Studies on function and origin of hepatic sinusoidal cytotoxic cells

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CONTENTS

ABBREVIATIONS

ADCC	—	Antibody cell mediated cytotoxicity
AGM1	—	Asialo gangliotetraglycosylceramide
APAAP	—	Alkaline Phosphatase anti-Alkaline Phosphatase
B–cell	—	Bone marrow derived immunoglobulin producing cell
BMC	—	Bone marrow cells
BSA	—	Bovine Serum Albumin
BUdR	—	Bromodeoxyuridine
C3bR	—	Third component of complement receptor
CD	—	Clusters of differentiation antigens on cells
CFU-GM	—	Granulocyte-macrophage colony forming units
CM	—	Conditioned medium
CMC	—	Cell mediated cytotoxicity
CRBC	—	Chicken red blood cells
CSF	—	Colony stimulating factor
DMEM	—	Dulbecco's minimum essential medium
DNA	—	Deoxyrybonucleid acid
EA–RFC	—	Cells forming rosettes with antibody
EAC–RFC	—	Cells forming rosettes with antibody and complement
EC	—	Endothelial cells
FcR	—	Fc receptor
H–2	—	Major histocompatibility locus in mouse
HBSS	—	Hanks balanced salt solution
HDC	—	High density cells
IFN	—	Interferon
Ig	—	Immunoglobulin
IL1–6	—	Interleukin
IVC	—	Inferior vena cava

K	—	Killer
KC	—	Kupffer cells
LAK	—	Lymphokine activated killer cells
LDC	—	Low density cells
LDL	—	Low density lipoprotein
LGL	—	Large granular lymphocytes
LPS	—	Lipopolysaccharide
MEM	—	Eagle's minimal essential medium
MNC	—	Mononuclear cells
MoAb	—	Monoclonal antibodies
NC	—	Natural cytotoxic
NK	—	Natural killer
NKCF	—	Natural killer cytotoxic factors
NK-CIA	—	Natural killer derived colony inhibition activity
PBL	—	Peripheral blood lymphocytes
PBMC	—	Peripheral blood mononuclear cells
rIL2	—	Recombinant IL2
RT1	—	Major histocompatibility locus in rat
sIg	—	Surface immunoglobulin
SRBC	—	Sheep red blood cells
T-cell	—	Thymus derived cell
TBS	—	Tris buffer solution
TNF	—	Tumor necrosis factor

INTRODUCTION

Liver is physiologically exposed to antigens absorbed into the portal venous system from the gastrointestinal tract.

The intestinal mucosa provides an extensive surface for potential absorbtion of antigens, such as microbes, nutrients, chemicals etc.. The intestine is richly populated with lymphoid tissues capable of initiating various immune reactions. Contact between antigens and the mucosal immunologic apparatus initiates a series of immunologic events. Absorbed antigens, primed intestinal and splenic lymphocytes, and products of gut immune reactions like cytokines, are transported with portal blood to the liver.

It has been known that liver plays a role in establishing oral tolerance [70]. Certain studies have suggested that the immune response to orally administered antigens may differ from that obtained following administration of antigen by other routes, and that this may be due to the effect of the liver function [103]. Food and microbial antigens in the portal circulation have been shown to induce specific unresponsiveness [102].

The cells lining liver sinusoids, are responsible for clearance of most autologous and foreign materials from the portal blood supply. They are situated at an interface with the blood stream. This particular location affords them constant exposure to antigens absorbed from the gut and causes them to be among the first cells to encounter immunogens from the oral route. Sinusoidal cells seem to lack the ability to interact with immunocompetent lymphocytes and may rather possess a function to sequester antigens, thereby preventing their dissemination to lymphoid organs and avoiding a general immune response. This function of the lining cells (Kupffer and endothelial cells) is important in induction of the suppressor responses.

Endothelial cells (EC) have the capacity to specifically remove some materials from the blood, as demonstrated, for example, by their ability to clear acetylated low density lipoprotein (LDL) [79]. These cells were shown to bind soluble and insoluble immune complexes via their Fc receptors [105]. Recently Nagura et al. [69] have demonstrated that hepatic sinusoidal endothelial cells share antigens with a peripheral blood monocyte subset. They demonstrated the presence of HLA–DR, OKM–5 molecules, and synthesis of IL–1. These findings suggest that EC are capable of presenting soluble anti-

gens and inducing immunoregulatory protein synthesis by hepatocytes as well as stimulating lymphokine production.

The experiments examining the antigen–presenting function of Kupffer cells (KC) have suggested that these cells can present Ia antigens and induce proliferative responses of allogeneic T lymphocytes [68,81]. In contrast, other experimental results indicate that antigens introduced into the portal system in vivo do not provoke immune responses, but rather may result in specific tolerance [103]. In addition, Rubinstein et al. [94] found that guinea pig liver sinusoidal lining cells express functional Ia, but are unable to trigger a response by unprimed T cells. The defective antigen presentation could be due to the inhibition of Ia expression by prostaglandins or other substances present in the hepatic microenvironment such as α–fetoprotein [23]. This putative deficiency may contribute in part to the diminished capacity of antigens introduced into the portal blood to initiate an immune response.

Clinical observations in transplantation immunity further support this presumption. It was shown that cardiac or renal allograft survival could be prolonged by draining venous blood from the transplanted organ to the portal vein [5,8,18,35,95]. A marked decrease of the graft–versus–host reaction induced by semiallogeneic lymphoid tissues was also observed when these tissues were implanted into the liver itself [28]. Despite these suggestive observations, the cellular basis for immunologic unresponsiveness, or tolerance induction, after hepatic uptake of antigen remains unclear. Phagocytic blockade of the KC by administration of gelatin did not change the clinical course of the mild graft-versus-host reaction obtained after implantation of semiallogeneic lymph nodes into the liver [106]. Similarly, KC blockade did not result in a clear prolongation of heart allograft survival [47].

Another predicted function of hepatic sinusoidal cells is the recognition and destruction of tumour cells arising spontaneously in the liver or metastasizing to the liver via the portal and/or arterial circulations.

The liver has been shown, under certain experimental conditions to phagocytize tumours and modify tumour growth in vivo [26,90, 91]. Other studies demonstrated that livers from nonimmunized animals can rapidly eliminate radiolabeled tumour cells in vivo [88,89]. On the basis of animal experiments [17,58] it seems likely that millions of cancer cells per day are released into the blood-stream from primary foci, yet comparatively few metastases arise. Since liver sinusoids, especially, the periportal ones, are very narrow [113] it is easy to conceive that any (metastasizing) tumour cell coming into

the liver must come in close contact with the sinusoidal cells.

Passage of cancer cells through the liver vasculature is for them substantially more traumatic than passage through that of the lungs [110].

The mechanism underlying tumour cell destruction in the liver is unknown. The observations associating the death of many cancer cells with their transit through the liver do not permit discrimination between the possible mechanisms involved. These could include mechanical trauma or damage consequent upon the cellular and humoral inflammatory responses to interactions of the cancer cells with liver sinusoidal cells.

In accordance with Cohen et al. experiments, the nonparenchymal liver cells are the predominant effector cells in tumour surveillance [21]. While recent studies have identified KC as a primary sinusoidal cell mediating hepatic anti–tumour immunity, other sinusoidal cells also, appear to have tumoricidal capacities and may play an important role in the anti–tumour defense of the liver. This observation is in agreement with the work of Malter et al. [65] which has shown that silica pretreatment of the rats left substantial residual cytotoxic activity, indicating that after the elimination of macrophages, other cells had survived. Elimination of macrophages via a Sephadex column led to a cell population still able to kill tumour cells.

Many of the other studies support the hypothesis that Kupffer cells possess only minimal activity in hepatic anti–tumor resistance [12,22,104] and NK cells play significant role in the clearance of tumour cells in the liver [6,40,112].

The liver is a major organ for the replication of many viruses and the sole organ for the replication of some. The sinusoidal cells constitute a barrier between the blood and the hepatocytes. Destruction of the sinusoidal lining may allow the hepatocytes to be infected by non-hepatotropic viruses [56] and dissemination of the virus to other cells in the organism. Given their position within the hepatic sinusoids, the Kupffer and endothelial cells play a major role in virus infections in the liver. However, the observations that high numbers of large granular lymphocytes accumulate in the liver during viral infection suggest their relative importance [14,66].

Previously the role of sinusoidal cells in the defence mechanisms against infectious agents and tumour cells has long been attributed exclusively to KC and EC phagocytic properties. Recently, NK cell activity in the liver sinusoids represents an important factor limiting the survival and hence the metastasis of neoplastic cells from a tumour, as well as virus spread into the

circulation.

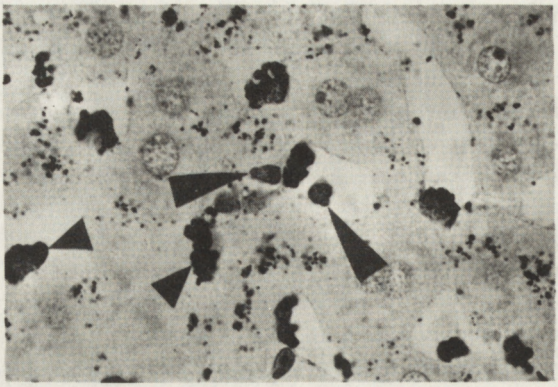

Figure 1. Histological picture of rat liver after i.v. injection of colloidal carbon. Note lymphocytes (large arrows) in hepatic sinusoids attached to the endothelial cells, Kupffer cells phagocytizing carbon particles (small arrows).

We have shown that under normal steady state conditions rat liver sinusoids harbours a pool of marginated cells [Fig.1] which express high natural cytotoxic activity [59]. These original findings were confirmed by the others [9,10,49,50,51,65]. These cells do not represent a constitutive liver non-parenchymal population and are rather sequestered from blood.

AIMS OF THE PRESENT STUDY

- To characterize functionally and phenotypically cells retained in the rat liver sinusoids
- To establish whether they are resident or blood–borne cells
- To evaluate the role of spleen and/or gut as potential suppliers of the cells to the liver

MATERIALS AND METHODS

Animals. Eight– to 10–week–old, conventionally housed rats of DA ($RT1^a$), Agus ($RT1^l$) and Lewis ($RT1^l$) strain and C57BL/6 ($H–2^b$) mice aged 8–12 weeks were routinely used in the experiments.

Tumour cell lines. The NK–sensitive cells used in this study were human K–562 leukaemic and mouse Moloney virus–induced YAC–1 lymphoma cells, and the NK–resistant cells were P–815, a methylcholantrene– induced mastocytoma, L–5178y, a methylcholantrene–induced lymphoma, and MBL–2, a Moloney virus–induced lymphoma. WEHI–164 fibrosarcoma was used as an NC–sensitive target, and WEHI–3 myelomonocytic cell line as a source of colony–stimulating factor (IL–3).

Radioisotopes. ^{51}Cr (sp.act. = 1 mCi/ml) and [^{111}In]oxine (sp.act = 20 mCi/ml) were obtained from Amersham (Arlington Heights).

Radiolabelling procedure. Briefly, 2×10^6 tumour cells were incubated for 60 min with 100 μCi ^{51}Cr at 37° C. Labelling with [^{111}In]ox was done by incubating 10^7 tumour cells with 30 μCi [^{111}In]ox for 10 min at room temperature.

Blood samples. Blood was drawn from the splenic, mesenteric, portal and hepatic veins, inferior vena cava (IVC) and aorta after 20 U of heparin had been injected intravenously (i.v.).

Isolation of cells from the spleen and mesenteric lymph nodes. The spleen and mesenteric lymph nodes were teased to release cells, which were washed once and resuspended in complete medium (CM) – RPMI–1640 supplemented with 10% fetal calf serum (FCS), 100 U penicillin and 100 μg streptomycin, sodium pyruvate and l–glutamine.

Vascular washout of liver, gut and hind limbs. Twenty units of heparin in 0.2 ml of saline solution were injected i.v. and the rats were exsanguinated. The portal and supradiaphragmatic vena cava were cannulated for liver perfusion, the superior mesenteric artery and vein for bowel perfusion

and the abdominal aorta and IVC for hind-limb perfusion. Forty milliliters of phosphate-buffered saline (PBS) at 20° C was infused under pressure, 25–30 mm Hg, and the effluent fluid was collected.

The vascular washout of gut and limbs served as control material for evaluation of the liver washout cell population.

Isolation of cells from the liver. Liver was excised after being perfused and minced into small pieces, forced through 50 gauge stainless steel mesh and placed in 50-ml tube with RPMI 1640. This extract was centrifuged at 600 g for 10 min and the supernatant was removed. Thirty mililiters of prewarmed enzyme solution, containing 0,01% collagenase type II (Sigma) and 1 mg DNA-se type I (Sigma) in RPMI 1640 medium + 10% FCS was added to the packed liver extract. The enzyme extract mixture was incubated at 37° C water bath for 8–10 min with constant agitation and the enzymatic digest washed two times in cold RPMI 1640 medium. The enzymatically digested liver was then resuspended in 20 ml of RPMI 1640 medium.

Isolation of intestinal mucosal lymphoid cells. Small intestine free of detritus was clumped at distal end and filled with 3% bovine serum albumin (BSA) in Hanks' balanced salt solution (HBSS) it was then rubbed gently between fingers and the contens was poured into siliconized tubes. Clumps of epithelial cells were spun down at 1000 rpm for 10 min and the pellet was resuspended in 25 ml of 3% BSA in HBSS. Cell suspension was passed over the glass beads column at room temperature. The column was washed once with 25 ml of HBSS. The eluate was collected and spun down at 1000 rpm for 10 min at 4° C. The pellet was resuspended with 5 ml of 35% BSA in HBSS and 2 ml of 28% BSA followed by 2 ml of 10% BSA in HBSS was placed on the top of cell suspension. Cells were centrifuged at 3000 rpm for 45 min at 4° C and the band between 10% and 28% BSA was collected and washed three times in RPMI complete medium.

Mononuclear cell isolation. Heparinized whole blood, spleen cell suspension, liver washout fluid and enzymatically digested liver mixture were separated on Lymphoprep gradient (Nycomed, Oslo, Norway) by centrifugation at 1600 rpm for 35 min. Cells obtained from the interface were washed 3 times in RPMI complete medium.

Morphological characterization of mononuclear cells. For the morphologic analysis 1.5×10^6 cells were centrifuged for 3 min at 400 rpm onto slides by using a Cytospin (Shandon Instruments Co, Sewickley, PA) centrifuge. Air–dried slides were fixed for 15 min in methanol and stained with May Grunwald Giemsa (Sigma). The large granular lymphocytes were recognized by azurophilic granules in the pale cytoplasm, a reniform nucleus, and a high cytoplasmic nuclear ratio, compared with other lymphocytes.

Fc and C3b receptors. Sheep red blood cells (SRBC), washed in HBSS were suspended at 2% volume and were opsonized with either IgG (7S) rabbit anti–SRBC antibody or with IgM (19S) rabbit anti SRBC–antibody (Cordis , Miami, FL) by incubating the SRBC for 30 min at 37° C with antibody diluted to a subagglutinating concentration. C3–opsonized SRBC were formed by incubating IgM–reacted SRBC for 30 min at 37° C with fresh mouse serum diluted 1/10 with saline. SRBC were then sedimented with mononuclear cells (MNC) by centrifugation at 800 rpm for 5 min at a ratio 75:1 and were incubated for 30 min at 20° C. Fc– and C3b–mediated adherence was examined by light microscopy.

sIg cells. Two million of MNC were spun down at 1300 rpm for 10 min. The supernatant was discarded, and 100 μl of fluorescein conjugated rabbit anti–rat IgG (Miles Lab.) were added to the pellet. After 30 min incubation at 20° C cells were washed three times in Eagle's minimal essential medium (MEM) and resuspended in 100 μl of medium. Two hundred cells were counted in a fluorescence microscope (Leitz Orthoplan).

Separation of adherent cells. To remove adherent cells from mononuclear cell suspension 2×10^6 cells were suspended in 10 ml CM. These cells settled for 2 hr at 37° C in tissue culture plates. Nonadherent cells were collected by vigorously rinsing the plates.

Phagocytosis assay. In vivo phagocytosis of effector cells was studied by injection of China–Ink intravenously and counting cells with engulfed particles. In vitro assay was performed according to the method by Taffet and Russel (100). Briefly, 1×10^6 MNC in 0.2 ml HBSS were incubated with 10 μl of a 1/6 dilution of coloidal carbon, were sedimented at 800 rpm for 5 min and were incubated at 37° C for 60 min. Cells were washed twice to

remove external carbon particles and were examined by light microscope.

Histochemical characteristics of cells. Liver perfusate MNC were identified histochemically by endogenous peroxidase, nonspecific esterase and PAS positive material in order to detect any contamination of sinusoidal washout with endothelial, and Kupffer cells.

Monoclonal antibody identification. Cytocentrifuge smears were made from vena porta and vena cava blood and from liver perfusate mononuclear cells, fixed in cold acetone for 90 seconds. Mouse MoAb: W3/13 (CD3), W3/25 (CD4), OX8 (CD8), OX6 (Ia), OX19 (CD5) (Sera Lab, Sussex, England) were added and incubated for 30 min at room temperature. Smears were washed 1–2 min in Tris–buffered saline (TBS) then incubated with anti–mouse Igs (Dakopatts, Copenhagen, Denmark) diluted 1/25, for 30 min at room temperature, and washed in TBS for 1–2 min. APAAP complex (Dakopatts) diluted 1/50 was added, incubated for 30 min at room temperature then washed for 1–2 min in TBS and alkaline phosphatase substrate was added and incubated for an additional 15–20 min at room temperature. Smears were washed in TBS and then in tap water, counterstained with haematoxylin, and mounted in a suitable aqueous mounting medium (Dako glycerol).

Cell depletion. The subsets of cells were depleted of OX8–positive cells (cytotoxic/suppressor subset) by the panning method. Briefly, mononuclear cells were incubated with mouse MoAb OX8 for 40 min on ice, washed three times with PBS+5% FCS, and added to the polystyrene sterile Petri dishes coated with goat anti–mouse IgG (Dakopatts). The Petri dishes were then incubated for 40 min at 4° C, swirled a few times, then incubated for another 30 min at 4° C. Nonadherent cells were aspirated and used as OX8–negative subsets. OX8–positive cells attached to the dishes were scraped by a rubber policeman into complete medium and the correct concentration was made for further functional studies.

Elimination of natural killer (NK) cells with anti–asialo–GM1 antiserum.

In vitro: 2×10^6 MNC were mixed in 0.5 ml of CM with 5 μl of anti–asialo–GM1 (AAGM1) antiserum (WAKO, Japan) and incubated for 30 min

at 4° C. After washing 1.25 ml of fresh rabbit serum diluted 1/15 was added to the pellet and incubated for 60 min at 37° C.

In vivo: 5 μl or 25 μl of AAGM1 was dissolved in saline and injected intravenously 24 hr prior to collecting blood samples.

Target binding cell assay. Two $\times 10^6$ MNC were mixed with 2×10^7 K–562 cells in 0.4 ml CM and centrifuged at 200 rpm for 15 min, at 4° C in round–bottomed plastic tubes. Tubes were placed on ice, cell suspension was separated vigorously with a Pasteur pipette. One drop of cell suspension was placed on a microscope slide and percentage of lymphoid cells binding to K–562 cells was determined.

Assay for NK cytotoxicity. The details of this test have been described previously [60] Briefly, 5×10^3 ^{51}Cr–labelled target cells in 0.1 ml volume were incubated with serial dilutions of effector cells at 40:1 to 5:1 (E/T ratio) in round–bottomed wells of micro titre plates (Limbro, Scientific Inc.) and the test was incubated for 4 hr or 18 hr at 37° C. The supernatant was removed from each well and the ^{51}Cr present in the supernatant was assessed in a Beckman gamma counter for 1 min. Supernatant harvested from wells containing target cells alone served as controls for baseline release of ^{51}Cr and 5×10^3 labelled target cells were also counted to determine total ^{51}Cr incorporated into the target cells. The percentage of cytotoxicity was calculated by the formula:

$$\frac{\text{cpm of test group} - \text{cpm in medium control}}{\text{Total cpm in } 5\times10^3 \text{ targets} - \text{cpm of medium control}} \times 100$$

All E/T ratios were measured in triplicate.

Antibody dependent cell cytotoxicity (ADCC) assay. One hundred ul of MNC at concentration 1×10^7/ml, 5×10^6/ml or 2.5×10^6/ml were mixed with 100 μl of chicken erythrocytes (CRBC) labelled with ^{51}Cr (5×10^4 /ml) and 100 μl of rabbit anti–chicken antiserum diluted 1/10000 was added. After 4 hr incubation at 37° C in an atmosphere of 5% CO_2 supernatant was collected and the activity of supernatant and supernatant + pellet was measured. The percentage of ^{51}Cr specific release was determined as in the NK assay.

Treatment with sugars. All sugars tested for inhibition of NK cyto-

toxicity (d–mannose, d–glucose, d–galactose, N–acetyl–d–glucosamine) were obtained from Sigma (St.Louis, Mo, USA) as sodium salts with a purity of 97–100%. Stock solutions (250 mM) were made in RPMI–1640 (pH 7–7.2, 290 mOsm). The sugars were chosen on the basis of previous studies of the role of sugars on the lysis of targets by NK cells. The possible inhibitory effects of the sugar solutions were examined in the cytotoxic assay by adding different monosaccharides to the effector phase of the cytotoxicity assay at the concentration of 25 mM.

Percoll fractionation of mononuclear cells. Cells were separated on a seven–step discontinuous Percoll gradient (Pharmacia Fine Chemicals, Uppsala, Sweden) as described elsewhere [64]. Percoll was adjusted to 285 mOsm with hyperosmolar PBS (10x) solution before preparing the gradient. Typically, a seven step gradient consisted of 44.2%, 47.5%, 50.8%, 52.5%, 55.0%, 57.5%, and 66.7% Percoll and complete medium. Up to 10^8 cells were centrifuged at 2000 rpm for 30 min at room temperature to obtain seven bands, which were carefully collected with a Pasteur pipette. The cells were washed twice with RPMI 1640 and then resuspended in complete medium for use as effectors.

Production of supernatant fluid from mononuclear cells cocultured with different tumour cell lines. Mononuclear cells from different sources were resuspended to a concentration of $5x10^6$/ml with complete medium and incubated for 48 h at 37° C in 5% CO_2, humidified atmosphere with $2x10^5$ K–562 or YAC–1 cells. After 48 h of incubation, the cells were centrifuged and cell–free supernatants were harvested, filtered through 0.22 μm Millex–GC filters and were either used immediately or kept frozen at –70° C until use.

Natural killer cytotoxic factor (NKCF) assay. Rat NKCF activity was assessed by measuring cytotoxicity against YAC–1 target cells labelled with [^{111}In]ox. The assay was set up in triplicate in 96–well U–bottomed sterile microtitre plates. Fifty microliters of target cell suspension ($8x10^4$ cells/ml) was added to each well. Different volumes of cell–free supernatant containing NKCF (100 and 50 μl) were added to each well and the final volume was adjusted to 0.2 ml by adding medium. After 18 hr of incubation at 37° C in 5% CO_2, the [^{111}In]ox released into 0.1 ml of supernatant from

each well was determined. Total counts per minute were determined by resuspending the target cells and counting the radioactivity in 0.1 ml of suspension. Spontaneous release was assessed from supernatants containing target cells alone in 0.2 ml medium. The percentage of cytotoxicity was calculated as follows:

$$\frac{\text{test cpm} - \text{spontaneous cpm}}{\text{total cpm} - \text{spontaneous cpm}} \times 100$$

Bone marrow cell (BMC) preparation. BMC were harvested from the femurs of C57BL/6 mice by flushing with sterile Dulbecco's minimum essential medium (DMEM) with l-glutamine, sodium pyruvate, antibiotics, and 10% FCS through 21 gauge needles. The cells were washed twice in DMEM and resuspended to a concentration of 10^6 BMC/ml.

Inhibition of bone marrow late granulocyte–macrophage colony–forming unit (CFU–GM) growth by cell–free supernatants. The effect of soluble factors released from mononuclear cells after stimulation with tumour cells on colony formation was tested by adding supernatants (100 μl) to 10^6 BMC from C57BL/6 mice and preincubation for 3 h at 37° C. The BMC were washed and plated for colony formation in 0.35% agarose for CFU–GM growth with a slight modification of the method by Pike & Robinson [78]. Briefly, a two–layer colony assay with WEHI–3 conditioned medium in the bottom layer as a source of colony stimulating factor (IL–3) and 10^5 BMC in the upper layer was used. Duplicate plates were incubated in 7% CO_2 at 37^2 C and the number of colonies containing more than 50 cells was counted under an inverted microscope after 7 days. DMEM with antibiotics, l–glutamine, sodium pyruvate, and 20% FCS was used. [Fig. 2]

Liver transplantation. Liver was transplanted orthotopically between syngeneic female (donor) and male (recipient) Lewis rats. The animals were used for the further experiments four weeks later after transplantation. The microsurgical technique described by Lee [57] was used with own modification of the perfusion: the entire animal was perfused with 15 ml of ice cold Ringer's solution following cannulation of the aorta. The graft was supplied only with blood from the portal vein, no hepatic artery anastomosis being made. The circulatory/respiratory system of the donor was intact the vena cava below the liver was opened. Prior to perfusion blood was withdrawn

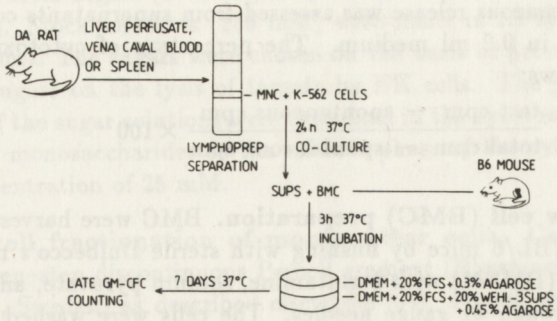

Figure 2. Schematic representation of the method used to inhibit granulocyte and macrophage colony forming cells (late GM–CFC) growth.

from the arterial system via the aorta. The organ ischemic time lasted between 25–30 min and the nonhepatic period did not exceed 15 min. An individually adapted portex tube was inserted into the common bile duct before implantation in the duodenum. During surgery, great care was taken to ensure that the blood volume remained constant. Before operation the animals received 2 ml of blood and 2 ml 5% glucose, during the nonhepatic period and revascularization 5 ml of Ringer's solution were given. Post–operatively 5 mg gentamicin were administered intraperitoneally.

Histologic studies. For routine histology, livers were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 6 μm, and stained with hematoxylin and eosin.

Identification of dividing cells. MNC in CM was incubated with Bromodeoxyuridine (BUdR) at final concentration 10 μg/ml (1–2x10^6 cells) for

2 hr at 37° C. After washing in CM the cytocentrifuge smears were made. Slides were dried before staining. Dissociation of histones and partial denaturation of DNA was carried. This involved treatment with 70% (v/v) formamide in 0.15 M trisodium citrate at 70° C for 40 min followed by extensive washing in staining/ washing buffer (0.05 M Tris–HCl saline, pH 7.6). BUdR was detected by staining with M–744 a mouse monoclonal antibody (Dako, Copenhagen,Denmark). This antibody was applied to smears at a dilution 1/20 and after washing was in turn detected with a rabbit anti–mouse Ig conjugated to alkaline phosphatase– D–314 (Dako) The rabbit anti–mouse Ig conjugate was diluted in Tris–HCl saline 1/30 prior to use. The reaction was developed with chromogenic substrate. Slides were counterstained in Mayer's hematoxylin. Controls of labelling specificity consisted of omitting the primary antibody.

Cytogenetic analysis of sex chromosomes. Chromosome preparations were carried out as described [93]. Briefly, MNC were resuspended in CM and exposed to colcemid at final concentration 0.07g/ml for 60 min, followed by treatment with hypotonic 0.075 M KCl for 10 min and extensive fixation in methanol/acetic combination (1:3). Fixed cells were spread on slides and dried before storage or staining. After 3 days slides were stained in Giemsa and analyzed directly under the light microscope. Some slides in each experiment were stained using C–banding procedure, described by Sumner [98].

Metaphases of good quality were analized under the light microscope. The sex chromosome of the cells were determined on the basis of presence or absence of hetero–Y chromosome, which is recognizable after C–banding.

Surgical procedures depriving liver of venous splenic or intestinal blood supply. Laparotomy was performed under ether anesthesia. In various groups splenectomy, porto–caval and mesentero–caval shunts and porto–caval transposition were performed [Fig.3]. Splenectomy deprived liver of potential source of cytotoxic cells, porto–caval shunt totally eliminated portal blood supply, mesentero–caval shunt diverted intestinal venous blood from the liver, porto–caval transposition eliminated portal flow but maintained total liver blood flow at physiological levels.

Statistical evaluation. For analysis of statistical significance of differ-

ences in results the Student t–test for pairs was used.

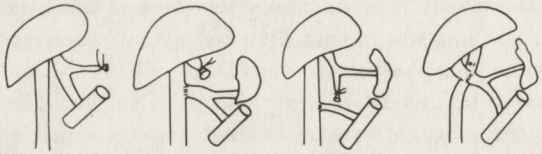

Figure 3. Types of microsurgical procedures performed to deprive liver of portal or splenic blood inflow.

RESULTS

Lymphocyte counts. In the splenic venous blood the lymphocyte count was $13.2 \pm 3.7 \times 10^9$/l; in the mesenteric vein, 6.9 ± 2.6; in the portal vein, 10.7 ± 1.7; in the hepatic artery, 4.2 ± 2.8; in the hepatic vein, 6.4 ± 2.4; and in the IVC $7.2 \pm 2.9 \times 10^9$ /l. There was a statistically significant difference in lymphocyte count between the splenic and other venous bloods /$p<0.05$/.

Histochemical characteristics. The percentage of cells with diffuse cytoplasmic staining for non–specific acid esterase was in the splenic, portal, and IVC blood within the range of 15–26% but in liver sinusoidal blood reached $51.8 \pm 14.8\%$ /$p<0.01$/.

The percentage of cells phagocytizing colloidal carbon remained between 2% and 6% in all blood sources. The blood and liver washout mononuclear cells was found to be peroxidase negative and PAS negative. No cells morphologically similar to the Kupffer and endothelial cells or hepatocytes could be found in the smears from liver washout [Fig.4].

The concentration of LGL was at a similar level in splenic, portal, and IVC blood (4–8%) but in the liver was as high as $28.8 \pm 10.2\%$ /$p<0.01$/.

It was interesting to observe that the percentage of LGL remained stable in the consecutive wash–out samples despite decreasing total numbers of cells harvested from the liver vasculature [Fig.5].

Mononuclear cells washed out from the liver sinusoids revealed high percentage of mitoses ($9.08 \pm 1.9\%$) [Fig.6], blood mononuclear cells derived from different sources did not contain dividing cells.

Cell surface receptors. The splanchnic venous blood contained more Fc+ cells ($12.4 \pm 2.9\%$) than the arterial ($5.1 \pm 1.8\%$) and peripheral venous blood ($7.4 \pm 1.9\%$) /$p<0.05$/. The highest percentage of EA–RFC was found in spleen, intestinal mucosal and liver sinusoidal washout cells ($22.8 \pm 4.9\%$, $16.7 \pm 2.4\%$ and $14.8\% \pm 2.8$, respectively). No evident differences in the percentage of Fc+ cells were observed between the portal ($11.3 \pm 3.4\%$) and hepatic venous blood ($11.5 \pm 3.4\%$) [Fig.7].

The mononuclear cells isolated from spleen, intestinal mucosa, mesenteric lymph nodes and liver perfusate revealed more C3b–positive cells ($33.5 \pm$

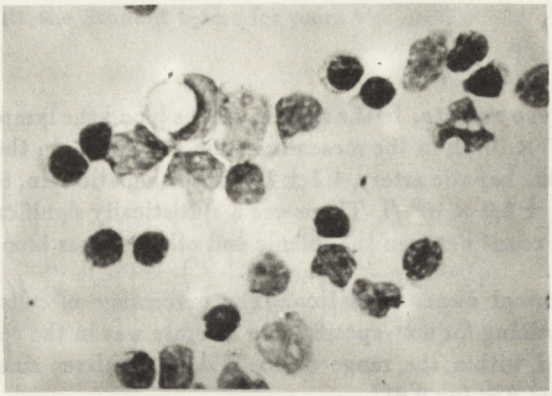

Figure 4. MGG–stained cytopreparation of rat liver washout.

5.0%, 24.1 ± 2.1%, 17.8 ± 1.8% and 11.6 ± 3.7%) than the splenic (5.8 ± 2.7%) and other venous blood (2.8 ± 1.6%)/$p<0.01$/. There were no statistically significant differences between the percentage of C3b–positive cells but a gradient was found for the EAC–RFC between portal and hepatic venous blood /$p<0.05$/ [Fig.8].

Splenic venous blood contained the highest number of sIg–positive cells (9.5 ± 1.7%) of all blood sources, but there was no difference in the concentration of these cells between portal (7.8 ± 2.6%) and hepatic venous blood (7.1 ± 2.4%). The highest percentage of sIg–positive cells was found in the intestinal mucosa (31.0 ± 1.8%) and spleen (16.6 ± 2.6%) mononuclear cells [Fig.9].

Phenotypic characteristics of functional subsets (MoAb). Splanchnic venous blood contained from 75.7 ± 3.5% to 84.6 ± 4.7% of W3/13–positive (CD3) cells. No significant difference in their concentration was found between the splenic, mesenteric, portal, and liver sinusoidal blood. The arterial and IVC blood had 80.6 ± 6.6 and 78.1 ± 6.5% of W3/13–positive cells, respectively [Fig.10].

The concentration of W3/25–positive (CD4) cells was similar in all blood

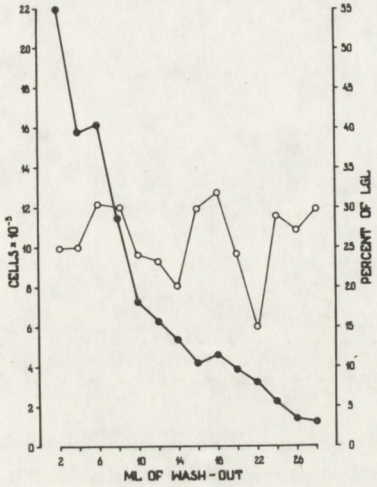

Figure 5. Cell yield (•) and percentage of LGL (o) in liver vascular washout. In the course of perfusion, the cell yield decreased, but the percentage of LGL remained unchanged.

sources (range of 53.4 ±3.4% to 65.0 ±6.0 (NS) except for the liver sinusoidal washout, where it was clearly decreased (39.5 ± 4.9%) /$p<0.05$/ [Fig.11].

The OX8–positive cells (CD8) were represented in the splenic and liver sinusoidal blood at a significantly higher level than in other splanchnic blood sources and in the hepatic artery and IVC /$p<0.05$/. The concentration in the splenic vein was 35.0 ± 3.4% and in the sinusoidal blood, 46.3 ± 6.3%. In other sources it ranged between 22.6 ± 4.4% and 26.6 ± 3.2% [Fig.12].

The level of OX19–positive (CD5) cells in the peripheral blood was 64.3 ± 7.6%. It was significantly decreased in portal blood (51.4 ± 3.6%) and liver sinusoidal blood (50.8 ± 10.9%, $p<0.05$).

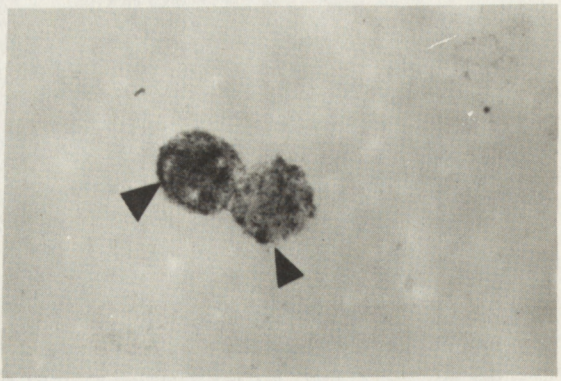

Figure 6. Freshly obtained liver sinusoidal washout cells incubated with BUdR and processed by BUdR MoAb with immuno–alkaline phosphatase staining technique. Arrows show positive cells.

The OX6–positive cells (Ia) was similar in blood from the splanchnic area and hepatic artery and IVC blood and ranged between 7.4 +2.8% and 10.0+4.0%. No difference between the splenic, mesenteric, portal and hepatic venous blood was observed [Fig.13].

Natural killer cell cytotoxicity (NKC). The highest NKC cytotoxicity (76.0 $\pm$ 7.4%) was observed in liver sinusoidal blood [Fig.14]. In blood from other splanchnic and nonsplanchnic sources, it remained in the mean range of 49 and 66%. No statistically significant differences could be found between splenic, portal, and hepatic venous blood.

The NKC cytotoxicity in the capillary washout of gut and limb was 32.5 $\pm$ 3.5 and 39.0 $\pm$ 4.8%, respectively, which are well below the level in the arterial and venous mesenteric blood (49.89 $\pm$ 12.28% and 60.79 $\pm$ 8.83%, respectively). Natural killer activity among mononuclear cells isolated from spleen, mesenteric lymph nodes and mucosa was low compare to the other sources and ranged between 11 and 36% [Fig.15].

Killer cell activity. The highest level of killer cell activity (87.3±10.2%) was found in the liver sinusoidal blood [Fig.16]. The cytotoxicity in blood from splenic, portal and hepatic veins, and IVC ranged between 46 and 53%, and the differences were not statistically significant.

The blood capillary wash–out of the gut and limb revealed 47.5±3.0% and 43.5±6.0% cytotoxicity, respectively.

Killer cell activity in spleen, mesenteric lymph nodes and mucosal mononuclear cell population was 26.6±1.4, 5.2±4.6, and 4.4±0.2%, respectively [Fig.17].

Natural killer–target binding. The percentage of lymphoid cells binding to K–562 cells was in the liver vascular wash–out population 10.6±0.5% whereas in blood from all other investigated sources was found at the level of around 5% /$p<0.05$/.

Elimination of NKC activity with anti–asialo GM1

In vivo. Treatment of rats with AAGM1 twenty four hours prior to testing of the NK activity, brought about no change in the percentage of LGL in liver wash–out population, but there was an evident decrease in portal /$p<0.05$/, and caval blood /$p<0.05$/ (Table I).

Table I. Large granular lymphocytes in liver sinusoidal washout, portal and caval blood before and after in vivo and in vitro treatment with anti-asialo-GM1 antiserum.

Source of cells	In vivo		In vitro	
	Control	AAGM1	Control	AAGM1
Liver washout	22.8±10.2*	16.7±7.1	28.5±8.0	28.5±10.5
Portal blood	6.2± 2.2	0.6±0.7#	3.1±2.7	1.9± 1.1#
Caval blood	3.7± 1.4	2.3±0.2#	5.4±3.1	0.4± 2.3#

* Values are means of percent ± SD., n=6

\# ASGM1 treated vs controls $p<0.05$

The NK cytotoxicity remained almost unchanged in the liver sinusoidal population (83.0±11.5% and 72.5±8.0%, NS, E/T 40:1); however, it declined evidently in portal (56.4 ±4.1% to 40.0±4.5%) /$p<0.05$/ and caval blood (53.0±8.1% to 32.0±9.1%) /$p<0.05$/ [Fig.18].

Treatment of rats with 5–times higher doses of AAGM1 (25 μl) almost totally eliminated the NK cytotoxicity in portal (36.9±7.4% to 4.7±0.4%) /$p<0.05$/ and caval blood (39.8±0.4% to 6.6±0.5%) /$p<0.05$/ but did not affect the cytotoxicity of liver washout population (76.7±5.5% to 73.0±5.0%) [Fig.19].

In vitro. Elimination of NK cells with AAGM1 and complement did not decrease the percentage of LGL in the liver wash–out population; however this procedure was highly effective in portal, and caval blood. Lack of changes in the percentage of LGL in the liver population was reflected in their natural cytotoxicity level. There were no changes observed (69.3±3.7% and 67.1±3.7%, NS, E/T 40:1). Portal and caval blood had a decrease of cytotoxicity from 45.8±10.5% to 7.6±5.2% /$p<0.05$/ and 39.4±7.1% to 11.2±9.6% /$p<0.05$/, respectively [Fig.20].

Cytotoxicity of rat mononuclear cells (MNC) against various target cells. MNC derived from rat PBL and liver washout were tested for cytotoxicity against six different allogeneic cell lines in 4 h and 18 h ^{51}Cr release assay. The effectors were titrated down from 20:1 to 5:1 in most cases and the results are expressed graphically in Fig.21. Of the two NK–sensitive targets tested, both K–562 and YAC–1 lines displayed appreciable susceptibility to lysis by PBL (28.52±11.25% and 26.41±14.47%, respectively at an E/T ratio of 20:1) however, the percentage of cytotoxicity was significantly higher with liver washout cells used as effectors (56.67±16.51% and 58.66±15.47%, respectively, $P<0.01$). In contrast, NK–resistant targets L–5178y, MBL–2, and P–815 lines were poor targets for PBL effectors (3.11±5.02%, 1.78±0.82%, and 4.10±5.6%, respectively at an E/T ratio of 20:1) but they were very sensitive to liver washout cells (54.55±28.95%, 36.63±9.23%, and 43.40±14.37%, respectively) The WEHI–164 displayed a weak sensitivity to rat PBL (13.51±8.91% at an E/T ratio of 20:1), but liver sinusoidal cells were responsible for the relatively high cytotoxicity against these NC–sensitive targets (33.79±10.12%).

Effects of sugars on interaction of NK cells with the target cells. Four different sugars were tested for their effects on lysis of ^{51}Cr–labelled K–562 target cells mediated by freshly isolated mononuclear cells. The results of these studies are presented in Table II. No appreciable inhibition of either PBL or liver washout cell cytotoxicity was observed by any of the tested

saccharides.

Table II. The effect of monosaccharides on natural cytotoxicity mediated by liver washout cells and PBL*

Monosaccharide	Liver washout cytotoxicity#(%)	PBL cytotoxicity# (%)
None	69.50±2.12	15.00±8.8
D–mannose	69.00±4.24	15.17±9.9
D–glucose	62.00±5.66	15.50±9.6
D–galactose	67.00±1.41	14.33±8.6
N–acetyl D–glucosamine	64.00±0	14.00±11.7

* Each value represents the arithmetic mean of 3–6 samples ± standard error of the mean

As measured against K–562 at E/T ratio 20:1

Enrichment of NK cells using seven–step Percoll gradient separation. It has been demonstrated that discontinuous Percoll gradient centrifugation can be used to enrich cells with NK activity. In the seven–step Percoll gradient rat NK cell activity was found in a denser fraction than NK–enriched fraction of human PBL. The mean data from five experiments are presented in Fig.22. Among the seven bands of liver washout cells the low–density fractions 2 to 5 contained the population of cells with significantly increased NK activity (70.0–76.5%) as measured in the CMC assay compared with the high–density fractions 6 (46.7±6.48%) and 7 (43.5±8.14%) or to the unseparated cells (63.3±8.9%). In the portal blood, high NK activity was associated with fractions 2 to 5 (24.0–42.3%) and in the peripheral blood from vena cava fractions 3 to 5 (19.5±38.2%) contained the most cells with increased NK cytotoxicity.

There was a high correlation between the level of NK activity and frequency of large granular lymphocytes (LGL) in the isolated fractions. In the liver washout cells, fractions 2, 3 and 4 revealed a higher frequency of LGL (43.0±22.0%, 46.0±9.7%, and 49.6±24.7%, respectively) than high–density fractions 6 and 7 (15.0±9.0% and 5.8±3.2%) ($P<0.01$). Fractions 2, 3, and 4 from portal blood were enriched in LGL (26.33±9.22%, 16.0±4.89%, and

13.28±6.77%, respectively) compared to the whole cell population (11.0 ± 1.0%).

Blood from vena cava contained higher percentage of LGL in fractions 2, 3, and 4 (13.3±4.41%, 11.5±5.5%, and 7,86±3.2%, respectively) than the whole unseparated cell population (6.0±2.45%) [Fig.23].

Function of OX8–positive cells in rat NK activity. To test whether most of the NK activity was associated with OX8–positive cells, cells from the vena cava and liver washout were carefully fractionated into OX8–positive and OX8–negative subpopulations.

The results are presented in Fig.24. In PBL the OX8–positive cells were highly cytotoxic compared with the whole population (21.3±7.54% and 8.0±4.5%, respectively) and with OX8–negative cells (21.3±7.54% to 4.0±0%) /$p<0.01$/, respectively at the ratio 10:1). Liver washout OX8–positive cells were slightly more cytotoxic than the unseparated cells, but the differences were not statistically significant. There was, however, a significant difference in the level of cytotoxicity with respect to the OX8–negative population /$p<0.01$/ although the level of the latter remained relatively high (26.6±6.4% at E/T ratio 10:1).

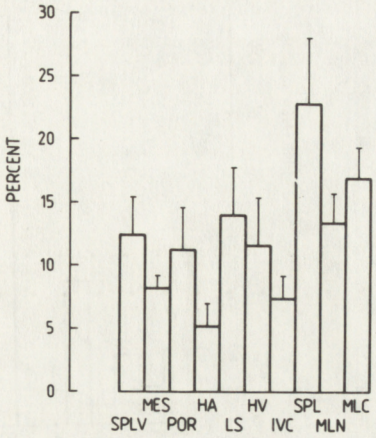

Figure 7. Mononuclear cells forming EA rosettes in blood and different organs in the splanchnic area. SPLV = splenic; MES = mesenteric; POR = portal; HA = hepatic arterial; LS = liver sinusoidal; HV = hepatic venous; IVC = inferior vena cava; SPL = spleen; MLN = mesenteric lymph nodes and MLC = mucosal lymphoid cells. Values are mean percentages $\pm$ SD, n = 5.

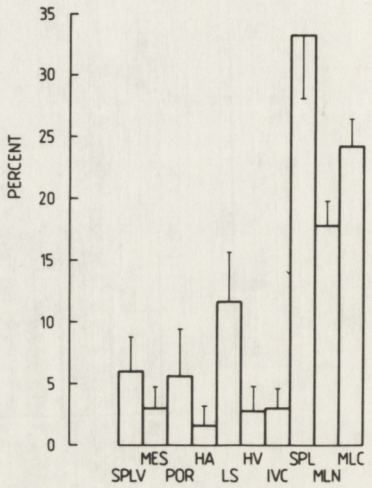

Figure 8. Mononuclear cells forming EAC rosettes in blood and different organs in splanchnic area. For details see Fig.5.

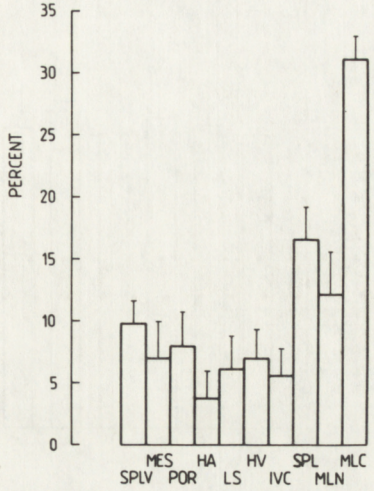

Figure 9. Mononuclear cells with surface immunoglobulins (sIg+) in blood and different organs in splanchnic area. For details see Fig.7.

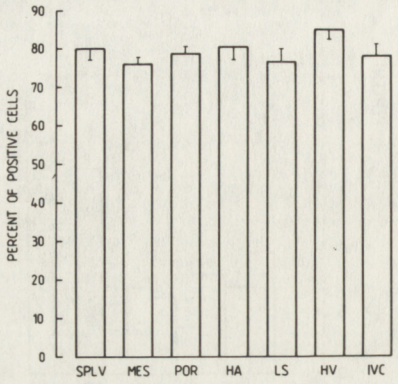

Figure 10. Monoclonal antibody (W3/13 = CD3) characterization of mononuclear cells in blood in the splanchnic area. For details see Fig.7.

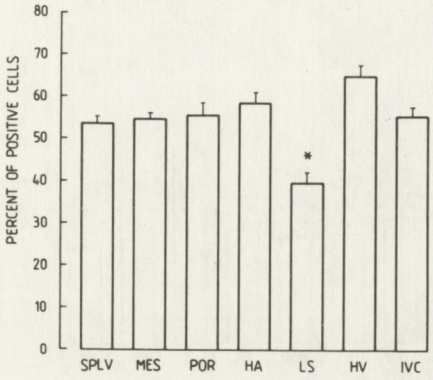

Figure 11. Monoclonal antibody (W3/25 = CD4) characterization of mononuclear cells in blood in the splanchnic area. For details see Fig.7. * Liver sinusoidal blood versus all others, $p<0.05$.

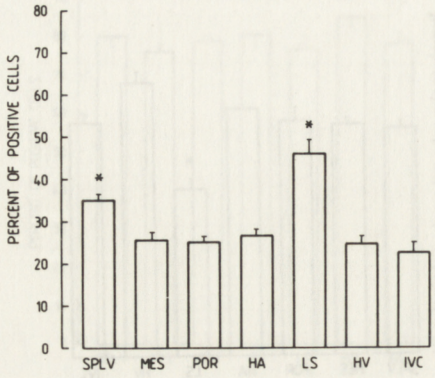

Figure 12. Monoclonal antibody (OX8 = CD8) characterization of mononuclear cells in blood in the splanchnic area. For details see Fig.7. * Splenic and liver sinusoidal blood versus all others, $p<0.05$.

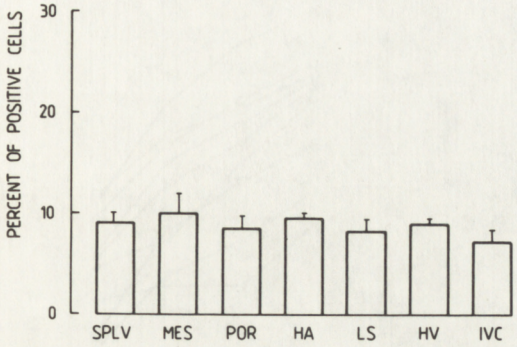

Figure 13. Monoclonal antibody (OX6 = Ia) characterization of mononuclear cells in blood in the splanchnic area. For details see Fig.7.

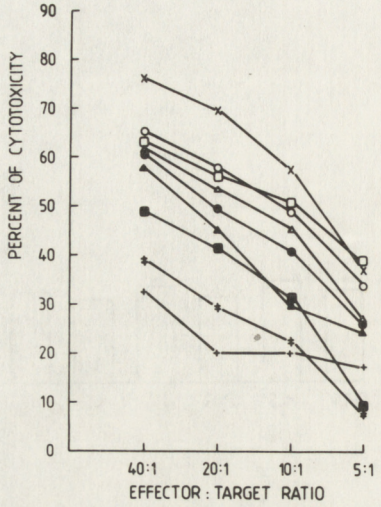

Figure 14. Natural killer cell cytotoxicity against K–562 target cells in blood in the splanchnic area. Splenic (△); mesenteric (●); portal (○); hepatic arterial (■); liver sinusoidal (×); hepatic venous (△); inferior vena cava blood (□); gut (+); and limb (‡) capillary washout. Values are means of six experiments. Note the high NK activity in liver sinusoidal washout compared with portal blood and gut and hind limb capillary washout ($p<0.05$) .

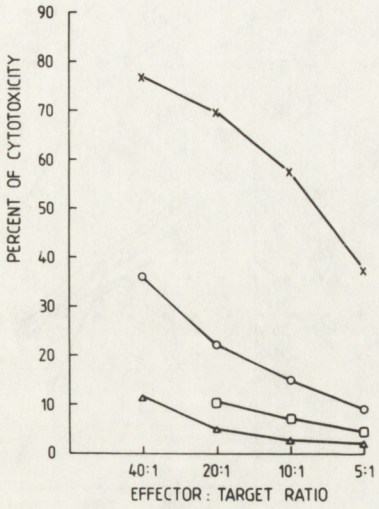

Figure 15. The natural killer activity in the mononuclear cell population isolated from spleen (○), mesenteric lymph nodes (△), mucosa (□) and liver vascular washout (×). Values are means of five experiments. Note the high NK activity in liver vascular washout compared with the other sources ($p<0.05$).

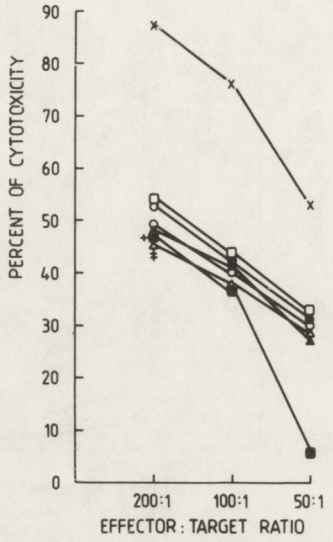

Figure 16. K–cell cytotoxicity in blood in the splanchnic area. For details, see Figure 14.

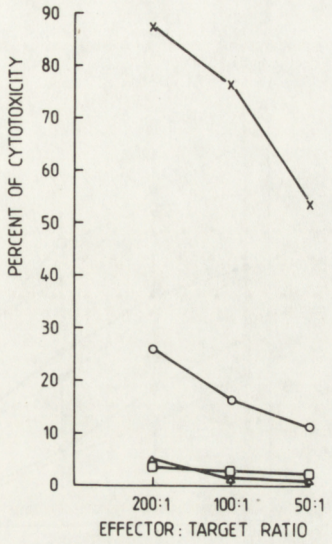

Figure 17. K–cell activity in mononuclear cell population isolated from spleen, mesenteric lymph nodes, mucosa and liver capillary washout. For details, see Figure 15.

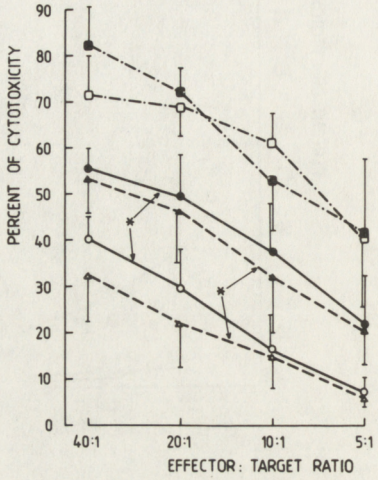

Figure 18. The NK cell cytotoxicity in portal (○●), caval (△▲) blood and liver capillary washout (□■) in normal (closed signs) and anti–AAGM1 antiserum (5 μl) (open signs) treated rats. Values are means $\pm$ SD, n = 5, *p<0.05.

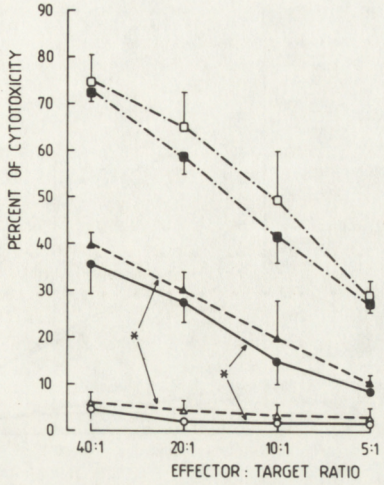

Figure 19. The NKC cytotoxicity in portal, caval blood and liver capillary washout in normal and anti-AAGM1 antiserum (25 μl) treated rats. For details, see Figure 18. Values are means $\pm$ SD, n = 5, *p<0.05.

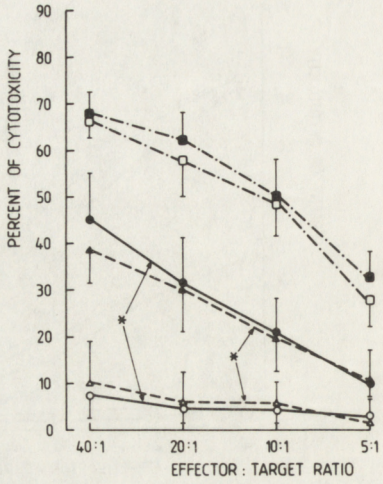

Figure 20. The NK cell cytotoxicity in portal (○●), caval (△▲) blood and liver capillary washout (□■) in normal (closed signs) and in vitro anti-AAGM1 antiserum + C'-treated (open signs) populations. Values are means ± SD, n = 5, *$p<0.05$.

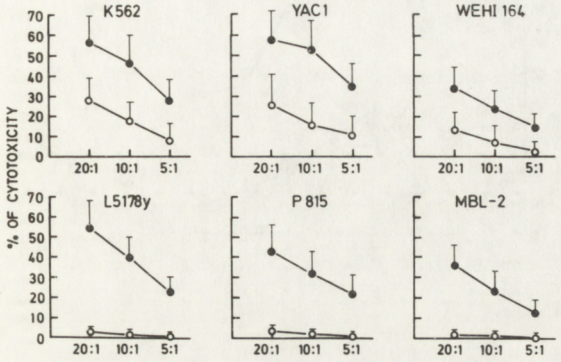

Figure 21. Natural cytotoxicity of rat PBL (○) and liver washout cells (●) against various target lines. All cytotoxicity values represent mean percentage ± SD, n = 5.

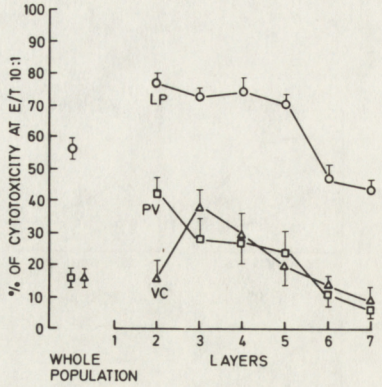

Figure 22. Enrichment of liver washout (o), vena portae (□) and vena cava blood (△) natural killer activity using seven step Percoll gradient. Mean values from five experiments ± SD.

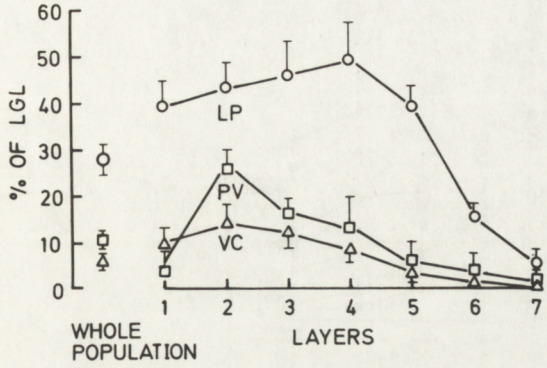

Figure 23. Large granular lymphocytes enrichment by discontinous Percoll gradient separation of liver washout cells (o), vena porta (□) and vena cava (△) blood cells. Mean percentage of six experiments ± SD.

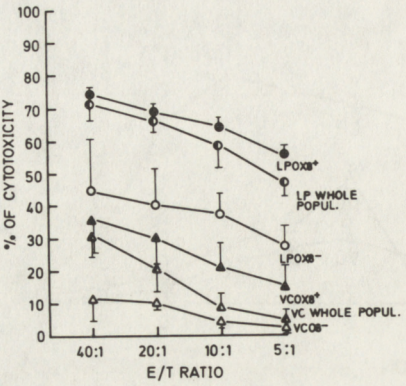

Figure 24. Natural cytotoxicity of the whole unseparated population of mononuclear cells from liver washout (LP) and vena cava (VC), OX8–positive cells and OX8–negative cells of LP and VC. Mean percentage of five experiments ± SD.

Table III. Natural cytotoxicity mediated by cell-free supernatants from PBL, spleen and liver washout cells*.

Effector cells	Cytotoxicity of NKCF (%)	
	100μl	50μl
Experiment 1		
PBL	10.42	7.76
Spleen	4.39	2.26
Liver washout	55.04	26.31
Experiment 2		
PBL	17.01	5.42
Spleen	12.29	0.83
Liver washout	51.05	20.11

* As measured against ^{111}In YAC-1 targets in 18-h assay

Production and lytic activity of natural killer cytotoxic factors (NKCF) by rat liver washout, peripheral blood lymphocytes (PBL) and spleen cells. Previous experiments have shown that liver perfusate contained a population of cells with significantly increased NK activity measured in the CMC assay as compared to PBL and spleen. Liver washout cells also released greater levels of NKCF (53.04±2.82%) than did PBL or spleen cells (13.62±4.78% and 8.49±5.79%) /$p<0.01$/, respectively). The results of two representative experiments are shown in Table III. These data suggest that liver sinusoidal washout cell populations with increased NK activity release more NKCF.

Inhibition of bone marrow hematopoietic colonies by cell supernatants. To evaluate the possibility that upon contact with sensitive targets NK cells release soluble factors that mediate inhibition of late CFU-GM growth, cell-free supernatants from liver washout, PBL, and spleen cells cultured with YAC-1 cells were tested for their effect on colony growth. The results are shown in Fig.25. Greater inhibition was displayed in supernatants from liver washout cells (28.69±12.11%) than in supernatants from

spleen cells (17.84±11.42%) but almost no inhibition of colony formation was observed using sups obtained from PBL cells, incubated with YAC–1 cells (3.42±7.15%).

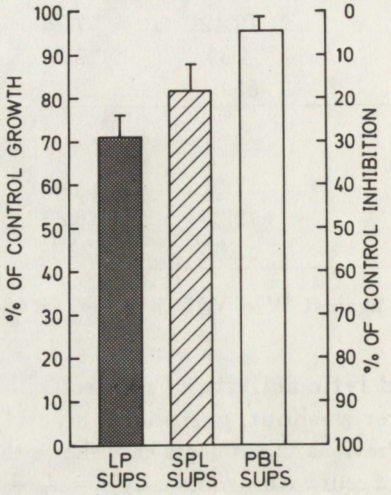

Figure 25. The effect of supernatants from liver washout, spleen cells and PBL co–cultured with YAC–1 cells on C57BL/6 mice bone marrow progenitors colony growth (late GM-CFU). Bars represent the mean percentage of colony growth or inhibition observed ± SD, (n = 4-5).

Natural killer cytotoxicity of MNC isolated from the liver after enzymatic treatment. The NKC cytotoxicity in the mononuclear cell population obtained from the liver after enzymatic treatment was 6.7±4.1% (against K–562 target cells) and 6.36±5.65% (against YAC–1 target cells) at

E/T 40:1, which are well below the level in liver sinusoidal blood (55.6±11.3% and 59.3±8.8%, respectively) (Table IV).

Table IV. Natural killer activity in liver mononuclear cells isolated after enzymatic treatment and liver washout cells in normal Agus rat*.

	Cytotoxicity against K–562 cells at E/T ratio		
Source of cells	40:1	20:1	10:1
Liver MNC after enzymatic treatment	6.79±4.11	2.86±1.34	1.70±2.27
Liver washout	55.60±11.53	50.68±9.84	32.32±12.89

	Cytotoxicity against YAC–1 cells at E:T ratio		
Source of cells	40:1	20:1	10:1
Liver MNC after enzymatic treatment	6.36±5.65	3.25±3.40	1.42±0.87
Liver washout	59.35±8.84	50.84±9.37	34.41±13.40

* Percent of specific ^{51}Cr release, values are means ± SD(n=5)

Karyotypic evaluation. Karyotypic studies were performed on mononuclear cells isolated from liver sinusoidal washout of eight liver transplants recipients. The examination was based on the identification of the Y chromosome in C–banded metaphases in which the Y chromosome stains differently from all other chromosomes. Dividing cells were found in preparations from 7 experiments. Among them in five cases the metaphases had a quality allowing C–banding analysis of the chromosomes. In each particular case 7–14 metaphases were analyzed. In all C–banded metaphases the Y chromosome was identified. The results clearly show the male (XY) karyotype of the analyzed cells. In parallel liver sinusoidal washout cells from control animals were analyzed for the sex chromosomes. The samples were coded and cytogenetic analysis was performed "blind". In three control rats the female (XX) karyotype and in the remaining four rats the male (XY) karyotype was identified. These results were in concordance with the real sex of the control animals. Beside the liver washout cells, peripheral blood mononuclear cells derived from the transplanted and control rats were examined. No dividing cells were found in the preparations.

Natural killer cytotoxicity of cells in liver vascular washout after depriving liver of portal blood supply. Five weeks after laparatomy, splenectomy, porto–caval shunts and porto–caval transposition, the NK activity was measured in the blood from the portal vein and inferior vena cava and in the liver, gut and hind limb vascular washout.

Splenectomy produced some decrease in the liver washout cellular cytotoxicity as compared with the control (70.2% to 54.8%,NS) but did not affect the level of NK cytotoxicity in the portal and caval blood [Fig. 27].

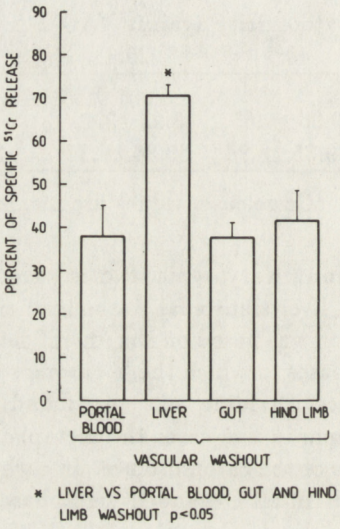

Figure 26. The natural killer cytotoxicity in portal blood and liver, gut and hind–limbs vascular washout five weeks after laparotomy (control group). Values are means $\pm$ SD, (n = 5). E/T ratio 20:1.

Porta–caval shunt evidently decreased the NK cytotoxicity in the liver

washout population (70.2% to 45.6%, $p<0.05$) without affecting the cytotoxicity level in the portal and peripheral blood [Fig. 28].

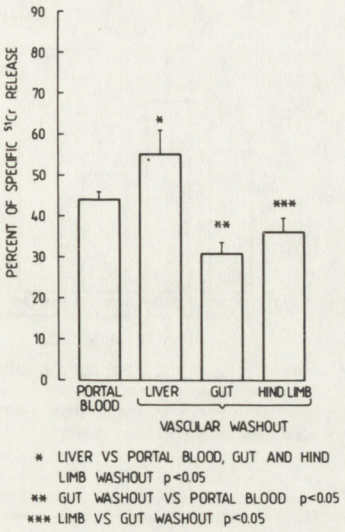

Figure 27. The natural killer cell cytotoxicity in liver, gut and hind–limb vascular washout and portal blood five weeks after splenectomy. Values are means $\pm$ SD (n = 5).E/T ratio 20:1.

Mesentero–caval shunt abrogated NKC activity of cells washed out from liver vasculature (70.2% to 32.5%,$p<0.05$), keeping the level of cytotoxicity in portal and inferior vena cava blood almost unchanged [Fig. 29].

The porto–caval transposition brought about a moderate decrease of cytotoxicity of liver vascular population compared with the control (70.2% to 55.2%,NS) [Fig. 30].

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

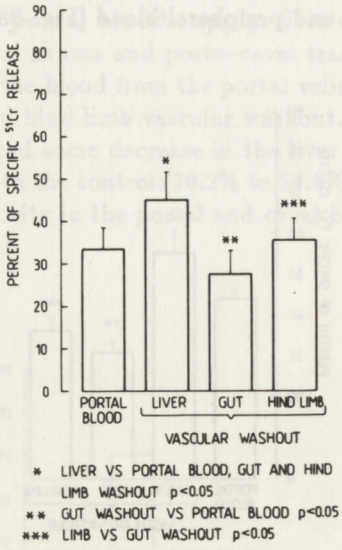

Figure 28. The natural killer cytotoxicity in portal blood and liver, gut and hind–limb vascular washout five weeks after porta–caval shunt. Values are means $\pm$ SD (n = 5).E/T ratio 20:1.

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 washout from the same animals. These data [Fig. 31] revealed that all procedures depriving liver of portal blood, also the porto–caval transposition maintaining high flow through the liver but of the non–portal blood, reduced significantly the liver vascular washout cytotoxicity.

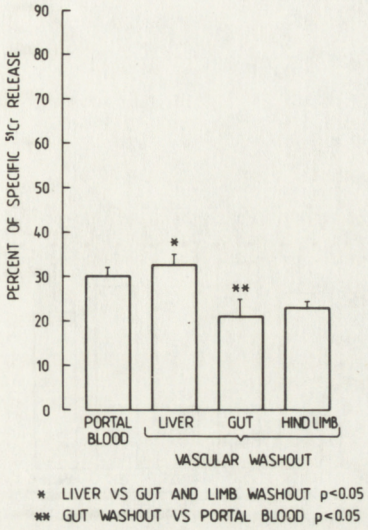

Figure 29. The natural killer cell cytotoxicity in portal blood and liver, gut and hind-limb vascular washout five weeks after mesentero-caval shunt. Values are means ± SD (n = 5). E/T ratio 20:1.

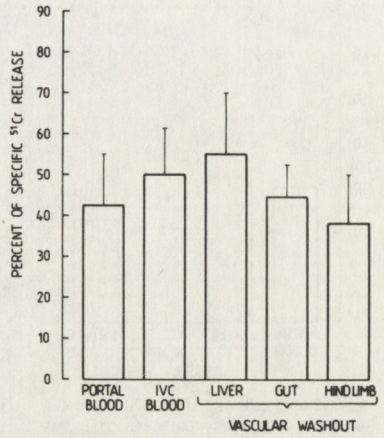

Figure 30. The natural killer cell cytotoxicity in liver, gut and hind-limb vascular washout and portal and caval blood five weeks after porto-caval transposition. Values are means of five experiments ± SD. E/T ratio 20:1.

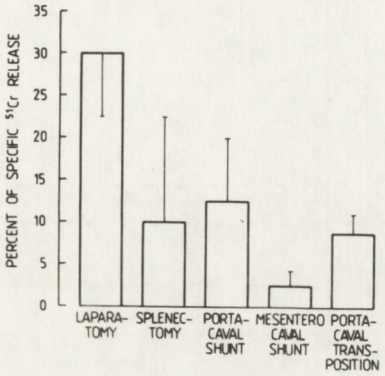

Figure 31. The gradients of natural killer cytotoxicity between the inflowing portal blood and liver vascular washout five weeks after procedures depriving liver of inflow of cells from spleen and gut. Mean values $\pm$ SD, n=5, E/T ratio 20:1, PC–, MC– and PC–X vs laparotomy $p<0.05$.

DISCUSSION

Our investigations have demonstrated 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 cytotoxic activity commonly attributed to the NK, NC and K cells. The level of cytotoxicity was significantly higher than in portal and systemic blood.

The population of cells was nonadherent, peroxidase negative and nonphagocytic, enriched in cells with Fc and C3b receptors, contained a high percentage of large granular lymphocytes and revealed high target cell binding. They were CD8–positive and CD4-negative and anti–asialo GM1–resistant. Soluble factors released from liver sinusoidal cells selectively lysed NK target cells and suppressed the growth of normal haematopoietic granulocyte-macrophage colony forming cells. The origin of cytotoxic cells in the liver sinusoids remains uncertain. Possibly, these cells could be immigrants from the spleen and/or from the gut.

The present study has demonstrated that, compared with peripheral venous blood, splanchnic venous blood is enriched in certain functional subclasses of lymphocytes. The lymphocyte content in the splenic vein was significantly higher than in IVC blood.

The release of splenic lymphocytes into the blood has been directly demonstrated in guinea pigs comparing the counts of lymphocytes in splenic efferent and afferent blood [30,96]. A small release has been found already soon after birth, but this increased with age and was quantitatively large in adult animals [30]. After immunization with pertussis vaccine there was a highly increased release of lymphocytes from the spleen cocurrent with pronounced lymphocytosis in blood [30]. During the immune response to SRBC there was a release of cells with surface receptors directed against this antigen [96].

Several questions arise concerning the process of release of cells from the spleen. First, what is the signal for the release?; second, which cell populations are released?; and third, what are the mechanisms for retaining spleen–derived cells in the liver and in which processes do they participate?

It has been found that noradrenaline [97] and steroids [29] cause a large

export of splenic lymphocytes. With respect to the characteristics of cell populations released from the spleen, little information can be found in the literature. Sandberg [97] reported that release is not confined to any particular subgroup of lymphocytes, as defined by mitochondrial content. Pabst et al. [76] found in their extracorporeal spleen perfusion system that, although both B and T migrating lymphocytes are produced in that organ, B cells show a much higher incidence of newly formed cells.

The organ distribution and homing areas in lymphoid and nonlymphoid tissues for spleen released cells have been studied previously [74,75,77]. Olszewski et al. showed [74] that about 12% of cells home to the liver, Pabst et al. [75] found 17.9% of spleen labeled cells in the liver 4 days after labelling.

Observations of high concentration of lymphocytes in the splenic vein and high rate of accumulation of i.v. injected labelled lymphocytes in the liver, prompted us to retrieve cells which supposedly were trapped in this organ. A simple lavage procedure was applied.

Evaluation of the cellular content of the liver sinusoidal washout carried out in our studies, revealed high proportions of cells with characteristics similar to those found in the spleen, and splenic vein. Significantly higher concentrations of cells with Fc receptors and C3b receptors in the liver sinusoidal than splenic blood indicate, that a certain number of these cells are temporarily retained in the liver.

These cells could be also immigrants from the gut. The mononuclear cells of intestinal mucosa and mesenteric lymph nodes had greater numbers of Fc and C3b positive cells, in comparison with that of peripheral blood. Significant differences in membrane properties between these two populations may be analogous to those observed by Bull and Brookman [15] among human intestinal mucosal and circulating lymphoid cells. However, previous studies of Olszewski et al [73] indicated that gut may rather produce a signal for release of splenic lymphocytes, than release cells which are retained in the liver sinusoids.

Our original findings show that liver sinusoids contain high numbers of sequestered cells. These population were found to be peroxidase–negative and PAS–negative, nonadherent and nonphagocytic, which rule out the possibility of Kupffer and endothelial cells contamination [62]. They are composed of lymphocytes and monocytes. The phenotypes of isolated liver washout cells

are clearly different from those of peripheral blood. The most remarkable difference was the presence of a large population of cells bearing the CD8 antigen and reduced number of CD4– and CD5–positive cells.

Recently, a significant population of CD8–positive/CD5–mononuclear cells has been described in the normal rat [9,10] and sheep [67] liver. This population was further characterized to be associated with LGL [45,85,87,109,114], a morphologic equivalent of the functionally defined natural killer cells.

In our studies LGL, made up a large proportion of liver washout mononuclear population. Substantial numbers of LGL isolated from vascular lavage of the liver has also been reported by Bouwens [10,11].

In addition to the high numbers of LGL observed in our studies, appreciable NK activity was detected in the liver washout population. The level of NK cytotoxicity was significantly higher than in portal and systemic blood mononuclear populations, as well as in cells washed out from the gut and peripheral tissues. The evidence of liver washout population to be enriched in cells with characteristics of the NK population was based on the correlation of the high level of cytotoxicity with high target binding activity by LGL, and high concentration of LGL.

It has been shown that enriched LGL population reveal augmented NK activity [84], 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 [86].

Large granular lymphocytes mediate both, natural killer and antibody–dependent cell mediated cytotoxic activities in rats [36,83,84].

The results of our study demonstrated a general correlation between the organ distribution of NK and ADCC activity. Consistently, liver perfusate cells gave the highest NK/K cytotoxicity with the PBL and spleen being intermediate, and MLN and MLC very low. Moreover, mononuclear cells obtained from peripheral blood, as well as from liver sinusoidal washout, revealed not only considerable NK activity but also NC activity against WEHI-164 cells, the former population being consistently less cytotoxic than the latter. These results suggest that in the rat there exist subsets of natural cytotoxic cells with different targets specificity and organ distribution. To our knowledge, there have been no consistent studies discriminating between rat NK and NC cells. It has been reported that D–mannose, D–glucose, D–galactose and D–glucosamine can inhibit murine natural cytotoxicity [13]. It may be possible to distinguish between NK and NC cells with the aid of simple sugars, since

D–mannose and D–galactose preferentially block NC lysis, while a range of other sugars inhibit NK cells [99]. We applied this method for differentiation of the rat NK and NC cells.

The obtained results demonstrate that neither D–glucose nor D–mannose, D–galactose or D–glucosamine affected the natural cytotoxicity of rat peripheral blood mononuclear cells (PBMC) and liver washout cells. The demonstration that neither NK cell nor NC cell activity were subject to modulation by certain monosaccharides has raised the question as to whether there are distinct effector populations of NK and NC in the rat at all. According to Zoller and Matzku, [120] there is no evidence in favour of diversity between rat NK and NC cells, and these cells represent identical cell populations with respect to target recognition structures.

Selectivity of target cell lysis has been one of the classic characteristics used for the definition of NK/NC activity and allows its discrimination from macrophages and cytotoxic T–cell mediated lysis. We tested mononuclear cells against a panel of NK– and NC–resistant tumour cell lines.

In our hands three NK–resistant tumour targets (P–815, L–5187y and MBL–2) were poorly lysed by PBMC even at the highest effector cell concentration used. Conversely, all of them were good targets for liver sinusoidal washout cells, and L–5178y targets proved as sensitive as K–562 cells. These experiments demonstrated that liver perfusate cells possess the cytotoxic specificity attributed to both NK and NC cells. This report suggests heterogeneity of natural cytotoxic populations derived from various tissues. The heterogeneity may reflect different maturational states of NK cells, or different activation states of a single lineage.

IFNs have been shown to rapidly augment the cytotoxicity of rat NK cells in vitro [33], by enhancing the lytic efficiency of natural killer cells. Murine mononuclear cells co–cultured with an allogeneic stimulus or with IL–2 can kill tumour cells regardless of whether the target cells are NK-sensitive or NK–resistant [4,31]. Moreover, IL-2 not only induces the rapid expression of broad antitumour cytolytic activity, but also induces a strong proliferative response resulting in the expansion of large numbers of highly active cytolytic cells [2,108].

Since we found the blast cells in the liver sinusoidal washout [63], it seems that blood mononuclear cells carried into the liver become activated, and entered the proliferation stage in the microvascular network of the liver. This process may be responsible for their high cytotoxic activity against different

tumour cell lines, regardless of whether the target cells are NK–sensitive or NK–resistant.

The reason why rat sinusoidal cells appear to be activated in situ may be due to the exposure of the liver to bacteria and endotoxin from enteric sources. Nolan et al.[72] have demonstrated that murine Kupffer cells produce IFN alpha/beta. Therefore, it seems that IFN may activate NK cells. It could also be related to some factors produced in the liver and released into the circulation, since spontaneous NK activity was substantially decreased in patients with chronic liver disease and hepatocellular carcinoma [46]. Mc Intyre et al.[55] reported that liver NK cells undergo blastogenesis and proliferation during the first few days of viral infection, or following injection of mice with IFN or with IFN inducers.

Rat LGL can be enriched by centrifugation on discontinous Percoll density gradients. Their high cytoplasmic to nuclear ratio makes them less dense than most lymhocytes and provides a basis for efficient separation. However, the purity of LGL isolated from rat mononuclear cells using Percoll gradients never exceeds 50–70%. This limitation is due to the high level of agranular lymphocytes that copurify with the LGL.

The results of the present study have demonstrated that discontinous Percoll density gradients can be used to enrich the liver washout population with LGL. The less dense Percoll fractions (Fr 2–4) contained more LGL than the unseparated liver washout or blood lymphoid cells. Conversely, the denser fractions (Fr 5–7) were almost totally depleted of LGL. The simultaneous appearance of large numbers of LGL and NK activity in the low density fractions again indicates a strong functional association between LGL and NK cells.

It has previously been shown that a high percentage of rat LGL express OX8 antigen [19,85,87]. We have demonstrated NK activity in the OX8–positive population of PBMC, but among the cells isolated from liver washout relatively high natural cytotoxicity remained in OX8–negative lymphocytes, in addition to that in the OX8-positive population.

The neutral glycolipid asialo–GM1 is displayed at large concentrations on the surface of murine cells with NK cell activity but not on cytotoxic T cells, making the serological display of AGM1 a useful means for distinguishing between two lymphoid cells with cytotoxic function [52,119]. Intravenous

injections of microliter amounts of anti–AGM1 into nude mice almost completely abolish NK activity [53].

We found that this antiserum was also extremely potent in rats, decreasing the number of LGL and abrogating NK cytotoxicity in peripheral blood after intravenous administration and after in vitro treatment with complement [61]. Surprisingly, the population of cells washed out from liver vasculature turned to be resistant to the in vivo, as well as in vitro treatment with anti–AGM1, retaining the number of LGL and keeping level of cytotoxicity almost unchanged.

The mechanism of this observation is unclear. It is likely that the surface glycolipids of lymphocytes undergo in the liver microvasculature enzymatic changes, making the NK cells non–susceptible to the AGM1 antiserum. AAGM1 resistance of liver sinusoidal cytotoxic cells may also suggest an activated state of these cells. The anti–AGM1–resistant NK cells, present in the liver have recently been detected by Wiltrout [112] and Bouwens [10].

Mononuclear cells co–cultured with NK–sensitive targets release factors that are selectively cytotoxic to NK targets. Since the original mouse, rat, and human studies, several lines of evidence have suggested that these factors may be involved in the mechanism of lysis by natural cytotoxic cells [116,117,118].

Our findings revealed that the liver washout cells, rich in NK effectors were evidently more active producers of NKCF than PBMC and spleen cells.

This confirms and extends previous findings, indicating that the highest levels of NK activity were related to the highest levels of NKCF [7,92,115].

In the past few years, a biologically significant role for NK cells has been proposed in regulation of haematopoiesis [54]. In humans, NK cells have been shown to react with cells from different lineages at early stages of differentiation, such as immature bone marrow cells [44]. Inhibition of haematopoiesis in vitro has been ascribed to the release of soluble factors from lymphocytes upon stimulation with NK–sensitive target cells that inhibit different types of haematopoietic colony growth in vitro [24]. This NK-derived colony inhibiting activity (NK–CIA) is simultaneously produced by NK cells with NKCF, since all supernatants containing NKCF also contained NK–CIA [25].

In our studies we analyzed the relationship between NK–CIA and NKCF, released by NK cells from different sources. The greatest inhibition of CFU–

GM growth was observed with supernatants obtained from liver washout cells incubated with YAC–1 cells. This demonstrates a highly significant positive correlation between NKCF and NK–CIA released from the liver washout cells. Slight inhibition was also observed when NK–CIA containing supernatants from spleen cells were tested. There was no significant inhibition of colony formation after stimulation of PBMC with NK–sensitive targets, although the cytotoxic effect of supernatants collected from peripheral blood cells stimulated with YAC–1 cells was evident. The relatively weak NK–CIA activity in the tested supernatants was probably due to the fact, that mononuclear cells releasing these factors were contaminated by cell types other than NK. Previous studies have shown that the effect of NK cells on colony formation is identical in the autologous and allogeneic combinations [3,54]. The ability of NK–CIA released from rat mononuclear cells to suppress murine CFU–GM growth indicates that the released factors were also effective in xenogeneic combinations.

Thus, our investigations have demonstrated that liver sinusoids contain high numbers of sequestered cells with cytotoxic activity commonly attributed to the NK and NC cells. The questions arise as to whether, first, these cells are blood–borne cytotoxic cells preferentially homing in the liver and not resident cells desquamated from the sinusoidal wall during the washout procedure, and second what is their source of origin.

The results of the present studies strongly suggest that liver sinusoidal cytotoxic cells are derived from extra hepatic antecedents. Cytogenetic analysis of metaphases of cells washed out from female liver transplanted into male rat, revealed the existence of Y chromosome which indicates recipient origin of these cells. Had they been resident cells they should have preserved their female karyotype. Since all metaphases among cells washed out from female liver transplanted to syngeneic male showed recipient (XY) karyotype, a short lasting harbouring of blood cells marginated in the liver microvasculature and their rapid physiological replacement by inflowing peripheral blood cells may be suggested.

These suggestions are in good agreement with Freudenberg et al. findings who described the replacement of liver sinusoidal cells from F1(H–2k–positive) bone marrow cells transplanted into irradiated (H–2k–negative) parental mice [34].

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. Spleen may be the source of liver cytotoxic cells since it contains a relatively high concentration of NK cells [59,80]. The gut seems to be a less probable source of NK cells detained in the liver, since NK cell activity in the population isolated from the gut mucosa had exceptionally low spontaneous cytotoxic activity [32,37,59]. Local activation should also be considered. It is not known whether natural cytotoxic cells migrate spontaneously away from the organs.

In our studies, splenectomy brought about a slight but evident decrease in the NK activity in the liver vascular washout population. Diverting portal or selectively mesenteric blood to the systemic circulation by means of surgical shunts, significantly decreased liver vascular washout cell 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 porto–caval transposition was followed by a reduction in liver washout cytotoxicity. This observation 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 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, they have shown that portal blood supply is necessary for keeping the natural cytotoxicity at a normal level.

Recent in vitro studies have demonstrated that various soluble factors i.e. IFN, TNF, IL–1, IL–2, LPS stimulate the endothelial cells to bind lymphocytes [20,38]. On the other hand, Vujanovic et al. [107] communicated that upon activation by rIL–2, LGL rapidly undergo surface changes that enable their adherence. Intravenous injection of these cells showed preferential accumulation in the liver and the spleen comprising about 90% of total radioactivity recovered 24 h after the injection [1]. The rate of homing of lymphokine activated killer (LAK) cells in the liver was similar to that reported by Wiltrout et al. They found increased accumulation of NK cells in the

liver after treatment with biological response modifiers [111]. The observed homing pattern has been attributed to the biochemical and morphological differences between the liver sinusoidal cells and endothelia of other tissue [48]. The existence of more or less specific receptor and acceptor molecules on both, activated LGL and the endothelial walls of the microvasculature in the liver might be an explanation. Considering the role of hemodynamic factors in cell homing, the pressure in liver capillaries is according to Guyton as low as 9 mm Hg but as high as 20 mm Hg in most other organs [41]. This observation may also explain the temporary margination of certain population of mononuclear cells in the liver sinusoids.

The strategic position of the liver within the circulation system should make it an efficient filter, and some experiments and observations confirm this view [27,65,110]. Sinusoidal cells of the liver are particularly good candidates for performing such in vivo roles because of the anatomical position, which provides encounters with different cells migrating from various tissue sites or dietary, bacterial, and viral antigens leaving the gut with the blood. They may be important for prevention of tumour spread and metastasis formation, since other studies suggest that active NK cells play their role during the intravascular phase of tumour metastasis [39,42,43,89]. Investigation on the distribution and elimination of tumour cells in animal systems have indicated, that most circulating cancer cells are rapidly destroyed shortly after entering the bloodstream. Several factors, both immunological and nonimmunological might be responsible for this destruction. Most of the studies support the hypothesis that NK cells play a significant role in the destruction of tumour cells in the lungs [39,42,71,82,101]. In contrast, information is limited about NK–dependent clearance of tumour cells from the liver. Burkart et al. reported that 85-95% of lymphoma cells Eb and ESb, injected via mesenteric vein were trapped in the liver and most of the metastasizing variants (ESb) were destroyed within 18 hours [16]. Basse et al. investigations strongly suggest that the role of clearance of tumour cells from the liver depends on the action of the NK cells [6]. Conversely, Wiltrout et al. [111], and Bouwens et al. [9] have found very low number of LGL in the normal, unstimulated animals. However, these cells were obtained by enzymatic dissociation of the liver after discarding the cells attached to the sinusoids. Comparison of the two methods for mononuclear cell isolation enabled us to elucidate, that natural killer activity of liver washout cells is 10 times higher

than cytotoxicity of cells isolated by using classic enzymatic liver dissociation.

Our investigations strongly suggest that the NK cells inside the organ parenchyma are of minor importance in natural defence system, compared with the significant role they play in the liver microvasculature.

Liver sinusoidal cytotoxic cells seem to be recruited from blood, activated in the liver microenvironment, and attached temporarily to the sinusoidal wall. It has been difficult to define clearly the exact role of the marginated liver sinusoidal cytotoxic cells, but since they have extensive site–related opportunities, they may be important for the prevention of bacterial, viral and tumour antigens spread, as well as metastasis formation.

SUMMARY AND CONCLUSIONS

Increasing attention has been focused in the last few years on the liver as an organ involved in the prevention of tumour spread and metastasis formation. However, the mechanism underlying tumour cell destruction in the liver is unknown. Previously, the role of liver sinusoidal cells in the defence mechanisms against infectious agents and tumour cells has long been ascribed to KC and EC phagocytic properties.

The present study has demonstrated that liver sinusoids contain high numbers of sequestered cells with cytotoxic activity commonly attributed to the NK, NC and K cells. The level of cytotoxicity was significantly higher than in portal and systemic blood. The population of cells was peroxidase–negative and PAS–negative, nonadherent and non–phagocytic, enriched in cells with Fc and C3b receptors.

It contained a high percentage of LGL and revealed high target cell binding. The simultaneous appearance of large number of LGL and high NK activity was found in low density cells in CD8–positive as well as CD8–negative populations.

They were composed of lymphocytes and monocytes. The phenotype of isolated liver washout cells was clearly different from those of peripheral blood. The most remarkable difference was the presence of a large population of CD8–positive cells, reduced number of CD4– and CD5–positive cells and resistance to anti–asialo GM1 serum.

The mononuclear cells washed out from the liver microvasculature could be stimulated with NK–sensitive targets to release soluble factors which selectively lyse YAC–1 tumour cells and inhibit growth of normal haematopoietic granulocyte macrophage colony forming cells in vitro.

The cytotoxic cell population in the liver turned out to be blood-borne in origin and not resident. These cells could be immigrant from the spleen and/or gut since diverting portal or mesenteric blood to the systemic circulation significantly decreased liver vascular washout cytotoxicity.

Our findings suggest that liver sinusoidal cytotoxic cells represent an NK population with a predilection for marginaton and activation in the liver.

These cells may be important in eliminating tumour or virus infected cells passing through the liver from the circulation.

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