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Natural Killer Lymphocytes in Rat Liver Sinusoidal Blood

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Summary

Blood-borne cells with the characteristics described for the natural killer (NK) lymphocytes were recovered from the liver sinusoids in rat. They had cytotoxic activity against K 562 cells in a 18 h ^{51}Cr release assay, target binding rate and large granular lymphocytes percentage higher than the inflowing portal blood cells. The cytotoxic effector population was non-adherent and peroxidase negative, enriched in cells with Fc- and C36-receptors. Deprivation of liver of portal or mesenteric blood supply significantly decreased the liver NK activity not affecting the level of cytotoxicity in the portal blood. Splenectomy produced only a moderate decrease of activity. These findings may indicate that liver sinusoids is the site of accumulation of blood-born NK cells or of maturation of the NK-precursors. One possible mechanism by which natural cytotoxicity controls tumor growth and spread might be the killing of circulating tumor cells arrested in the liver by the blood-born NK-cells.

There is an experimental evidence that, under certain experimental conditions, liver may phagocytize tumors and modify tumor growth in vivo (3, 11). Moreover, livers from nonimmunized animals can rapidly eliminate radiolabelled tumor cells in vivo which suggest that the liver may contain cells with natural cytotoxic activity against tumors (9, 10). Another evidence that liver may exert anti-tumor cytotoxic activity was provided by *Gorelik* et al. (4) who found that mice treated with rabbit anti-asialo GM 1 serum which selectively inhibited the cytotoxic activity of NK cells, developed multiple metastatic foci in the liver after i.v. injection of B16 melanoma cells, whereas in the control mice no extrapulmonary metastases were found. The phagocytic system widely represented in the liver sinusoids seems to be responsible for tumor cells elimination and growth modification under experimental conditions (1, 11). This system is composed of resident Kupffer cells and in part of immigrant blood mononuclears. The various functional specificities of Kupffer cells have been documented (2, 13), however, little is known about the cytotoxic properties of population of blood mononuclear cells marginating and extravasating in liver sinusoids.

The purpose of the present study was to characterize the natural cytotoxic properties of cells in liver sinusoidal blood. In order to investigate the role of spleen and gut as potential suppliers of cytotoxic cells to the liver, experiments were carried out in animals after procedures depriving liver of splenic or intestinal blood inflow.

Materials and Methods

Animals

DA male rats at the age of 4 to 6 weeks were used.

Tumor cell lines

The K562 human erythroleukemic cell line obtained from Karolinska Instituted, Stockholm was used.

Blood samples

Blood was drawn from the splenic, mesenteric portal and hepatic veins and aorta into syringes with 5 μ of heparin in 0.1 ml of saline.

Vascular wash-out of liver, gut and hind-limbs

Twenty units of heparin in 1 ml of saline solution were injected i.v. Animals were exsanguinated. The portal and hepatic veins were cannulated for liver perfusion, the superior mesenteric artery and vein for bowel perfusion and the abdominal aorta and inferior vena cava for hind-limbs' perfusion. Forty ml of RPMI at 20 °C was infused under the pressure of 25–30 mmHg and the effluent fluid was collected. The vascular wash-outs of gut and limbs served as controls for evaluation of liver wash-out cell population.

Isolation of mononuclear cells

Mononuclear cells were separated by centrifugation at 1600 rpm for 35 min on Lymphoprep gradient (Nyegaard Co A/S, Oslo, Norway). Cells were washed three times in RPMI 1640 and resuspended to adjust the concentration to 10^6 cells/ml.

EA-rosette forming cells

One hundred microliters of cell suspension were mixed with 100 µl of 1% suspension of SRBC coated with rabbit-anti-SRBC antibody (Cordis Lab., Miami). This mixture was spun down at 1000 rpm for 5 min and followed by incubation at 20 °C for 30 min. The pellet was resuspended and 200 lymphoid cells were counted for rosette formation.

EAC-rosette forming cells

One hundred microliters of cell suspension were mixed with 100 µl of trypsinized SRBC coated with anti-SRBC-IgM antibody (Cordis, Miami, Fla) and fresh AKR mouse serum as a source of complement. The mixture was incubated at 37 °C for 30 min and then centrifuged at 1000 rpm for 5 min. After resuspension 200 cells were counted to determine the number of rosette forming cells.

Sig^+ cells

One ml of cell suspension was spun down at 1300 rpm for 10 min. The supernatant was discarded and 100 µl of rabbit anti-rat IgG fluorescein conjugated (Miles Lab.) were added to the pellet. After 30 min incubation at 20 °C cells were washed 3 times in Eagle MEM and resuspended in 100 µl of medium. Two hundred cells were counted with fluorescent microscope.

Histochemical characteristics of cells

Cytocentrifuged smears were stained with May-Grünwald-Giemsa method. The large granular lymphocytes (LGL) usually associated with the natural killer activity (12) were recognized by the azurophilic granulation in the pale cytoplasm, a reniform nucleus, and a high cytoplasmic nuclear ratio, compared with other lymphocytes. Two hundred cells were counted for the presence of LGL. Stainings for demonstration of peroxydase and PAS-positive material were also performed in order to detect any contamination of sinusoidal blood population with endothelial Kupffer cells.

Natural killer (NK) cell cytotoxicity assay

Target cells (3×10^6) were labelled for 60 min at 37 °C using 100 µCi of sodium^{51} chromate (Institute for Atomenergi, Kjeller, Norway and then washed 4 times in RPMI 1640. Labelled cells were resuspended in RPMI 1640 + 10% FCS to a concentration of 2.5×10^4 ml. 0.2 ml of lymphocytes were added to round bottom small culture tubes together with 0.2 ml of K562 cells to make the effector to target ratio 40:1, 20:1, 10:1 and 5:1. All tests were done in triplicate and incubated for 18 hr at 37 °C in an atmosphere of 5% CO_2 in air. After incubation the tubes were centrifuged at 1600 rpm for 10 min and 0.2 ml samples of supernatant were transferred to the other tubes and both aliquots counted on Intertechnique gamma counter. The percentage of ^{51}Cr release was determined for each tube using the mean values of the triplicate tubes

$$\frac{^{51}\text{Cr release in sample} - {}^{51}\text{Cr release in control}}{^{51}\text{Cr release in Triton} - {}^{51}\text{Cr release in control}} \times 100$$

Target binding cell assay

Two x 10^6 K562 cells were mixed with 2×10^5 of lymphoid cells in 0.4 ml culture medium and centrifuged at 25 g for 15 min, 4 °C in 10 x 55 mm round bottom plastic tubes. Tubes were placed on ice and separated vigorously with a

Table 1 The natural killer activity in liver and gut vascular wash-outs and blood percolating liver in normal DA rats

Vascular wash-out				
Liver		Gut		Hind-limbs
69.0 ± 7.6[1,2,3]		37.1 ± 12.9		32.0 ± 8.5
Source of blood				
Splenic v.	mesenteric v.	portal v.	hepatic v.	hepatic art.
53.4 ± 12.3[1]	49.9 ± 12.5	56.7 ± 7.5	45.6 ± 12.2	41.3 ± 14.0

[1]Percent of specific ^{51}Cr release, effector to target ratio 20 : 1, values are means ± SD/n = 6–9/. In the NK cytotoxicity assay the targets were K 562 cells.
[2]NK cells cytotoxicity in liver wash-out vs portal vein $p < 0.05$.
[3]NK cell activity in liver wash-out vs gut and hind-lim wash-out $p < 0.05$

Pasteur pipette. One drop of cell suspension was placed on a microscope slide and percentage of lymphoid cell binding to K 562 cells determined.

Surgical procedures depriving liver of venous splenic or

Laparotomy was performed under ether anesthesia. In various groups splenectomy, porta-caval and mesentero-caval shunts and portal-caval transposition were performed (Fig. 1).

Fig. 1 Types of microsurgical procedures performed to deprive liver of portal or splenic blood inflow.

Splenectomy deprived liver of potential source of cytotoxic cells, porta-caval shunt totally eliminated portal blood supply, mesentero-caval shunt diverted intestinal venous blood from the liver, porta-caval transplantation eliminated portal inflow but maintained total liver blood flow at physiological levels.

Statistical evaluation

For evaluation of statistical significance of differences in results the Student t-test for pairs was used.

Results

A. Characteristics of cells in liver vascular wash-out in normal rats

Natural killer lymphocyte activity

The cytotoxic activity of cells from the liver vascular wash-out against K 562 tumor cells was found significantly higher (65 to 80 % of ^{51}Cr release) than from the portal vein of the same animals ($p < 0.05$) (Table 1). It was found to be twice as high as of cells from the vascular wash-out of the gut and hind-lims (Fig. 2). Removal of adherent cells slightly in-

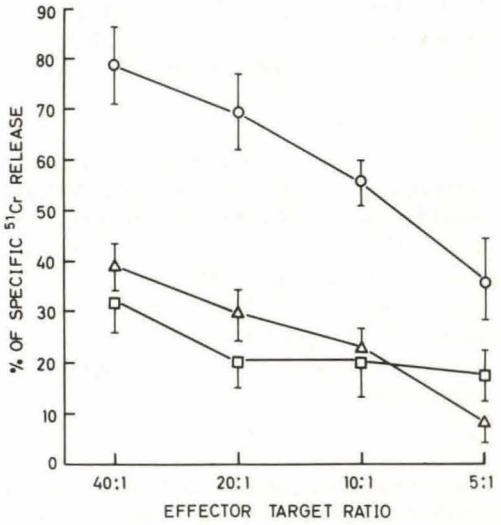

Fig. 2 Natural killer lymphocyte cytotoxicity against K 562 cells in the liver (o), gut (—□—) and hind-limbs' (—△—) vascular wash-out. Values are means ± SD (n = 5–8)

Table 2 Surface characteristics of mononuclear cells in liver wash-out, portal, splenic and mesenteric blood

Source of cells	EA-RFC	EAC-RFC	SIg^+
Liver wash-out	14.9 ± 4.1[1,2]	11.6 ± 3.9[2]	6.0 ± 4.6
Portal	11.3 ± 3.4	5.6 ± 3.6	7.8 ± 2.7
Splenic	12.0 ± 2.9	5.9 ± 2.7	9.5 ± 1.3
Mesenteric	8.1 ± 1.2	2.9 ± 1.7	6.9 ± 2.9

[1] Values are mean percent ± SD (n = 6–9)
[2] Liver wash-out vs portal and mesenteric cells $p < 0.05$

Table 3 The natural killer cell cytotoxicity in liver and gut vascular wash-outs and portal blood 5 weeks after splenectomy, porta-caval and mesentero-caval shunts and porta-caval transposition

Source of cells	Laparotomy	Splenectomy	Porta-caval shunt	Mesentero-caval shunt	Porta-caval transposition
Portal vein	37.8 ± 14.0[1]	44.1 ± 3.4	29.5 ± 6.0[2]	24.4 ± 4.0[3]	43.5 ± 13.6
Liver wash-out	70.2 ± 6.5[4]	54.8 ± 11.5[4]	35.6 ± 9.9[5]	32.5 ± 5.4[5]	55.2 ± 20.6
Gut wash-out	37.0 ± 12.9	31.0 ± 5.2	24.6 ± 7.2	20.7 ± 8.2	49.1 ± 8.3

[1] Specific ^{51}Cr release, effector to target ratio 20 : 1, values are means ± S.D. (n = 3–5).
[2] Sample taken from distal stump of portal vein.
[3] Sample taken from mesenteric vein.
[4] Liver vs portal vein and gut wash-out cells after laparotomy and splenectomy $p < 0.05$.
[5] Liver wash-out cells after PC- and MC-shunt vs laparotomy $p < 0.05$

creased the NK cytotoxicity in the liver wash-out cellular population which indicates that the non-adherent population and not macrophages was responsible for the high cytotoxicity level.

The NK cytotoxic activity of cells obtained from the splenic, mesenteric, portal and hepatic veins and hepatic artery was found at a similar level, between 40 and 55 % of specific ^{51}Cr release at ET 20 : 1 (Table 1). It was in all cases below the level of liver wash-out population.

Natural killer-target binding

The percentage of lymphoid cells binding to the K 562 cells was in the liver vascular wash-out population 10.6 ± 0.5 (n = 5) whereas in blood from all other investigated sources was found at the level of around 5 % ($p < 0.05$).

Morphological evaluation

The large granular lymphocytes (LGL) were found in the liver vascular wash-out in 22 ± 3 %, in the gut and limb wash-out in 5 ± 2 % and 7 ± 2 %, respectively, and in the portal and systemic blood in the range of 3 to 7 %. No cells morphologically similar to the Kupffer and endothelial cells or hepatocytes could be found in the smears from liver wash-out.

Surface characteristics

The percentage of cells with the FC and C3b receptor was found evidently higher in the population of cells from liver wash-out (mean values 14.9 and 11.6 %, respectively) than in other sources (Table 2). The mean percentage of SIg^+ cells in liver wash-out was 6.0 % and did not differ from that of portal blood.

B. Characteristics of cells in liver vascular wash-out after depriving liver of portal blood supply

Natural killer lymphocyte activity

Five weeks after laparotomy, splenectomy, porta-caval and mesentero-caval shunts and

porta-caval transposition, the NK cell activity was measured in the blood from the portal and inferior vena cava and in the liver, gut and hind-limb vascular wash-out.

Splenectomy produced some decrease in the liver wash-out cellular cytotoxicity as compared with the control (70.2 % to 54.8 %, NS) (Table 3), but did not affect the level of NK cytotoxicity in the portal and vena caval blood. Both porta-caval and mesentero-caval shunts evidently decreased the NK cytotoxicity in the liver wash-out population (to 35.6 % and 32.5 %, respectively ($p < 0.05$) without affecting the cytotoxicity level in the portal and peripheral blood. The porta-caval transposition brought about a moderate decrease of cytotoxicity of liver vascular population compared with control (to 55.2 %). Since the level of natural cytotoxicity may differ from day to day due to e.g. viral infections, comparison of data from animals investigated at different periods carries the risk of misinterpretation. In order to avoid it, we decided to measure the gradient of cytotoxicity between the portal blood and liver wash-out from the same animals. These data (Fig. 3) revealed that all procedures depriving liver of portal blood, also the porta-caval transposition maintaining high flow through the liver but of the non-portal blood, reduced significantly the liver vascular wash-out cytotoxicity. The liver cell-target cell binding was found 10.6 ± 0.5 % after laparatomy, 10 ± 3 % after splenectomy. 10.7 ± 0.9 % after the mesentero-caval shunt and 6.6 ± 1.1 %, after the porta-caval shunt.

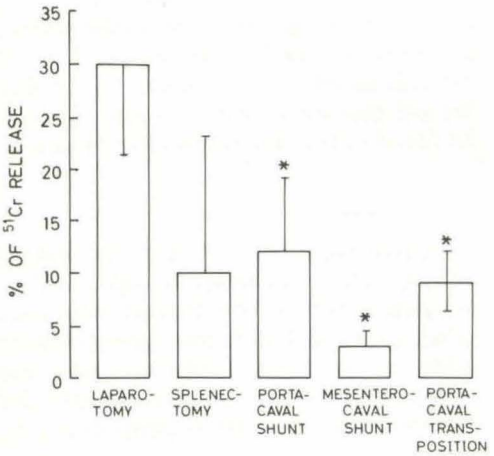

Fig. 3 The gradients of natural killer cytotoxicity between the inflowing portal blood and liver vascular wash-out 5 weeks after procedures depriving liver of inflow of cells from spleen and gut. Mean values ± SD, n = 3–5, E/T ratio 20 : 1. *PC-, MC- and PC-X vs laparotory $p < 0.05$

Discussion

The present study has yielded the following observations: a) a high natural killer lymphocyte cytotoxic activity in the non-adherent population of cells washed out from the liver microvasculature. The level of cytotoxicity was significantly higher than in the portal and systemic blood mononuclear populations, as well as in cells from the gut and peripheral tissues vascular wash-outs, b) the population of cells from the liver vascular wash-out had a high lymphocytes-K562 cell binding ability and contained a high percentage of large granular lymphocytes and cells possessing Fc and C3b receptors, c) the distribution of natural cytotoxicity in the liver wash-out and blood from other sources closely followed that of LGL and target cell binding, d) depriving liver of portal or mesenteric blood supply markedly decreased the NK cell cytotoxicity in the liver wash-out. Splenectomy had a moderate effect.

Our findings show that liver sinusoids contain, in contradistinction to the capillaries of the gut and other tissues like skin and muscles, high numbers of sequestered cells with NK activity. The questions arise as to whether, firstly these cells are blood-borne cytotoxic cells preferentially homing in the liver and not resident cells desquamated from the sinusoidal wall during the wash-out procedure, and secondly, what is their source of origin. The liver effector cell population was found to be peroxidase negative and its cytotoxicity was not reduced by removal of adherent cells, which rule out the possibility that Kupffer cells could account for the observed activity. The perfusion procedure with culture medium without enzymes, under low pressure and with low volume should not produce detachment of any significant number of resident non-parenchymatous cells. This was confirmed in our electron-microscopical studies of the perfused

liver of various species for transplant preservation (unpublished observations).

The evidence of liver wash-out population to be enriched in cells characteristics for the NK population was based on the correlation of the high level of cytotoxicity with high target-binding activity by the LGL and the high concentration of LGL. It has been shown that LGL, enriched by centrifugation on discontinuous Percoll density gradient reveal augmented NK activity (7) and also that athymic (nude) rats with 3- to 5-fold higher NK activity than euthymic rats have 2- to 7-fold higher concentration of LGL in peripheral blood and spleen (8).

The literature on the in vitro tumor-cell cytotoxicity by liver effectors in scarce. *Cohen* et al. (1) found that parenchymal liver cells (hepatocytes) had little tumoricidal activity, however, when nonparenchymal cell suspensions were separated, they demonstrated significant spontaneous killing of tumor cells. The authors presumed that nonparenchymal macrophages were most likely the predominant effector cells. However, they did not indicate whether these cells belonged only to the resident or also to the blood-borne population.

The origin of cytotoxic cells in the liver sinusoids may be diverse. These cells could be immigrants from the spleen and/or from the gut, Local activation or proliferation of NK precursor marginated in liver sinusoids should also be considered.

Spleen may be the source of liver cytotoxic cells since it contains a relatively high concentration of NK cells (6). It is, however, not known whether these cells migrate spontaneously away from that organ. The level of NK activity in the splenic vein blood did not differ from that of systemic venous blood. Nevertheless, splenectomy brought about a slight but evident decrease in the NK-activity in the liver vascular wash-out population. The gut seems to be a less probable source of NK cells detained in the liver. The NK cell activity in the population isolated from the gut mucosa had an exceptionally low spontaneous cytotoxic activity (5).

Diverting portal or selectively mesenteric blood to the systemic circulation by means of surgical shunts significantly decrased liver vascular wash-out cellular cytotoxicity. This might be accounted for by a low liver blood flow and mononuclear cell input. However, since the same concentration of cells was used in the cytotoxic tests, irrespective of the cellular yield from the liver microvasculature, a decrease in percentage of natural killer cells seems to be the most likely explanation. Maintaining of high blood flow through the portal vein in rats with porta-caval transposition was also followed by a reduction in liver wash-out cytotoxicity. These observations may indicate that either some subsets of cytotoxic cells which originate from spleen and/or gut preferentially home in the liver or humoral factors originating in the gut (e.g. interferon) and released to the portal blood stimulate the natural cytotoxicity of lymphocytes trafficking through the liver sinusoids.

Our studies did not give definite answer to the question of origin of liver sinusoidal blood NK cells, however, have shown that portal blood supply is necessary for keeping the natural cytotoxicity level at a normal level.

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