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Natural cytotoxicity of rat hepatic natural killer cells and macrophages against a syngeneic colon adenocarcinoma

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Summary. The in vitro cytotoxic activity of two types of hepatic sinusoidal cells, i.e., natural killer (NK) cells and macrophages (Kupffer cells), was tested against a syngeneic rat colon adenocarcinoma cell line (DHD-K12). Purified hepatic NK cells (85% cells with large granular lymphocyte morphology) were spontaneously cytolytic, whereas Kupffer cells (90% pure) were not able to kill the DHD-K12 cells. This carcinoma cell line was found to be resistant to the action of mouse recombinant tumor necrosis factor which is considered as the major cytolytic molecule secreted by macrophages. However, colon carcinoma cells were readily lysed by soluble factors present in the culture supernatant of NK cells. It is postulated that hepatic NK cells, which are strategically located within the lumen of the sinusoids, may form a first line of defense to metastasizing colon carcinoma cells.

Introduction

The liver of normal rats contains more natural killer (NK) cells than the peripheral blood [2, 10, 11], and also harbours the largest population of fixed macrophages, i.e., the Kupffer cells [7]. Both cell types have been reported to have natural cytotoxic activity against several tumor cell lines [2, 3, 4, 16, 21]. In addition, hepatic NK cells, also called pit cells [2], and Kupffer cells [19] are located strategically with the major portion of their cytoplasm in the sinusoidal lumen. Since liver sinusoids, especially the periportal ones, are very narrow [20] it is easy to conceive that any (metastasizing) tumor cell coming into the liver must come in close contact with these sinusoidal cells. The present study aims at determining the involvement of hepatic NK cells and Kupffer cells in the natural defense against a syngeneic colon carcinoma cell line by investigating their in vitro interactions.

Materials and methods

Animals. Inbred male BD-IX rats, raised locally, with an average body weight of 300 g were used, and were maintained under conventional conditions. For some experiments, specific pathogen-free male Wistar rats were used (Proefdierencentrum, Leuven, Belgium).

Tumor cell lines and media. The syngeneic cell line DHD-K12 originating from a 1,2-dimethylhydrazine-induced colon adenocarcinoma in BD-IX rats [12] was kindly provided by Prof. G. Willems (Cancer Research Unit, Free University of Brussels). Other cell lines used were YAC-1 (murine lymphoma) and L929 (murine fibrosarcoma). The tumor cell lines were cultured in 50% RPMI-1640/50% Dulbecco's minimal essential medium supplemented with 10% fetal calf serum and 1% penicillin-streptomycin. This medium was also used for all other in vitro tests and manipulations. Cells were kept at 37° C in a humid atmosphere of 5% CO_2.

Isolation of hepatic NK cells. Hepatic NK cells were isolated by the sinusoidal lavage method as described previously [1, 2]. Briefly, the liver was perfused with Dulbecco's phosphate-buffered saline (PBS) supplemented with 0.1% EDTA via the portal vein. The perfusion pressure was raised to the pressure of a 50 cm water column and the perfusate was collected by cannulation of the vena cava. The perfusate (300 ml) was concentrated by centrifugation and mononuclears were subsequently separated on a Ficoll-Paque density gradient, then representing the unfractionated preparation. To further purify the NK cells, the cells were filtered over a nylon wool column whereafter the nonadherent cells were centrifuged on a 45% Percoll gradient at 550 *g* for 25 min, leaving the enriched NK cells on top of the gradient. The NK cells were identified by their large granular lymphocyte (LGL) morphology in Giemsa-stained cytosmears or by electron microscopy in pellet sections as described previously [1, 2].

Isolation of Kupffer cells. Nonparenchymal liver cells were isolated by enzymatic liver dissociation according to the method of Knook et al. [8]. Briefly, the liver was perfused and subsequently incubated in a 0.05% pronase/0.05% collagenase mixture in warm Gey's balanced salt solution. Nonparenchymal cells were separated on a 30% Nycodenz gradient after centrifugation at 1400 *g* (15 min). Kupffer cells were then purified by centrifugal elutriation at a rotor speed of 2500 rpm (750 *g*), between counterflow rates of 23 and 42 ml/min.

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Cytolysis assays. Adherent target cells were obtained by trypsinization (DHD-K12) or by vigorous flushing with a pipette (L929) of confluent monolayers. Cytolysis was assayed by the ^{51}Cr release method after incubation of target cells for 80 min with 250 μCi $Na_2\ ^{51}CrO_4$ (Amersham). For NK cells, 10^4 labeled target cells were added to varying numbers of effectors in a total volume of 0.2 ml medium in U-shaped 96-well plates. After 4 h or 16 h coincubation (37° C, 5% CO_2), 0.1 ml of supernatant was removed and ^{51}Cr release was measured in a gamma counter. Tests at 6 different effector/target ratios were performed in triplicate.

Macrophages obtained by enzymatic liver dissociation were allowed to adhere and recover from the isolation procedure in flat-bottomed 96-well plates for 24 h at a concentration of 2×10^5 cells/well. At that time, the cells had spread and formed a confluent monolayer. The macrophages were then stimulated by activating factors and after 24 h, 10^4 ^{51}Cr-labeled target cells were added per well. Release of ^{51}Cr was measured after 18 h coincubation. These tests were done ×6.

Spontaneous release was measured by incubation of labeled target cells with medium in the absence of effector cells. Maximal release was measured after treatment of target cells with sodium dodecyl sulfate detergent. Specific lysis was calculated as follows:

% cytolysis = (test release – spontaneous release)/(maximal release – spontaneous release) × 100

In some cases, the results were expressed as lytic units (LU)/10^7 cells with an LU being the number of effector cells required to cause 20% lysis of target cells.

Photometric cytotoxicity assay. A photometric assay was developed according to Fransen et al. [5] to measure the cytotoxic activity of NK cell supernatant or mouse recombinant tumor necrosis factor (r-TNF) on target cell monolayers. Either NK cell supernatant or r-TNF in medium was added to target cell monolayers (10^4 cells per microwell) which were plated the previous day. The cells were incubated for 16 or 48 h at 37° C in 5% CO_2 before they were fixed and stained for 10 min with a mixture of 0.5% crystal violet, 8% v/v 40% formaldehyde, 0.17% NaCl, and 22.3% v/v ethanol. The adherent cells were thoroughly washed with running tap water and then dissolved in 33% acetic acid (0.1 ml/well). The released dye was measured spectrophotometrically using a Titertek micro Elisa reader. The cytotoxicity (a combination of cytolysis and cytostasis) was calculated as follows:

% cytotoxicity = [1 – (test O. D./control O. D.)] × 100

with O. D. being the optical density at 540 nm wavelength and controls being target cells incubated in medium alone.

Macrophage activation. Macrophages were activated by the addition of endotoxin (lipopolysaccharide, LPS) at a concentration of 0.1 μg/ml, a crude lymphokine supernatant of concanavalin A-stimulated spleen cells (10% v/v), or mouse recombinant gama interferon (r-IFNγ), or by a combination of LPS and these cytokines. Murine r-IFNγ (10^6 units/ml) was kindly provided by Dr. A. Billiau (Rega Institute, Leuven, Belgium) and Dr. H. Schellekens (T. N. O., Rijswijk, The Netherlands). The LPS, kindly provided by Dr. H. Van Bossuyt (our group), was extracted from *Salmonella abortus equi* bacteria as described elsewhere [17].

Natural killer cytotoxic factors and r-TNF. Natural Killer cytotoxic factors (NKCFs) were prepared by adding sinusoidal lavage cells to adherent DHD-K12 cells at an effector-to-target ratio of 20:1 and a final concentration of 2.5×10^6 cells/ml medium. After 16 h coincubation, the cell free supernatant was used as a source of NKCFs [13]. The r-TNFα, sp. act. 10^8 units/ml, was kindly provided by Innogenetics (Ghent, Belgium).

Electron microscopy. For the characterization of effector cells, cell suspensions were pelleted, fixed, and prepared for electron microscopy as described previously [2, 17].

Results

Cytolytic activity of hepatic NK cells

The sinusoidal lavage was composed of 31% cells with LGL morphology, previously shown to represent NK cells [1, 2], and contained further agranular lymphocytes, and less than 10% monocytes, as determined by light and electron microscopy. After nylon wool filtration the cell preparations contained about 50% LGL and were practically free of contaminating monocytes (<4%), the other cells being dense agranular (T) lymphocytes. These preparations were strongly cytotoxic to YAC-1 lymphoma cells in a comparable way to DHD-K12 colon carcinoma cells. However, whereas maximal YAC-1 lysis was obtained at 4 h coincubation, DHD-K12 lysis required an incubation time of 16 h (Fig. 1). Preparations that were enriched for LGL by fractionation on a Percoll gradient (up to 90% pure LGL) still contained high cytotoxicity to DHD-K12 cells. As judged from the lytic activity at an effector-to-target ratio of 10:1, and from the lytic units, these LGL-enriched cell preparations were more cytotoxic than the unfractionated cell preparations (Table 1). When sinusoidal

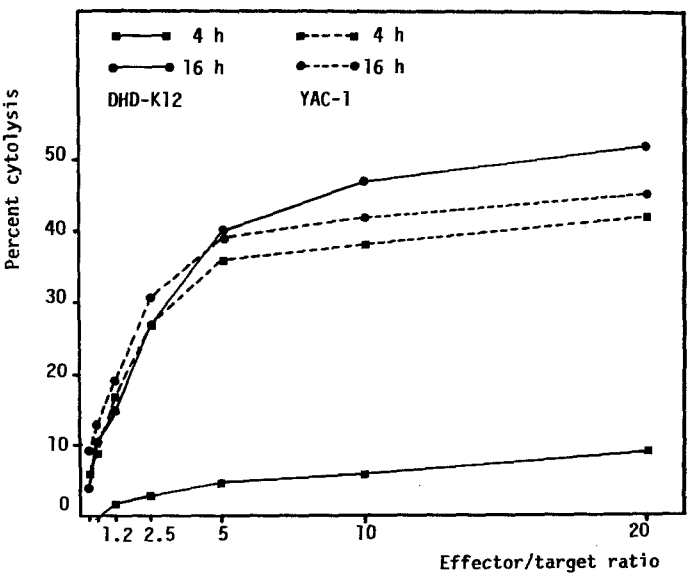

Fig. 1. Cytolytic activity of nonadherent LGL (large granular lymphocytes) against DHD-K12 colon carcinoma cells (*full lines*) and YAC-1 lymphoma cells (*dashed lines*). Specific ^{51}Cr release was measured after 4 h (*squares*) and 16 h (*dots*) of coincubation

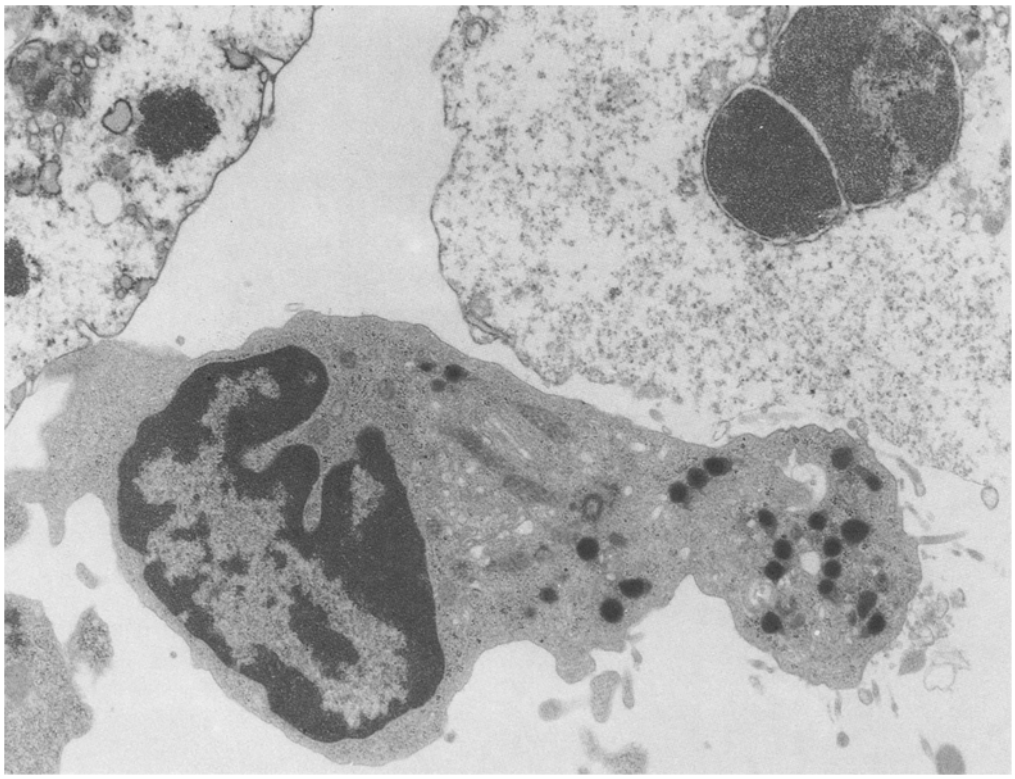

Fig. 2. Electron micrograph of a hepatic LGL/NK cells which is bound to two killed DHD-K12 target cells. Note the dense granules of the NK cell. 12,473 ×

Table 1. Cytolysis of DHD-K12 colon carcinoma cells by sinusoidal lavage cells

Cell preparation	Number of experiments	% Large LGL	% Lysis (E : T = 20)	% Lysis (E : T = 10)	$LU_{20\%}/10^7$
Unfractionated	6	31 ± 3	41 ± 11	30 ± 12	181 ± 126
Nonadherent Percoll (45%) low-density	4	85 ± 6	48 ± 10	45 ± 9	433 ± 81

Mean values from 4–6 different rats are given with the SE. Percentage cytolysis was assayed using the ^{51}Cr release method after 16 h coincubation of effectors and targets (E : T = effector-to-target ratio). $LU_{20\%}$ = lytic units causing lysis of 20% of the target cells

lavage preparations were mixed with DHD-K12 cells in suspension, conjugate formation between LGL and target cells was observed (Fig. 2). Dense agranular lymphocytes, most probably T cells, were also seen to bind the colon carcinoma targets but Percoll high-density cell preparations were not cytolytic (data not shown).

Cytotoxic activity of NKCFs

The supernatants of nonadherent sinusoidal lavage cells (stimulated by the presence of DHD-K12 cells) were cytotoxic to DHD-K12 target cell monolayers. Control medium of NK cells alone was not significantly cytotoxic (Table 2). These results were confirmed by microscopic examination of the target cell monolayers: the treated colon carcinoma monolayers regressed strongly and eventually disappeared completely (beyond 24 h). However, these supernatants were not cytotoxic to macrophage monolayers or to normal fibroblasts (data not shown).

Cytolytic activity of Kupffer cells

At least 90% pure Kupffer cells preparations were used, as characterized by electron microscopy (Fig. 3). Kupffer cells, either unstimulated or after stimulation with LPS, lymphokines, r-IFNγ or a combination of these factors, were not cytolytic to DHD-K12 cells in an 18-h assay. They were able, however, to lyse the macrophage-sensitive L929 cell line (Table 3). In pilot experiments, pronase was

Table 2. Cytotoxicity against DHD-K12 colon carcinoma cells of recombinant tumor necrosis factor (r-TNF) and natural killer (NK) cell supernatant

Factor added to DHD-K12 cultures	% Cytotoxicity[a]
Supernatant of NK cells + DHD-K12	66 ± 8[b]
Supernatant of NK cells alone	18 ± 14[b]
r-TNF 1000 units/ml	4 ± 0.2[c]
r-TNF 500 units/ml	0.5 ± 0.01[c]
r-TNF 250 units/ml	0.4 ± 0.01[c]

[a] Cytotoxicity (= cytolysis + cytostasis) was measured by a colorimetric assay (see *Materials and methods*)
[b] Cytotoxicity was measured after 16 h coincubation
[c] Cytotoxicity was measured after 48 h coincubation
The SEs are given for 12 replicate samples

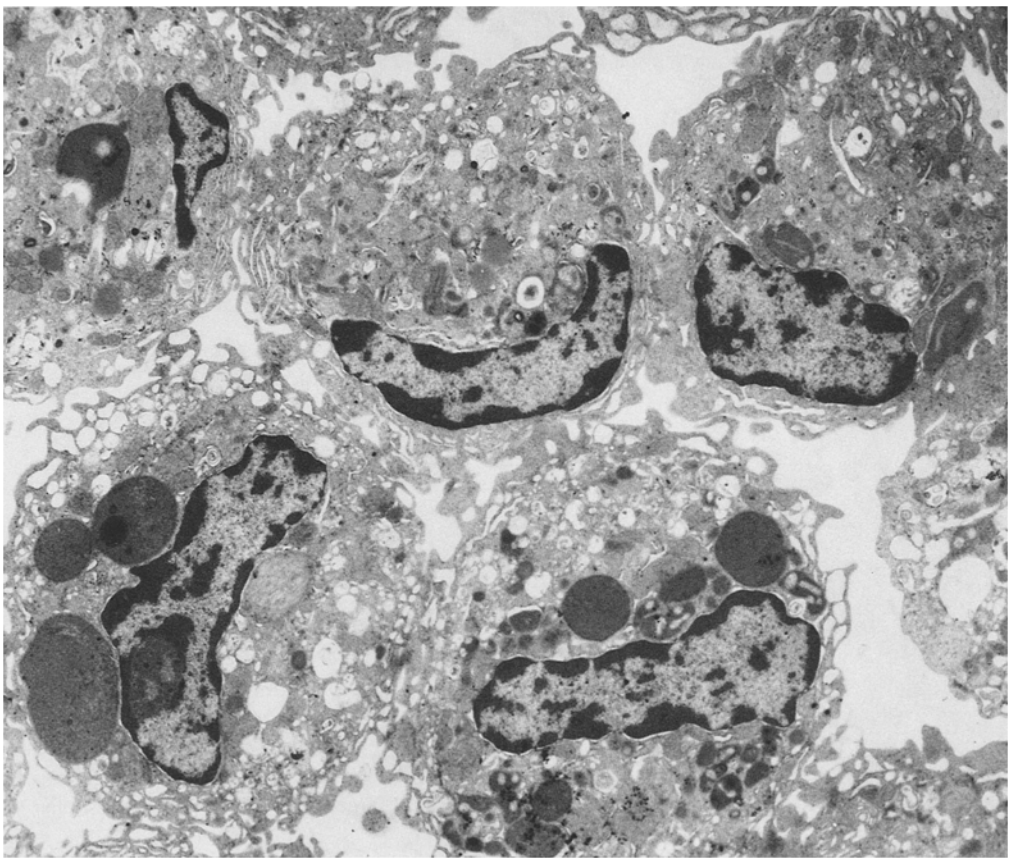

Fig. 3. Electron micrograph of a purified Kupffer cell suspension. Note the numerous surface extensions, vacuoles, and phagosomes of these macrophages. 5,612×

Table 3. Cytolysis of DHD-K12 colon carcinoma and L929 fibrosarcoma target cells by in vitro-activated Kupffer cells

Activators[a]	DHD-K12	L929
LPS + lymphokines	3±1[b]	25±3[b]
LPS + r-IFN$_\gamma$	0±2	30±3
LPS	0±0	20±5
Lymphokines	3±2	34±5
r-IFN$_\gamma$	0±2	21±3
None	0±2	10±4

[a] Macrophage activators were added to the culture medium, 24 h before the cytolysis assay, in the following concentrations: lymphokines (concanavalin A supernatant) 10% v/v; lipopolysaccharide (LPS) 100 ng/ml; recombinant gamma interferon r-IFN$_\gamma$ (rat) 100 units/ml

[b] Percentage of specific cytolysis ± SE of × 6 cultures in an 18 h ^{51}Cr release assay (E:T=20:1). Data are representative of one experiment of four

omitted from the isolation procedure because of its potential harmful effects on Kupffer cell function. This procedure gave lower Kupffer cell yields but did not influence the results of the cytolysis experiments. In addition, resident peritoneal macrophages isolated by washing off the abdominal cavity with sterile PBS, were also unable to lyse the DHD-K12 cells (nonsignificant specific ^{51}Cr release of less than 10%).

Sensitivity of DHD-K12 cells to r-TNF

The DHD-K12 cells were found to be resistant to the action of 250–1000 units/ml of r-TNF (Table 2). L929 monolayers were completely lysed at these r-TNF doses (1 unit of TNF being defined as the amount necessary to cause the lysis of 50% of L929 target cells).

Importance of rat strain

Cytolysis experiments with NK cells and macrophages were also performed using effector cells derived from specific pathogen-free Wistar rats. Hepatic NK cells, but not macrophages, were cytolytic to the colon carcinoma cells in a comparable way as with effector cells from syngeneic BD-IX rats (data not shown).

Discussion

We have shown previously that rat liver harbours a numerically and topographically important population of NK cells, characterized by their LGL morphology in light and electron microscopy [2], surface antigens [1], and spontaneous natural cytotoxicity to NK-sensitive targets [2]. In the present paper we demonstrated that these hepatic NK cells are also able to kill syngeneic, solid tumor-derived target cells. The cytolysis of colon carcinoma cells required a coincubation of effector and target cells longer than the standard 4–6 h time required for classical NK assays with lymphoma cells (YAC-1) as targets. Thus, in a strict sense,

the cytolytic activity against these colon carcinoma cells was not NK activity but rather natural cytotoxic (NC) activity, defined as the cytotoxicity to solid tumor cells requiring at least 16 h coincubation time for the assay [9, 14]. Although it is widely accepted that the majority, if not all, of the NK activity in normal animals is confined to the LGL, the exact nature of cells with NC activity is less clear [9, 14]. We showed that hepatic LGL, purified by nylon wool filtration and Percoll density fractionation, have NC activity. We characterized these effector cells previously by their ultrastructure and immunophenotype, showing that they are OX8 (CD8) positive and OX19 (CD5) negative [1]. This NC activity was not only exerted against the colon carcinoma cells but also against other adherent cell lines such as murine L929 fibrosarcoma cells and 3LL Lewis lung carcinoma cells (unpublished observation). These observations are in accordance with the recently published results of Pelletier et al. [15] who found that DHD-K12 colon carcinoma cells are sensitive to the spontaneous lytic activity of nonadherent spleen cells, possibly NK cells. In addition, we showed that this NC activity was mediated, at least partly, by soluble factors that can be found in the culture supernatant of NK cells stimulated by target cells, probably representing NKCFs [13].

In contrast to the hepatic NK cells, the liver macrophages or Kupffer cells were not able to kill syngeneic colon carcinoma cells, not even after activation. Nevertheless, they were able to kill macrophage-sensitive L929 target cells. The latter target cells are known to be very sensitive to TNF and are actually used in bioassays of TNF activity. Generally, macrophages from different body compartments including Kupffer cells are not spontaneously tumoricidal but require activation by cytokines, biological response modifiers, and/or endotoxin [3, 4, 16, 21]. It is generally believed that this macrophage-mediated cytotoxicity is mainly the result of TNF secretion and this has also been demonstrated for Kupffer cells [4]. We found that the DHD-K12 colon carcinoma is not sensitive to r-TNF, which explains its resistance to Kupffer cell and macrophage-mediated cytotoxicity.

DHD-K12 colon carcinoma cells form artificial metastases in the liver when the defense of the host is suppressed by treatment with cyclosporin [18]. Since it is known that cyclosporin strongly supresses the activity of NK cells but not of macrophages [6], these observations support the biological significance of hepatic NK cells in the prevention of metastases and indicate that our in vitro findings may also rule in the in vivo situation. Although the liver sinusoids harbour two populations of potentially tumoricidal cells, i.e., Kupffer cells and NK cells, we conclude that only the latter are of importance in natural resistance to colon carcinoma cells, at least in the present rat model. Although the total number of hepatic NK cells is relatively low, e.g., ten times lower than the number of Kupffer cells [2], their strategic position in the narrow liver sinusoids gives them the opportunity to efficiently control the hepatic blood for the presence of metastasizing tumor cells. Experiments are under way in our laboratory to further investigate the in vivo role of NK cells in metastatic disease.

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