Normal Inflammatory Bowel Disease Mucosa Conceals Alterations in Natural Killer Cell Activity


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Non-major histocompatibility complex-restricted cytotoxicity or natural killer (NK) activity could be detected in all intestinal lamina propria mononuclear cell preparations of histologically normal mucosa from 57 patients with gastrointestinal disease. Similar levels of NK activity were detected among the different disease groups. Within the inflammatory bowel disease patient group, however, Crohn's disease patients showed a threefold higher level of NK activity than detected in ulcerative colitis patients. Cytotoxicity levels in Crohn's disease patients were also higher than in the control carcinoma patients, whereas ulcerative colitis patients had considerably lower cytotoxicity levels than the carcinoma patients. Thus, unaffected normal inflammatory bowel disease mucosa conceals alterations in NK activity which might occur before the inflammation. The colon adenocarcinoma cell line Caco-2 was found to be a representative target for detecting individual differences in NK activity of lamina propria mononuclear cells compared with standard K-562 targets. The latter can be of relevance when studying mucosal immunoregulatory mechanisms in intestinal disease.

Key words: Crohn's disease; inflammatory bowel disease; natural killer activity; normal mucosa; ulcerative colitis

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Current trends in inflammatory bowel disease (IBD) research do not seem to cover the natural killer (NK) cell in the gut as a participant in the etiopathology of this disease. Nowadays, with the availability of more specific monoclonal antibodies against NK-cell surface antigens and using adapted assay conditions for measuring cytotoxicity, these cells are found to be commonly present in the lamina propria of normal gut mucosa (1–3). Their immunohistochemical detection, in both tissue sections and isolated mononuclear cell preparations, has shown that NK cells comprise only 1% to 3% of the total intestinal lamina propria mononuclear cell (LPMC) population depending on the monoclonal antibodies used (3–6). Intriguingly, the distribution of cells with NK activity in the lamina propria of the gut is proportionally low compared with peripheral blood, and they are of a different phenotype than their peripheral blood counterparts (3, 6–8). Our group has previously described LPMC exerting spontaneous or non-major histocompatibility complex (MHC) restricted cytotoxicity in both short and prolonged chromium-release assays to be NK cells, whereas T cells did not participate in this type of cellular cytotoxicity (3). NK cells have important implications in disease with regard to the killing of microorganisms and virus-infected cells, modulation of the immunoglobulin secretion, and cytotoxicity against malignant cells or epithelial cells that have undergone changes of surface antigen expression (9–11).

In general, studying non-MHC-restricted cytotoxicity or NK activity in LPMC preparations from IBD patients has been discouraging. Cytotoxicity levels were found to be absent or low, and this finding was accompanied by the inability to detect cells with NK-cell markers immunohistochemically or only at very low percentages (12–15). Studies of mucosal NK activity in IBD usually involved cells isolated from inflamed specimens, whereas control LPMC were isolated from normal mucosa of patients with carcinoma. To define the appropriate control mucosal specimens to study the role of NK cells in IBD seems to be of major relevance. Presumably, LPMC isolated from normal mucosa adjacent to the inflamed region represent an appropriate control population by eliminating differences inherent in individuals, disease, or tissue characteristics. In the present study the NK activity of LPMC isolated from histologically normal mucosa was assessed in particular with regard to inflammatory bowel disease. Moreover, the sensitivity of a colonic target cell line for NK activity of LPMC was studied and compared with the standard NK-sensitive K-562 cell line. Immunomodulation of NK activity by inflammatory mediators or gastrointestinal peptides may also require the use of colonic cell lines as exemplary targets for mucosal NK activity.

MATERIALS AND METHODS

Specimens studied
In this study intestinal specimens were obtained from 57 patients, comprising 28 women and 29 men, ranging in age.
from 21 to 89 years, with a mean of 59 ± 2 years, with various diagnoses who underwent a bowel resection in our hospital. The population consisted of 28 patients with colorectal carcinoma (mean age, 67 ± 3 years), 17 individuals with IBD, divided into 9 Crohn’s disease (CD) (mean age, 48 ± 4 years) and 8 ulcerative colitis (UC) (mean age, 45 ± 3 years) patients, and 12 patients with miscellaneous diseases (mean age, 57 ± 4 years)—that is, diverticular disease (n = 5), sigmoid volvulus (n = 2), familial adenomatous polyposis coli (n = 2), Hirschsprung’s disease (n = 1), rectopexy (n = 1), and colon interposition (n = 1). Diagnosis was based on a combination of established clinical, radiologic, endoscopic, and histologic criteria. In the IBD group 3 patients had no medication (2 CD, 1 UC), and 14 received 5-acetylsalicylic acid (5-ASA)-containing drugs, steroid therapy, or a combination of these drugs (7 CD, 7 UC). Macroscopically normal mucosal specimens were taken distant from the tumour or inflamed or otherwise abnormal region. Immediately after resection and dissection by the pathologist the tissue was transported to the laboratory and processed for isolation of LPMC. All mucosal tissue samples were retrospectively judged as being normal according to histologic evaluation by the pathologist. In this study the origin of the intestinal specimens obtained from the various patient groups was divided as follows: all specimens in the carcinoma patients group comprised colonic tissue; in the CD group we obtained seven ileal and two colonic tissues; in the UC group seven colonic and one ileal specimen; and in the miscellaneous diseases group all samples were colonic tissue except for one ileal specimen.

**Isolation of effector cells**

LPMC were isolated by the method described by Bull & Bookman (16) and Fiocchi et al. (17) with slight modifications. In brief, the mucosa was carefully dissected from the underlying submucosa, cut into small pieces of 0.5 to 1 cm², and incubated in 1 mM dithiothreitol (DTT) (Sigma, St. Louis, Mo., USA) in calcium- and magnesium-free Hanks’ balanced salt solution (CMF-HBSS) to remove mucus. Then the tissue was collected, washed extensively and rapidly cut into small fragments. Four to five sequential incubations in ethylenediaminetetraacetic acid (EDTA) (0.75 mM; Merck, Darmstadt, Germany) in CMF-HBSS were performed at 37°C for 1-h periods to remove epithelial cells, followed by overnight incubation at 37°C in medium supplemented with 20 U/ml collagenase CLSPA (Worthington, Freehold, Mass., USA) and 278 U/ml DNase (Worthington). The next morning the cell suspension was

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**Fig. 1A.** Cytotoxicity of freshly isolated lamina propria mononuclear cells (LPMC) from histologically normal mucosa of patients with intestinal disease against K-562 target cells in both 4-h and 18-h [51Cr]-release assays. Mean cytotoxicity levels of the entire patient group studied (total), carcinoma patients (carc.), inflammatory bowel disease patients (IBD), and patients with miscellaneous disease (misc.) are shown. Number of individuals studied in each group as indicated within bars. **Fig. 1B.** Cytotoxic activity of freshly isolated LPMC from normal mucosa of patients with inflammatory bowel disease (CD = Crohn’s disease, UC = ulcerative colitis) against K-562 target cells in both 4-h and 18-h [51Cr] release. Significant difference between IBD subgroups: *p = 0.005, **p = 0.001. Number of individuals as indicated within bars.
filtered through a 50-µm mesh, washed in medium, counted, and analyzed for viability. This heterogeneous cell population was further enriched for mononuclear cells by density gradient centrifugation using Ficoll-Isopaque (1.077 g/ml). Viability of this cell population was approximately 90%, whereas contamination with epithelial cells was rarely found.

**Target cells**

K-562 erythroleukemia cells and Caco-2 colon adenocarcinoma cells were used as targets in the chromium-release assay. The cells were cultured at 37°C in a 5% CO₂/95% air incubator and kept in the growing phase by transferring approximately 1:5 twice a week. Caco-2 cells were used in the cytotoxicity assay when they had reached confluence in culture.

**Cytotoxicity assay**

Both 4-h and 18-h assays were performed using freshly isolated LPMC and target cells labeled with sodium chromate-

\( ^{51} \text{Cr} \) (100 µCi/5 x 10⁶ cells, Amersham Laboratories, U.K.) for 1 h at 37°C. LPMC were tested at a 500:1 culture. NK activity in the gut mucosa according to Gibson et al. (1). This high E/T ratio in mucosal cytotoxicity experiments is used to study differences in activity levels between patient groups and enables the use of less susceptible target

ments is used to study differences in activity levels between patient groups and enables the use of less susceptible target cells, such as Caco-2. Incubations for spontaneous and maximum release of chromium by the target cells were performed in sextuplicate, whereas the incubations with effector cells were done in triplicate. Specific cytotoxicity was calculated by the following formula:

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\text{Cytotoxicity (\%)} = \frac{\text{Experimental release} - \text{Spontaneous release}}{\text{Maximal release} - \text{Spontaneous release}} \times 100.
\]

The intra-assay coefficient of variation of our cytotoxicity assay detecting NK activity by LPMC was 5.2 ± 0.4%.

**Analyses of data**

Statistical analyses were performed using SPSS/PC+ (Microsoft Corp., Redmond, Wash., USA). Data are expressed as the mean ± SEM unless stated otherwise. Student’s t test or separate variance analysis was used to calculate the significance of the differences between groups. Correlations were described using linear regression analysis.

**RESULTS**

The mean cytotoxic activity of LPMC suspensions against K-562 target cells was 13 ± 2% in the 4-h assay (n = 55) and 28 ± 3% in the 18-h assay (n = 54). The cytotoxic activity of LPMC against Caco-2 target cells showed relatively greater differences between the two incubation periods than found using K-562 targets: 4 h, 4 ± 1% (n = 27), and 18 h, 23 ± 4% (n = 26). NK activity of LPMC against K-562 within the different patient groups is shown in Fig. 1A. Considerable NK activity was detected in all patient groups when tested at a 500:1 effector to target cell ratio. Interestingly, the NK activity of LPMC from normal mucosa was not found to differ when the various patient groups were compared in both 4-h and 18-h assays; that is, patients with carcinomas, miscellaneous diseases, or IBD had cytotoxicity levels similar to those calculated for the entire population. However, when further differentiating the IBD group in patients with Crohn’s disease and those with ulcerative colitis, considerable differences in cytotoxicity levels were found in both 4-h (21 ± 4% versus 7 ± 2%, p = 0.005) and 18-h (49 ± 7% versus 16 ± 3%, p = 0.001) assays, hence the level in the Crohn’s disease patient group was threefold the level detected in the ulcerative colitis patient group (Fig. 1B). In addition, the cytotoxicity level in the Crohn’s disease group was higher than the carcinoma patient group (4 h, 21 ± 4% versus 13 ± 2%, p = 0.059; 18 h, 49 ± 7% versus 28 ± 4%, p < 0.02) and the miscellaneous diseases group in the prolonged assay (18 h, 49 ± 7% versus 23 ± 6%, p < 0.01), whereas a lower level of cytotoxic activity was detected in the ulcerative colitis group than in the carcinoma group (4 h, 7 ± 2% versus 13 ± 2%, p < 0.05; 18 h, 16 ± 3% versus 28 ± 4%, p < 0.05). Of importance was the observation that two patients with Crohn’s colitis had high levels of NK activity by LPMC isolated from the normal colonic mucosa (mean NK activity in the 4-h assay, 21%; in the 18-h assay, 47%), whereas cells from small-bowel tissue of a single UC patient showed low NK activity (4-h assay, 8%; 18-h assay, 17%).

No relation was found between drug therapy and the apparent differences in NK activity as detected in LPMC preparations from CD and UC patients in particular. Furthermore, both UC and CD groups were age-matched, and no relation was found between age and NK activity levels either in the entire study group or in the disease subgroups. Further stratifying the miscellaneous diseases group did not show differences in NK activity between patients with mucosal or physical changes of the intestine.

Linear regression analysis was performed to evaluate individual differences in NK activity against Caco-2 target cells compared with the activity measured against the standard NK-sensitive K-562 cells in both 4-h and 18-h assays. Levels of individual cytotoxicity between 4-h and 18-h assays, irrespective of using either K-562 or Caco-2 target cells, were found to correlate remarkably well (Fig. 2). Hence individuals with high or low levels of cytotoxicity in the standard 4-h \(^{51}\text{Cr}\)-release assay against K-562 were also found to have high or low cytotoxicity levels, respectively, in the K-562 18-h, Caco-2 4-h, and Caco-2 18-h assays as well.

**DISCUSSION**

Non-MHC-restricted cytotoxicity or NK activity in LPMC preparations from intestinal mucosa could be demonstrated...
in each individual patient by the use of a high effector to
target cell ratio. This was earlier described by Gibson et al.
(1), but curiously, it has not yet been introduced as a common
procedure to study mucosal NK activity despite immuno-
histochemical and functional experiments demonstrating NK
cells to be present in low proportions. It therefore remains
difficult to understand the biologic significance of such a
sparsely distributed cell in mucosal immunopathology. So
far, most studies have reported difficulties in detecting NK
activity in LPMC or LPL preparations, or only very low
levels have been found when using regular (50:1) effector
to target cell ratios (6, 12, 14, 15). Significant levels of
cytotoxicity, however, were obtained not only by introducing
high effector to target ratios but also by preincubating the
effector cells, prolonging the assay duration (2, 13, 18), and
through enriching for NK cells by panning (6). The use of a
high E/T ratio enabled us to detect differences in the func-
tional contribution of lamina propria NK cells with regard
to inflammatory bowel diseases. Such an observation could
have been obscured in previous studies due to the use of
low E/T ratios, thus failing to detect significant NK activity
levels.

Our group recently described the cell responsible for the
spontaneous cytotoxic activity by LPMC preparations in
both 4-h and extended 18-h assays as having NK-cell charac-
teristics (3). Depletion of cells with specific NK-cell markers
from LPMC resulted in abrogation of cytotoxicity in both
4-h and 18-h assays. However, these effector cells are of a
somewhat different phenotype—that is, all CD56+ and some
CD16+—than the equivalent functional cells in peripheral
blood which co-express both CD56 and CD16 differentiation
antigens. Moreover, CD3+ T cells were not found to con-
tribute to this type of non-MHC restricted cytotoxicity in
the intestinal lamina propria, as demonstrated by functional
depletion experiments.

Cytotoxicity levels by LPMC were reported to be low in
IBD when using low E/T ratios, as shown by Fiocchi et al.
(5) and MacDermott et al. (13), and no differences were
reported between Crohn’s disease and ulcerative colitis. By
using the low effector to target ratio in a prolonged 24-h
assay, Beeken et al. (18) found significant levels of cyto-
xicity in LPMC preparations of both normal and inflamed
mucosa from Crohn’s disease patients. In addition, when
combining the high effector to target cell ratio and a pro-
longed 24-h assay, Gibson & Jewell (4) detected significant
levels of NK activity in LPMC preparations, but they were
found to be independent of the underlying disease, drug
therapy, or anatomic origin of the effector cells. In contrast
to our study presented here, they did not describe differences
between Crohn’s disease and ulcerative colitis patients, and
almost all tissue samples included in their study were taken
from inflamed mucosa. In general, studies on NK activity in
IBD comprised inflamed mucosa, and LPMC from unaf-
Aected normal mucosa from carcinoma patients or from
patients of a miscellaneous group served as controls. In the
present study LPMC isolated from normal mucosa distant
from the tumor region in carcinoma patients as well as
normal mucosa from a miscellaneous disease group were
both found to be appropriate control LPMC populations to
compare with normal IBD mucosa.

We found that even histologically normal IBD mucosa
shows alterations in NK activity—that is, high levels in
Crohn’s disease versus low levels in patients with ulcerative
colitis. These observed abnormalities were strengthened by
the findings that NK activity in Crohn’s disease patients or
ulcerative colitis patients were also higher or lower, respect-
ively, than in the control carcinoma patients. Beeken et al.
(18) did not find differences in cytotoxicity levels between
normal and abnormal mucosal tissue in Crohn’s disease,
which might favor our observation that even normal mucosa
conceals alterations in NK activity in this disease, perhaps
preceding inflammation. In a preliminary study we found
differences in the proportions of both CD16- and CD56-
positive cells between Crohn’s disease and ulcerative colitis
which were suggestive for the difference in NK activity that
we report here. In addition, Hirata et al. (19) found higher
proportions of Leu7+ NK cells by immunohistochemical
detection in lamina lymphocyte populations from both nor-
mal and inflamed mucosa in IBD. There is evidence from
peripheral blood studies that differences in antigen
expression designate different NK-cell subsets with regard
to their lytic efficacy (7, 20, 21). In relation to the immu-
nopathology of IBD it is important to observe that consi-
derable differences in cytotoxic activity as reported here
may be associated with selective compartmentalization of
NK cells in the intestine, a phenomenon that has been
described earlier by Gibson et al. (22). It needs to be
recognized, however, that despite interesting differences in
NK activity within the IBD patient group, the biologic
relevance of such a sparsely distributed cell in the intestinal
mucosa remains to be elucidated, in particular with regard
to the pathogenesis of IBD. Nevertheless, the present finding
of a difference in NK activity between UC and CD patients
may support the concept that we are dealing with different
disease processes. At this stage it cannot be completely ruled
out that the origin of the tissue from which the LPMC have
been isolated contributes to the observed differences in NK
activity between CD and UC. However, a few important
observations prompted us to consider the differences to be
disease-related. First, colonic cells from two patients with
Crohn’s colitis also showed markedly higher NK activity
levels than those from control colonic tissue of the carcinoma
or miscellaneous diseases group and much higher activity
than those of the UC group. Secondly, cells from small-

Fig. 2. Correlations between cytotoxic activity of individual lamina
propria mononuclear cell preparations against K-562 and Caco-
d2 target cells in 4-h or 18-h 51Cr-release assays. (n = number of
individuals tested; R = correlation coefficient; p = significance of
correlation.)
bowel mucosa of a single UC patient showed much lower NK activity than the LPMC isolated from normal ileum of CD patients.

Autologous epithelial cells from the colon have been used as ‘physiologic targets’ to study spontaneous or antibody-armed cytotoxicity (23, 24), but colon target cells are rarely used in peripheral blood or mucosal NK-activity studies. Another colon tumor target cell line, RPMI-4788, was used in studies by MacDermott et al. (13). Despite the low levels of cytotoxicity against K-562 targets, they obtained similar results when using these colon target cells. The latter cell line has been reported to be more sensitive for LPMC cytotoxicity than K-562 in studies performed by Beeken et al. (18). They postulated that RPMI-4788 cells were better targets for evaluation of mucosal cytotoxicity. The lower susceptibility of Caco-2 cells to NK activity in the short 4-h assay of the present study seems to be in accordance with previous findings using peripheral blood mononuclear effector cells (25). Despite the difference in susceptibility of Caco-2 cells to NK activity as reported here, we found nice correlations between the activities against both target cell lines in both 4-h and 18-h assays. Caco-2 cells can therefore be used as target for detecting differences in individual NK activity by LPMC, which may be of interest in mucosal cytotoxicity studies because these cells originate from colonic epithelium, a putative target for local NK activity. Our observation and those reported by Beeken et al. (18) and Taunk et al. (26) indicate that the mucosal NK cell has a different profile of target cell recognition from its peripheral blood counterpart. Furthermore, changes of mucosal nervous innervation and peptide concentration have been reported to be associated especially with Crohn’s disease (27, 28). Since we found gastrointestinal peptides to be able to modulate NK activity (29, 30), it would be interesting to include target cells from intestinal origin as well in studies of mucosal NK activity in IBD.

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REFERENCES


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