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Impaired local natural killer cell activity in human colorectal carcinomas

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Summary. The present study was undertaken to study natural killer (NK) cell activity in patients with colorectal cancer at peripheral and local levels. Mononuclear cells were isolated from uninvolved colorectal mucosa, tumor tissue and peripheral blood, and tested against the colon carcinoma cell line CaCo-2 and the erythroleukemia cell line K-562. Peripheral blood NK cell activity from the patients showed similar levels compared with healthy controls, whereas, mononuclear cells of tumor tissue were found to have a significantly decreased NK cell activity compared to the normal intestinal mucosa ($P < 0.01$). No relation was found between the NK cell activity and the advancement of the disease according to the Duke's stage. Interferon-γ (IFN-γ) stimulated the NK cell activity of the mononuclear cells from blood, mucosa and tumor. However, the increase of NK cell activity after IFN-γ stimulation was lower in the tumor compared to the mucosa ($P < 0.02$). The lectin, phytohaemagglutinin, increased the cytotoxicity of mononuclear cells from blood, mucosa and tumor to a similar level. These results suggest that patients with colorectal tumors exhibit a normal NK cell activity in peripheral blood and intestinal mucosa; however, a diminished NK cell activity exists at the tumor level. Although mononuclear cells isolated from the tumor have a normal response to lectin stimulation they show hyporesponsiveness to IFN-γ stimulation with regard to their NK cell activity.

Introduction

Natural killer (NK) cells play an important role in many physiological functions including defence against tumor [11]. There is a considerable controversy in the literature with regard to NK cell activity of peripheral blood determined in cancer patients. Depressed NK cell activity has been related to advanced disease [15, 17, 19]. In contrast, other investigators have reported normal NK cell activity independent of the Duke's grading in patients with colon cancer [23]. When circulating numbers of NK cells were studied with HNK-1 (Leu-7) in patients with colon cancer they were found to be reduced in comparison to control subjects [2, 3b].

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Intestinal immunity can also play a role in the local immune surveillance. Although low percentages of NK cells are found in the normal intestinal mucosa, functional natural killer and lymphokine-activated killer cells are both present in the human gut mucosa [7, 9, 12, 13, 18]. At the tumor level, the NK cell activity has been reported to be absent or not measurable [3b, 4, 16]. However, the existence of an increased number of NK cells has been shown in tumor-infiltrating lymphocytes in patients with colon cancer [6]. Also it has been reported that human and animal tumors contain a high proportion of cytotoxic T lymphocytes [6, 16, 24].

The purpose of this study was to analyse the NK cell activity under optimal conditions in peripheral blood, intestinal mucosa and tumor from patients with colorectal carcinomas. We have also evaluated the response of these NK cell populations to the immunomodulator IFN-γ and the lectin phytohaemagglutinin.

Materials and methods

Patients. Fresh heparinized blood was obtained from 15 patients with colorectal cancer prior to surgery and from 15 healthy controls. Specimens of colorectal tumors and intestinal mucosa were obtained from 11 patients undergoing surgical resection of large-bowel adenocarcinoma (1 coecum, 4 colon ascendens, 3 colon transversum, 2 colon sigmoid and 1 rectum). The Duke's stages of these patients were: A ($n = 1$), B ($n = 4$), C ($n = 4$), and D ($n = 2$). The adjacent intestinal tissue was taken at least 5 cm from the tumor and was examined histologically to ensure the absence of tumor cells.

Mononuclear cell isolation. We have applied a modified technique from Bull and Bookman [5], as described previously [22], for the separation of mononuclear cells from intestinal mucosa and colorectal tumors using enzymatic dispersion and separation on the basis of density and size by Ficoll-Hypaque. In brief, after resection, tumor and intestinal mucosa tissues were transferred in ice-cold Hank's balanced salt solution (HBSS) supplemented with antibiotics and were processed immediately under sterile conditions. Necrotic and connective tissue was removed from the tumor and the remaining tissue was cut into small pieces of 5 mm^2 and washed twice with calcium/magnesium-free HBSS (Gibco Europe). Normal mucosa was dissected free of submucosa, washed and cut into pieces of

5 mm^2 as well. The tissues were incubated in 1 mM dithiothreitol (Sigma) at room temperature for 15 min to remove mucus and washed three times more in Ca/Mg-free HBSS. The tissue specimens were then cut into smaller pieces of 1–2 mm^2 and incubated sequentially in 0.75 mM EDTA at 37° C, with stirring in a Wheaton chamber, five or six times for 1-h periods to dissociate epithelial/tumor cells. The remaining lamina propria and tumor fragments were washed twice in RPMI 1640 tissue-culture medium (Gibco Europe) supplemented with 5% fetal calf serum (FCS, Gibco Europe) and antibiotics. Enzymatic dissociation of the remaining tissue was subsequently carried out by continuous incubation of the tissues in 100 ml RPMI 1640, supplemented with 10% FCS, 2000–4000 U collagenase (CLSPA Worthington Laboratories Freehold, NJ), 15000 U DNase (Worthington Laboratories) 20000 U penicillin, 20 mg streptomycin, 5 mg gentamycin and 0.05 mg amphotericin B, overnight at 37° C to dissociate lamina propria or tumor constituents into monodispersed cells. The resultant cell suspensions were filtered through a nylon mesh to remove clumps and were washed. The cell suspensions were further separated by Ficoll/Hypaque density gradient centrifugation. The interface of mononuclear cells was harvested and washed three times with culture medium. Mononuclear cells from heparinized peripheral blood were obtained by Ficoll/Hypaque density gradient centrifugation. Differential cell counts were done on cytospin preparations of the dissociated cell suspensions from all the specimens. They were fixed and stained with May-Grünwald/Giemsa. The percentage of different type of cells was determined by counting at least 500 cells by light microscopy.

Target cells. The target cells used were the erythroleukemia cell line K-562, and the human colon cancer cell line Ca-Co-2. The K-562 cell line was cultured in suspension in RPMI 1640 medium supplemented with 10% FCS. The Ca-Co-2 cell line was cultured as an adherent monolayer in RPMI 1640 with 20% FCS, and was kept in growth phase by transferring 1:3 twice a week. Single-cell suspensions for the cytotoxic assays were obtained by trypsinization of the monolayer.

Cytotoxicity assay. The effector cells, mononuclear cells from peripheral blood, mucosa and tumor were cultured for 24 h at 37° C in a 5% CO_2/95% air mixture in culture medium to reverse any possible adverse effects of the isolation procedure before functional assay [10]. The NK cell activity was measured using an 18-h chromium-release assay as described previously [1]. The target cells were harvested and labelled with sodium ^{51}chromate (100 µCi/5×10^6 cells) for 1 h at 37° C. The effector:target (E:T) ratio used was 50:1 for peripheral blood and 50:1 and/or 500:1 for intestinal mononuclear cells and tumor-infiltrating mononuclear cells. IFN-γ was diluted in culture medium with a final assay concentration of 500 U/ml. Phytohaemagglutinin was dissolved directly in culture medium and used at a final concentration of 1 µg/ml. Cytotoxicity was determined using 100 µl effector cells, 50 µl stimulus or medium and 50 µl target cells. The assays were performed in triplicate with a final volume of 200 µl/well. After the target cells had been added, the plates were centrifuged for 5 min, 500 *g*, and incubated for 18 h at 37° C in a 5% CO_2/95% air atmosphere. Incubation was terminated after 18 h by centrifugation and supernatant collection by a harvesting system (supernatant collection system, Skatron). The released label was subsequently counted in a gamma counter. The spontaneous release (20%–29%) was determined by incubation of the target cells with culture medium, IFN-γ or phytohaemagglutinin. At the concentrations used in this assay, IFN-γ and phytohaemagglutinin did not affect the spontaneous release. Maximal release was obtained by incubation of the targets with saponin (80%–82%).

Cellular cytotoxicity was calculated by the following formula:

$$\text{cytotoxicity (\%)} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximal release} - \text{spontaneous release}} \times 100$$

Statistical analysis. All data are expressed as mean ± standard error of the mean. The statistical significances of the differences were evaluated with the paired and unpaired non-parametrical two-tailed Wilcoxon's rank-sum test.

Results

Intestinal cell isolation

The total numbers of cells obtained after enzymatic dispersion were for adjacent intestinal mucosae $33 \pm 8 \times 10^6$ cells/g tissue and for the tumors $34 \pm 6 \times 10^6$ cells/g. From these initial cell suspensions, the recovery of mononuclear cells after Ficoll/Hypaque separation was 29% (range 8%–69%) in the case of those cells infiltrating the tumor and 49% (29%–69%) in the case of the adjacent intestinal mucosa yielding respectively $9 \pm 2 \times 10^6$ cells/g and $15 \pm 3 \times 10^6$ cells/g. The mononuclear cell from the tumors contained 52 ± 4% lymphocytes and those from the intestinal mucosae 63 ± 4%, contamination with epithelial/malignant cells was less than 10%. The viabilities of the mononuclear cells of intestinal mucosae and of tumors were respectively 89 ± 1% and 84 ± 3%.

NK cell activity

Peripheral blood mononuclear cells from the patients showed a similar level of NK cell activity, compared with those from the controls, against both targets (K-562, 52 ± 11 vs 58 ± 2 and CaCo-2, 25 ± 4 vs 28 ± 3).

Mononuclear cells of the intestinal mucosa and of the tumor showed a low NK activity against CaCo-2 and K-562 tested with the ratio 50:1 (9 ± 2 vs 4 ± 1 and 2 ± 3 vs 1 ± 1 respectively). Cytotoxicity against the CaCo-2 cell line showed a significant difference between mononuclear cells from the intestinal mucosa and from the tumor ($P < 0.05$) (Fig. 1). With an effector:target ratio of 500:1, the cytotoxicity of the mononuclear cells of the intestinal mucosa and tumor increased significantly against the two cell lines tested, CaCo-2 and K-562 ($P < 0.02$). Using this ratio, the NK cell activity of the mononuclear cells infiltrating the tumor was found to be markedly decreased compared to the that of the adjacent intestinal mucosa ($P < 0.01$) (Fig. 1). No relation was observed between NK cell activity and the Duke's stage with the tumors nor with the normal mucosa as illustrated in Table 1.

Phytohaemagglutinin increased significantly the cytotoxicity of the mononuclear cells from blood, mucosa and tumor ($P < 0.02$). However, there was no difference in the

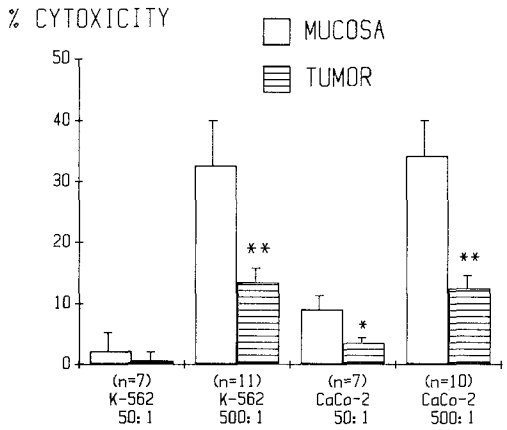

Fig. 1. Spontaneous NK cell activity of mononuclear cells of the normal intestinal mucosa and the tumor from patients with colorectal cancer. NK activity in two different effector:target cell ratios, 50:1 and 500:1, tested against K-562 and CaCo-2 cell lines. * $P < 0.05$, ** $P < 0.01$; significance of difference intestinal mucosa vs tumor

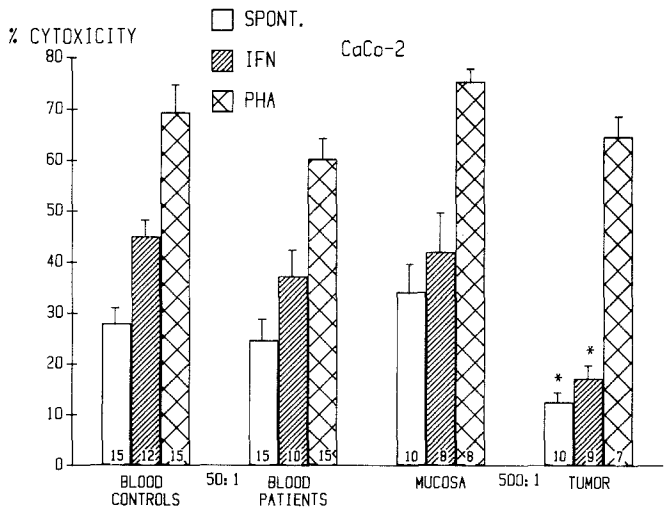

Fig. 2. Spontaneous NK cell activity and after stimulation with IFN-γ and phytohaemagglutinin of mononuclear cells of blood, normal intestinal mucosa and tumor from patients with colorectal cancer tested against the CaCo-2 cell line. Similar results were obtained with the K-562 targets. * $P < 0.01$; significance of difference intestinal mucosa vs tumor

cytotoxicity levels after phytohaemagglutinin stimulation between peripheral blood mononuclear cells from patients and controls nor between mononuclear cells from mucosa and tumor. Also no difference was found in the increase in the cytotoxicity of blood mononuclear cells between patients and controls nor between those of intestinal mucosa and tumor (Fig. 2).

IFN-γ stimulated significantly the cytotoxicity of mononuclear cells from blood, tumor and normal adjacent intestinal mucosa ($P < 0.02$). However, the increase of the cytotoxicity after IFN-γ stimulation of the tumor was significantly less when compared to the intestinal mucosa (4 ± 1 vs 10 ± 1, $P < 0.02$), and no difference was found with the peripheral blood mononuclear cells.

Discussion

This study shows that patients with colorectal carcinoma have a defect of the NK cell activity at the tumor level compared to the adjacent intestinal mucosa, which is unrelated to the Duke's stage, with a normal peripheral blood NK cell activity.

Although the number of NK cells is markedly decreased in intestinal mucosa [9, 18] NK cell activity at the intestinal level can be studied when optimal methods are applied. Thus, to be able to compare NK cell activity from blood with mucosa, other effector:target cell ratios should be used to obtain the same proportions of cytotoxic cells [10]. For these reasons, previous reports of generally minimal or absent NK cell activity cannot be validly interpreted [4, 7]. In this study freshly isolated mononuclear cells of the intestinal mucosa from all patients showed significant cytotoxicity against both cell lines, CaCo-2 and K-562. Using these assay conditions, which overcome most of the methodological difficulties, by culturing cells for 24 h before testing and using a high E:T ratio [10a, b] a defect of NK cell activity at the tumor level, has been demonstrated.

Table 1. NK cell activity, as percentage cytotoxicity, of mononuclear cells from normal intestinal mucosa and tumor divided according to Duke's stage

Duke's stage	Targets			
	K-562		CaCo-2	
	Mucosa	Tumor	Mucosa	Tumor
A and B, $n = 5$	32 ± 10	15 ± 4	31 ± 7	12 ± 1
C and D, $n = 6$	33 ± 12	12 ± 3	36 ± 9	13 ± 3

The depressed NK cell activity found in the colorectal tumor appears to be a general finding for tumors of different localizations [16, 24]. Mononuclear cells infiltrating tumors have been shown to have a higher proportion of HNK-1 (Leu-7) and/or CD8 (OKT-8) cells compared to the normal intestinal mucosa [6, 16]. The suppression of the activity of the NK cells may thus be related to the environment of the tumor itself. For example, it is known that tumors contain more macrophages and high prostaglandin E_2 production may be a possible mechanism for the depression of the NK cell activity at the tumor level [3a].

IFN-γ, a potent immunomodulator, has been shown to stimulate monocytes and NK cells [20, 21]. In this study mononuclear cells of the tumor were hyporesponders whereas those of blood and mucosa showed a normal response with IFN-γ. Other studies, using other types of interferons, have also shown stimulation of the NK cell activity [8, 9, 13].

Phytohaemagglutinin has been shown to increase cellular cytotoxicity by inducing interleukin-2 production, receptor expression and lymphocyte activation [1, 14]. In this study it increased the cytotoxicity of mononuclear cells of peripheral blood, tumor, and normal adjacent intestinal mucosa in the same fashion. The mononuclear cells of the tumor are capable of becoming fully activated.

We conclude that patients with colorectal cancer have a defect in the NK cell activity at tumor level. The mononuclear cells of the tumor are hyporesponsive to IFN-γ but have high potential lytic capabilities. It seems, therefore, possible that within the tumor, lymphocytes capable of being stimulated to an optimal killing are present but are

suppressed by the tumor cells or products produced in the tumor environment. Development of approaches to overcome this local malfunction of the NK cells might provide better local control of tumor growth.

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