Inflammatory bowel disease: study of cell mediated cytotoxicity for isolated human colonic epithelial cells

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SUMMARY A better understanding of the mechanism(s) of cell mediated toxicity for colon cells *in vitro* may help clarify the pathogenesis of inflammatory bowel disease (IBD). We have examined both the cytotoxicity of IBD peripheral blood mononuclear cells and the kinetics of induction of such toxicity by soluble plasma factors. Peripheral blood mononuclear cells from IBD patients were found to be cytotoxic for the colon cells. With the use of Chang cells, this cytotoxicity was shown not to be due to an increase in spontaneous cell mediated cytotoxicity. Colon cell toxicity *in vitro* did not correlate with site of disease or severity, but decreased toxicity appeared to be associated with *in vivo* steroid administration. Plasma from some IBD patients was capable of inducing normal peripheral blood mononuclear cells to be toxic to colon cells. This ability was not affected by steroid therapy. The induction capacity of IBD plasma was not associated with the presence of circulating immune complexes, as measured by Raji RIA, suggesting that large complement fixing complexes are not the inducing and directing factors. Unlike findings in other systems, induction could be demonstrated after a one hour preincubation of mononuclear cells with IBD plasma. The kinetics of induction are consistent with the hypothesis that either cytophilic antibody or small circulating immune complexes arm K cells for specific colon cell lysis.

The many alterations in the reactivity of the cellular and humoral elements of patients with inflammatory bowel disease (IBD) initially led to the hypothesis that the immune system is involved in the pathogenesis of IBD. To date, the most compelling support for this hypothesis remains the demonstration of the *in vitro* cytotoxicity of IBD peripheral blood lymphocytes (PBL) for trypsinised colon cells.¹⁻³

The precise mechanism of this *in vitro* cytotoxicity, however, remains poorly defined. Although initial studies suggested that lymphocytes from patients with IBD were intrinsically committed to colon cell lysis,¹ more recent work suggests that there may be an interaction between uncommitted effector cells and soluble 'arming' factors.^{4,5}

It has been suggested that the soluble arming factor(s) could be either (1) cytophilic antibodies (CAb) or (2) circulating immune complexes (CIC) having an antibody specificity for colon cell antigens.

Evidence for this hypothesis stems from a number of observations. First, CAb and CIC have been shown to be capable of inducing specific cytotoxicity in other *in vitro* systems.^{6,7} Second, a previous study has presented some data to suggest that colon cell toxicity induction is produced by a high molecular weight substance, the activity of which is blocked by an anti-immunoglobulin.⁵ Third, the effector cells in the *in vitro* reaction apparently possess surface receptors for the Fc portion of immunoglobulins,⁸ so that either CAb or CIC could theoretically bind to the effector cell and direct its activity. Finally, there is evidence to suggest that CIC occur in patients with IBD,^{9,10} and that the composition of these complexes may be disease specific.¹¹

The kinetics of induction as presently delineated, however, appear inconsistent with the hypothesis that cytophilic antibody or circulating immune complexes initiate and direct cell mediated cytotoxicity for colon cells *in vitro*. Both cytophilic antibody and CIC generally produce rapid induction of cytotoxicity,^{6,7} because the major requirement is binding of the immunoglobulin to the effector cells *via* Fc receptors. In contrast, in the previously re-

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ported cytotoxicity assay, induction of toxicity for colon cells was shown to occur only after four days of preincubation of normal PBL with IBD sera.⁴

There are many factors which influence the demonstration of cytotoxicity, such as the nature of the target cells, effector: target cell ratio, and the duration of the assay. As these assay parameters will also affect the detection of the induction of cytotoxicity, the long induction period previously reported might be an artefact of the assay conditions. Therefore, in the present study we developed a modification of the colon cell cytotoxicity assay to examine the ability of IBD peripheral blood mononuclear cells (PBMC) to produce colon cell toxicity. We studied the kinetics of the induction of cytotoxicity by IBD plasma, to determine if the time course is compatible with an induction mechanism involving either cytophilic antibody or circulating immune complexes. Finally, we looked for an association between the presence of CIC and the ability of plasma to induce cytotoxicity.

Methods

ISOLATION OF PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMC)

PBMC were obtained from 16 healthy controls and 20 IBD patients. The diagnosis of IBD was confirmed by a typical clinical history, contrast x-ray examinations, and histology; at least two of the three being positive in each case. Thirteen patients had ulcerative colitis and seven patients had Crohn's disease. Three of the CD patients had involvement limited to the small bowel by radiography and sigmoidoscopy. Six of the IBD patients had severe, 10 moderate, and four mild disease as defined by the criteria of Truelove *et al.*¹² and deDombal *et al.*¹³ for ulcerative colitis and Crohn's disease, respectively. Eleven of the 20 IBD patients were receiving corticosteroid therapy at the time of the study.

PBMC were isolated using the method of Boyum.¹⁴ Viable lymphocytes comprised 90% of the mononuclear cell population. Unless specifically noted, cells were maintained in Dulbecco's minimal essential media (DMEM) supplemented with 10% heat inactivated foetal calf serum, 100 U/ml penicillin, and 100 µg/ml streptomycin (complete media).

PREPARATION OF COLON EPITHELIAL TARGET CELLS

Surgical specimens from patients undergoing colonic resection for malignancy were the source of epithelial cells. Immediately after resection of the colon, a 4×4 cm piece of adjacent but normal mucosa was excised and placed in complete medium maintained at 4°C. This was rapidly transferred to the laboratory

where it was incubated in 1 mM dithiothreitol (DTT) for 30 minutes at 25°C. The specimens were placed on a dissection tray with the epithelial surface exposed and the remaining mucus and blood removed by flushing the mucosal surface with DMEM and very gentle brushing with forceps. The epithelial surface was removed by gentle scraping with a scalpel blade, continually moistened with the complete medium containing 1% collagenase (CLS 11, Worthington). The colon epithelial cell clumps were incubated in the collagenase DMEM solution for approximately three hours at 25°C, with gentle agitation every 15 minutes. The epithelial cells were then washed several times with complete medium and the cell number and viability were determined using a Neubauer haemocytometer, phase contrast microscopy (Fig. 1) and 0.4% trypan blue exclusion. Approximately 2–3×10⁶ epithelial cells could be obtained from a single specimen with greater than 97% viability. Cytological examination with modified Papanicolaou stain and thin section microscopy confirmed the cells to be epithelial in origin.

ASSAY FOR COLON CELL TOXICITY

Colon epithelial target cells (1×10⁵ in 150 µl of complete medium) were placed in 2.5 ml plastic Eppendorf tubes. Effector PBMC (1×10⁶ in 150 µl complete medium) were then added to the targets. Overnight incubation (10–12 hours) was performed in a 10% CO₂ incubator at 37°C. In each experiment, control tubes consisted of 1×10⁵ targets in a total volume of 300 µl of complete medium. Heat inactivated test plasma, at a 15% concentration, was substituted for the foetal calf serum to examine direct plasma toxicity. All test and control reactions were run in triplicate. One hundred colon epithelial cells from each tube—that is, a total of 300 cells per effector or control incubation—were examined blindly for viability by trypan blue exclusion. Spontaneous colon cell death was approximately 10%. Specific cytotoxicity was defined as the mean cell death occurring in the presence of specific effector cells or test plasma minus the mean spontaneous cell death occurring in the reactions containing only targets and complete medium. There was little variation in cytotoxicity of PBMC from a given individual. A normal individual tested against six different colon cell targets showed a range in cytotoxicity of only 2%.

SPONTANEOUS CELL MEDIATED CYTOTOXICITY (SCMC)

Chang liver cells (Microbiological Associates), maintained in continuous tissue culture, were harvested by treatment with 0.25% trypsin and gentle scraping with a rubber policeman. These cells

Fig. 1 Colonic epithelial cells, under phase contrast microscopy ($400\times$), at different time points during the isolation procedure. (Left) Cells with normal intercellular apposition after one hour of collagenase. (Middle) Cells beginning to lose intercellular junctions after two hours of collagenase. (Right) A typical colonic epithelial cell at the end of the isolation procedure. The short brush border can be seen on the cell.

were washed thoroughly and kept in suspension with complete medium for two hours. Chromium labelling was performed by incubating 1×10^6 cells with $100 \mu\text{Ci}$ ^{51}Cr for 45 minutes at 37°C . After washing three times in 15 ml of medium, 1×10^6 cells in 150 μl complete medium were placed in Eppendorf tubes. One $\times 10^6$ PBMC in 150 μl complete medium were then added and the reaction incubated overnight (10–12 hours) at 37°C . Control tubes containing only target cells in 300 μl of complete medium were used to determine spontaneous release. After incubation the tubes were centrifuged at 900 g for 10 minutes and the supernatants counted for released ^{51}Cr by gamma spectrometry. Spontaneous release after 12 hours was approximately 30%. Maximum release was obtained by multiple freeze-thaw procedures. SCMC was defined as follows:

$$\frac{\text{test cpm} - \text{spontaneous cpm}}{\text{max. releasable cpm} - \text{spontaneous cpm}}$$

PREINCUBATION OF NORMAL PBMC WITH HEAT INACTIVATED PLASMA

Plasma was obtained from six healthy volunteers and 28 IBD patients. Fourteen had ulcerative colitis and 14 had Crohn's disease. Five of the patients with Crohn's disease had disease limited to the small bowel. Ten patients had severe, nine moderate, and nine mild IBD. Thirteen of the patients from whom plasma was obtained were receiving corticosteroids. The plasma from each subject was divided into

multiple 1 ml aliquots and stored at -70°C until use.

Aliquots of frozen plasma were defrosted just before preincubation studies and complement was inactivated by heating for 30 minutes at 56°C . Heat inactivated plasma (300 μl) was added to 4×10^6 normal PBMC suspended in 2 ml DMEM with penicillin and streptomycin and incubated at 37°C in a CO_2 incubator for either one, 24, or 96 hours. The PBMC were then washed three times in 15 ml of complete medium, recounted and the final concentration adjusted to yield 1×10^6 PBMC/150 μl medium. Cytotoxicity against the colon epithelial target cells was assayed as described above. Heat inactivated plasmas without PBMC were also tested in the cytotoxicity assay to determine if plasma carryover might account for colon cell toxicity.

CIRCULATING IMMUNE COMPLEX (CIC) LEVELS

Plasma from IBD and control patients were tested for CIC by Raji cell radioimmune assay as previously described.¹⁵ Briefly, 25 μl of a 1:4 dilution of the test plasma were incubated with 3×10^6 Raji cells at 37° for 45 minutes, followed by washing three times with Hank's buffered salt solution without calcium or magnesium. The washed cells were incubated with 25 μl of ^{125}I -labelled rabbit antihuman IgG (containing 15 μg rabbit IgG), at 4°C for 30 minutes. The cells were washed and the amounts of ^{125}I bound to the Raji cells was determined by crystal scintillation

Table 1 Spontaneous cell mediated cytotoxicity (SCMC) for Chang liver cells

Source of PBMC	Subjects (no.)	SCMC	
		Mean (%)	Range (%)
Healthy controls	6	11.5	5-19
IBD patients	9	12.0	4-22

effector:target ratios identical with those used in the colon cytotoxicity studies, displayed normal SCMC levels.

INDUCTION OF PBMC CYTOTOXICITY FOR COLON EPITHELIUM BY PREINCUBATION WITH HEAT INACTIVATED IBD PLASMA

The results of the induction studies are presented in Fig. 3. Normal PBMC preincubated for 96 hours with inactivated IBD plasma produced significantly greater colon cell toxicity ($P < 0.025$) than PBMC similarly preincubated with normal allogeneic plasma.

To examine the kinetics of this induction, shorter periods of preincubation of normal PBMC with

Fig. 3 Induction of normal peripheral blood mononuclear cell (PBMC) toxicity for colonic epithelial cells by preincubation with heat inactivated plasma. Each point represents the mean cytotoxicity from triplicate assays performed with normal PBMC after preincubation with six healthy control and nine IBD plasmas for 96, 24, and one hour periods. The IBD plasmas induced significantly more cytotoxicity at each preincubation period than plasmas from controls ($P < 0.025$, < 0.005 , and < 0.005 at 96, 24, and one hours, respectively).

heat inactivated plasma were performed. These studies demonstrated that PBMC preincubated with IBD plasma for 24 hours also produced significantly greater colon epithelial cell toxicity ($P < 0.005$) than the same PBMC preincubated with normal plasma (Fig. 3). In fact, as illustrated in the same figure, IBD plasma pretreatment of normal PBMC for as little as one hour resulted in a statistically significant increase in cytotoxicity ($P < 0.005$). It should be noted, however, that heat inactivated IBD plasma, in the absence of PBMC, did not produce colon cell toxicity (Table 2).

Unlike the direct cytotoxicity of inflammatory bowel disease PBMC for colon epithelium, the ability of IBD plasma to induce toxicity was not associated with corticosteroid administration. Furthermore, there was no readily apparent association between disease severity and the ability to induce toxicity.

Four of the IBD plasmas which resulted in PBMC cytotoxic activity were fractionated by Sephadex G-200 column chromatography. The capacity to produce PBMC toxicity for colon epithelial cells eluted in the high molecular weight void volume fractions.

Plasma from 18 IBD patients was examined for both its ability to induce PBMC mediated colon cell toxicity and the presence of CIC by Raji RIA. Only three of 10 plasmas inducing cytotoxicity contained detectable CIC. Furthermore, two plasmas with raised CIC lacked induction capability. Thus, there was no association between the induction of cytotoxicity and the presence of raised levels of Raji detectable immune complexes.

Discussion

The aetiology and pathogenesis of inflammatory bowel disease (IBD) remain unknown. It has been hypothesised that the host immune system is activated, either by external stimuli or by loss of internal suppression, and that it secondarily attacks normal intestinal epithelium. To date the strongest evidence suggesting a role for the immune system in the pathogenesis of IBD has been the demonstration of IBD peripheral blood lymphocyte (PBL) cytotoxicity for trypsinised colon cells *in vitro*.¹⁻³

In the current study, peripheral blood mono-

Table 2 Plasma cytotoxicity for colon epithelial cells

Source of plasma	Subjects (no.)	Plasma cytotoxicity	
		Mean (%)	Range (%)
Healthy controls	7	0.3	0-2
IBD patients	12	0.8	0-6

nuclear cells (PBMC) from patients with IBD again produced significantly greater *in vitro* toxicity for colon epithelial cells than did PBMC from healthy controls. The colon epithelial cells in this study were obtained by mechanical separation and gentle collagenase digestion. Thus, these results not only confirm published findings, but also suggest that the reported cytotoxicity is unrelated to trypsin induced changes in the colon cell surface membrane.

Although the PBMC cytotoxicity for colon cells was significantly increased in the IBD patient group, PBMC from some IBD patients showed no demonstrable cytotoxicity. Patients failing to display cytotoxicity were primarily those receiving corticosteroid therapy, while patients with increased colon cell toxicity were receiving either other forms of therapy or no treatment for their IBD. These data suggest that steroids administered *in vivo* may block IBD PBMC toxicity for colon epithelium *in vitro*. Although the mechanism of this effect was not clearly defined, the above results, coupled with the apparent lack of effect of steroids on induction capabilities of IBD plasma, suggest several potential mechanisms. Steroids may decrease the absolute number of cells capable of undergoing activation, inhibit the ability of lymphocytes to be armed *in vivo*, or suppress the cytotoxic expression of activated lymphocytes. They apparently do not decrease the production of arming factor(s).

The cytotoxic activity of PBMC did not correlate with the clinical activity of IBD. A similar lack of correlation was reported in a study in which a single individual was followed over a seven month period of variable clinical activity.¹⁶ The reported loss of cytotoxicity 10 days after resection of diseased bowel^{3,4} initially appears to be inconsistent with the above findings. However, while those results suggest that diseased bowel must be present for cytotoxicity to be demonstrable, they do not really provide direct evidence regarding the relationship of severity of clinical disease and toxicity of IBD lymphocytes for colon epithelial cells.

It is unclear precisely how the *in vitro* assays relate to the pathogenesis of IBD. In our study, and in the studies of Shorter *et al.*,^{3,4} patients with Crohn's disease as well as those with ulcerative colitis had PBMC toxicity for colon epithelial cells *in vitro*. In fact, PBMC from one of our patients with Crohn's disease limited to the small bowel displayed one of the highest levels of colon cell toxicity. One possible explanation is that the patient with regional enteritis has subclinical disease distal to the small bowel. As clinical methods for detection of disease are relatively crude, a substantial number of patients with no radiographic or sigmoidoscopic evidence of large bowel disease may indeed have mild sub-

clinical colonic involvement. Alternatively, it is possible that the same cytotoxic potential actually occurs in both ulcerative colitis and Crohn's disease but that local cellular or humoral factors alter or regulate the immunological expression *in vivo*.

The restriction of *in vitro* colon cell toxicity to lymphocytes obtained from patients with IBD¹⁷ has been important in implicating cellular immune responses in the pathogenesis of IBD. However, equally important is the demonstration that such toxicity does not merely reflect a generalised increase in spontaneous cell toxicity for cultured human cells. The lack of toxicity of IBD PBMC for Chang liver cells documented in this study suggests that the cytotoxicity for colon cells is not due to increased SCMC in IBD patients.

The present results indicate that heat inactivated plasma from some IBD patients, though itself not toxic, is capable of inducing normal PBMC to become toxic for colon epithelial cells in culture, after 96 hours of preincubation. Furthermore, some IBD plasmas can also induce toxicity after considerably shorter preincubation periods. These rapid induction results are in contrast with the reports of Perlmann and Broberger¹ and Shorter *et al.*⁴ We used plasma rather than serum for induction, but this difference is not likely to be responsible for the disparate results. The most likely explanation for the disparity may be the differences in the cytotoxicity assays. The target cells are prepared differently and thus the population of cells, their antigenic determinants and their susceptibility to lysis may be dissimilar. Furthermore, the effector:target cell ratio, a parameter known to be of great importance in *in vitro* cytotoxicity studies,¹⁸ was greater in our experiments than either of the other two investigations. If only a small percentage of the total effector cell population were inducible, the other studies may have had less than optimal 'induced effector': target cell ratios for observing cytotoxicity. Finally, our assay incubation was performed overnight compared with four hours or less in the other studies. As it is known that certain effector cells can make multiple 'hits',^{19,20} the increased reaction time may have allowed an amplification phenomenon to occur in our system to produce identifiable cytotoxicity by very few 'induced effector' cells. Studies performed over shorter periods would lack such an amplification and therefore fail to demonstrate toxicity.

The previously demonstrated loss of cytotoxicity after colectomy^{3,4} is most compatible with CIC activation and direction of normal PBL, as complexes, but not specific antibody or committed T cells, would be expected to disappear rapidly after resection. The high molecular weight of the 'in-

ducing substance', as suggested by gel chromatography, is also consistent with immune complex induction of cytotoxicity. To further examine the role of CIC, plasmas examined for the induction of cytotoxicity were also examined for the presence of CIC by a Raji cell radioimmunoassay. This technique, which measures a wide range of complement fixing CIC with considerable sensitivity,²¹ demonstrated complexes in several patients. However, there was no demonstrable association between the presence of CIC and the induction capabilities of plasma samples.

Complexes which do not activate the complement sequence or are relatively small in size will not be detected by the Raji radioimmunoassay.²¹ Therefore, while the above data exclude a large population of complexes, they do not preclude the possibility that a particular subset of CIC might be responsible for the induction of cytotoxicity. Further characterisation of the inducing factor should lead to a better understanding of its possible role in the immunopathogenesis of IBD.

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