 7: Effect of intra-epithelial on secretion of IgA by lamina propria lymphocytes from eight control subjects and six patients with inflammatory bowel disease, cultured with or without pokeweed mitogen. (Lamina propria lymphocytes 5×10^5 cells $\pm$ intraepithelial lymphocytes 5×10^5 cells.) There was significant suppression of IgA secretion and total Ig secretion (Fig 8) both in the presence and absence of pokeweed mitogen, but no difference between control subjects and patients with inflammatory bowel disease. The results expressed as ng/ml of culture supernatant.

Figure 8: Effect of IEL on secretion of total Ig by lamina propria lymphocytes from eight controls and six patients with inflammatory bowel disease, cultured with or without pokeweed mitogen. (See legend to Figure 7.)

Figure 9: Effect of the addition of 0.5 ml of supernatant from 6 days' culture of intraepithelial to 5×10^5 peripheral blood lymphocytes from healthy volunteers on total immunoglobulin secretion. Peripheral blood lymphocytes were cultured for 9 days with or without pokeweed mitogen. The results expressed as ng/ml of culture supernatant.

total Ig and IgA secretion is significantly suppressed.

Compared with controls, PBL from patients with inflammatory bowel disease produced significantly more total Ig, IgA, and IgM. IgG synthesis was also higher in the inflammatory bowel disease group, but not significantly so. The addition of pokeweed mitogen failed to augment Ig synthesis in these patients, confirming previous observations.^{12,13} The reason for this is not clear, but it could be that in inflammatory bowel disease the B lymphocytes are already in an activated state.

In addition to their ability to down regulate IgA synthesis, IEL suppress proliferative responses of LPL stimulated with phytohaemagglutinin, and the magnitude of suppression is similar in both. The mechanism of action of IEL mediated suppression is not known. The data presented here suggest that it may be caused by a soluble factor released by the IEL into the culture supernatant. Hoang *et al* made a similar observation and also found that IEL mediated suppression was CD8 dependent.⁶ A possible cytokine causing this suppression is interleukin 10, which is known to down regulate a variety of immunological phenomena.¹⁴ Studies are currently in progress to characterise the responsible soluble factor.

IEL down regulate proliferative responses of autologous LPL in vitro,⁶ and this may be an important mechanism by which the gut tolerates the antigen rich environment of the lumen. This effect is also mediated by a soluble factor, which

Figure 10: Effect of the addition of 0.5 ml of supernatant from 6 days' culture of intraepithelial lymphocytes to 5×10^5 peripheral blood lymphocytes from healthy volunteers on IgA secretion. (See legend to Figure 9.)

presumably acts locally in the superficial layers of the mucosa. It is possible, although not proved, that IEL are involved in cell 'trafficking' which would allow them to modulate immune reactions deeper in the lamina propria.

It is interesting that IEL seem to suppress selectively IgA synthesis: the relevance of this in vitro observation to mucosal immune events remains speculative, however. There are several possible explanations for this effect. IgA is the most predominant immunoglobulin spontaneously produced by PBL or LPL,^{8,12} suggesting that IgA precursors are already in a stimulated state or are getting maximal T cell help in the culture. The addition of IEL, which are of suppressor phenotype, check the effect of helper T cells thus reducing the secretion of IgA by B cells. This view is supported by the fact that addition of a mitogen (pokeweed mitogen) to the culture medium tended to suppress IgA. This effect was possibly the result of pokeweed mitogen-stimulation of suppressor T cells in PBL or LPL as removal of CD8⁺ cells from PBL using anti-CD8⁺ coated dynabeads abolishes this effect (unpublished observation). An alternative explanation could be that IEL are actually the down regulating T cells specific for IgA precursor cells, as it has already been shown that separate sets of T cells regulate IgA synthesis by B cells in the mouse,¹⁵ and possibly also in humans.^{16,17}

An alternative explanation for IEL's apparent ability to down regulate IgA secretion specifically is that, given the fact that both PBL and LPL spontaneously secrete more IgA than IgG or IgM, any non-specific suppressive effect of IEL is more likely to be reflected in reduced IgA secretion.

Immune activation in the gastrointestinal mucosa in inflammatory bowel disease is well documented.¹⁸ Given the fact that IEL have potent suppressive functions, a reasonable explanation for this mucosal activation is that there is a defect in IEL mediated suppression in inflammatory bowel disease. However, the present data do not support this view, as IgA synthesis was suppressed equally in control and inflammatory bowel disease subjects. It is possible that the defect in inflammatory bowel disease lies in the IEL's response to specific antigen(s). This is an even more attractive possibility in coeliac disease where the numbers of IEL are greatly increased and a specific antigen is known to be involved in the pathogenesis. Further studies are currently in progress to assess antigen specific suppression in IEL.

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