Changes of Liver-Resident NK Cells During Liver Regeneration in Rats[1]

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To determine the role of NK cells in regulation of tissue growth, the phenotype and function of liver-resident NK cells were studied after 70% partial hepatectomy in rats. The process of liver regeneration was generally completed by day 14. In contrast, the number of liver-resident NK cells (NKR-P1^{bright}) was restored as early as day 3 after partial hepatectomy. However, spontaneous functions of liver-resident NK cells, including killing of YAC-1 and P815 targets, Ab-dependent cellular cytotoxicity, and redirected killing via NKR-P1, were continuously suppressed throughout the entire period of liver regeneration (from 3 h to 14 days). Augmentation of NK cytotoxicity against P815 targets and induction of NK cell adherence to plastic following 24 h of IL-2 stimulation showed a similar pattern of suppression. However, IL-2-induced augmentation of YAC-1 killing, proliferation and generation of adherent NK cells, and LAK activity in 5- to 7-day cultures were found to be suppressed only during the first 24 h and increased between days 2 and 7 after hepatectomy. Sorted NK cells (≥95% NKR-P1^{bright}) from liver-resident mononuclear leukocytes 24 h after partial hepatectomy showed the same pattern of suppression as unsorted mononuclear leukocytes. In contrast to liver-resident NK cells, no significant changes were detected in peripheral blood or spleen NK cells of rats following partial hepatectomy. Of particular interest, in normal liver, hepatocytes were resistant to NK lysis, while resident NK cells were cytotoxic for various NK-sensitive targets. In contrast, during the early period of liver regeneration, when hepatocytes were sensitive to lysis by liver-resident NK cells of normal rats, NK cells obtained from regenerating liver tissues were unable to mediate cytotoxicity. At the final phase of liver regeneration (days 7–14 after hepatectomy), both resistance of hepatocytes to killing by NK cells and cytotoxicity of liver-resident lymphocytes against hepatocytes from regenerating liver were simultaneously restored. In vivo depletion of NK cells by injection of rats with anti-NKR-P1 mAb resulted in a significant augmentation of liver regeneration subsequent to partial hepatectomy. Our data suggest that liver-resident NK cells may be involved in regulation of the extent of liver regeneration. *The Journal of Immunology,* 1995, 154: 6324–6338.

Physical, chemical, or biologic injuries of parenchymal organs, including trauma, surgery, nonphysiologic temperature, intoxications, infections, or cancer, are generally followed by tissue regeneration. Liver regeneration after partial hepatectomy in rodents has been a commonly used experimental model in studies of organ regeneration (1, 2). The natural history of liver regeneration after partial hepatectomy involves a rapid parenchymal cell proliferation and reconstruction of the organ to normal size, structure, and functions. When liver reaches its initial size, regeneration rapidly stops.

Mechanisms involved in liver regeneration have been only partially elucidated (2–5). It is known that liver regeneration is a multistep process in which hormones (6–10), growth factors (11–14), and growth inhibitory factors (15, 16) are involved. Recent in vivo data indicate that hormones, along with ions and nutrients, may represent the initiators of regeneration, which is then sustained by growth factors (16).

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In contrast to this relatively well defined understanding of initiation and progression of liver regeneration, little is known of the mechanisms that limit or turn off the regenerative process. Recently, data on the effects of immunophilins on liver regeneration have indicated that these cytosolic receptors for immunosuppressant drugs such as FK506, rapamycin, and cyclosporin are able to modulate liver growth through a variety of calcium-dependent signal transduction pathways (17, 18). This knowledge, combined with supporting studies of the potential influence of the immune system on liver regeneration, previously described in the literature (18–20), suggests the existence of a possible linkage between the two systems. In this paper, the behavior of NK cells extracted from liver at various times after partial hepatectomy is reported.

NK cells are a phenotypically and functionally distinct population of lymphocytes with the characteristic morphology of large granular lymphocytes (21, 22). NK cells do not rearrange the TCR genes or express the CD3-TCR complex (21, 22). NK cells selectively express several other triggering receptors, including the low affinity receptor for Fc fragment of IgG, FcγRIII, and NKR-P1, defined in humans and rodents (21, 23–26). The NKR-P1s are transmembrane homodimers of disulfide-linked glycoproteins of the C-type lectin superfamily (26, 27), which seem to be related to the putative NK cell receptor(s) for recognition and killing of target cells (27, 28). In rats, high density cell surface expression of NKR-P1 Ag (i.e., NKR-P1^{bright}) is an exclusive property of all mature NK cells (23, 29). NKR-P1 is also expressed on granulocytes and on a subset of T cells, but with a 2- to 10-fold lower density (i.e., NKR-P1^{dim}) than on NK cells. This difference in expression of NKR-P1 on NK cells and T cells or granulocytes makes it possible to discriminate accurately between these cell types by flow cytometry (23, 29). In contrast to most other peripheral lymphocytes, NK cells constitutively express functional intermediate affinity IL-2 receptor (IL-2R) and, therefore, are the main lymphocyte population directly responsive to IL-2 (30, 31). Functionally, NK cells have been defined as the major effector cells of nonadaptive immunity (21, 22). Without prior sensitization or antigen recognition in the context of the MHC, NK cells are able to mediate cytotoxicity against selected tumor and virus-infected cells (21, 22). NK cells are also the major effector cells of Ab-dependent cellular cytotoxicity (ADCC)[3] against Ab-sensitized target cells (21). Furthermore, NK cells are capable of secreting a variety of cytokines, and they may regulate adaptive immune responses as well as hematopoiesis and contribute to resistance against microbial infections and tumor growth (21, 22, 32). In contrast to the well defined role of NK cells in pathophysiologic processes, little is known about their involvement in the regulation of normal cell growth.

Recent studies have provided indications that NK cells may be involved in growth and regeneration of the liver. For instance, it has been shown that a substantial number of NK cells are present in normal rodent and human livers. They are located both in liver sinusoids (pit cells) and in the liver parenchyma between hepatocytes (33–35). Studies in mice have shown that an age-dependent increase in the number of liver-resident NK cells coincides with the slowdown of the process of rapid increase in liver weight (36). It has also been observed in mice that a decrease of normal liver mass follows IL-2 infusions (37) and that an inhibition of hepatocyte proliferation in the regenerating liver takes place after IL-2 or LAK cell infusions (18). Furthermore, it has been reported that regenerating liver hepatocytes become sensitive to cytotoxic activity of normal liver-resident mononuclear leukocytes (MNL) (36) as well as splenocytes from partially hepatectomized mice (38). In contrast, immunosuppressive drugs, such as cyclosporin A (CsA) or FK506, have been found to augment hepatocyte proliferation in regenerating mouse liver (17, 18). However, the available data do not provide information about the exact role of NK cells in liver regeneration or explain how liver regeneration can occur in the presence of cytotoxic NK cells that are capable of killing regenerating liver hepatocytes (36).

One possible explanation of how liver regeneration might occur in the presence of liver-resident NK cells is that soon after liver injury the effector functions of these NK cells might become temporarily suppressed, i.e., they become noncytotoxic for regenerating liver hepatocytes. To test this hypothesis, we analyzed the phenotypic and functional changes of liver-resident NK cells in rats after partial hepatectomy. We demonstrate here that after partial hepatectomy a prompt and profound suppression of NK-cell functions, including killing of regenerating liver hepatocytes, occurs. Furthermore, this immunosuppression is restricted to NK cells in the regenerating liver. Also, the susceptibility of regenerating liver hepatocytes to lysis by liver-resident NK cells is transient, as it is limited to the period of rapid liver growth immediately after partial hepatectomy. In vivo elimination of NK cells by a specific mAb results in augmented liver regeneration 7 days after partial hepatectomy. Our data indicate that liver-resident NK cells have a potential role in regulation of liver regeneration.

Materials and Methods

Animals

Male Fisher F344 rats and male NIH-RNU nude rats (180–200 g) were purchased from Hilltop Lab Animals, Inc. (Scottdale, PA) and from the National Cancer Institute (Frederick, MD), respectively. The animals were housed in a pathogen-free animal facility of the Pittsburgh Cancer Institute and fed with standard rat chow and water ad libitum.

[3] Abbreviations used in this paper: ADCC, antibody-dependent cellular cytotoxicity; FBS, fetal bovine serum; MNL, mononuclear leukocytes; A-NK, adherent NK; LAK, lymphokine-activated killer (cell); PE, phycoerythrin; RPMI-FBS, RPMI 1640 medium containing 10% fetal bovine serum; TCM, tissue culture medium.

Partial hepatectomy

Partial hepatectomy (70%) was performed in anesthetized rats as previously described (17). Sham hepatectomy consisted of laparotomy and gentle manipulation of the liver in anesthetized rats. All surgical procedures were performed between 8:00 and 10:00 a.m.

Tumor cell lines

An NK-sensitive, Moloney virus-induced YAC-1 lymphoma of A/Sn mouse origin was used as a tumor target for testing NK activity. An NK-resistant P815 mastocytoma of DBA/2 mouse origin was used as the target cell for detection of both LAK activity of freshly isolated or IL-2-activated NK cells and ADCC or redirected killing mediated by resting NK cells. The cell lines were grown in RPMI 1640 medium containing 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% (v/v) heat-inactivated fetal bovine serum (FBS), all purchased from Life Technologies, Inc. (Grand Island, NY).

Reagents and Abs

Collagenase type V, DNAse and hyaluronidase type I-S, used for the preparation of liver cell suspensions, were obtained from Sigma Chemical Co. (St. Louis, MO). Human rIL-2, containing 3.0×10^6 Cetus U (18×10^6 IU) of IL-2 per 1 mg of protein, was kindly provided by the Chiron-Cetus Corp. (Emeryville, CA). The phycoerythrin (PE)-conjugated mouse mAbs against rat CD45 (leukocyte common Ag) (OX-1, IgG1), CD3 (G4.18, IgG3), and CD45RA or CD45A/B (rat B-cell marker) (OX-33, IgG1) were obtained from PharMingen (San Diego, CA). The FITC-labeled mouse mAbs against rat lymphocyte surface structures, including anti-CD5 (OX19, IgG1) and anti-CD3 (G.4.1.A, IgG3), were purchased from PharMingen, and anti-Ia (OX6, IgG1) and anti-TCR-$\alpha\beta$ (R7.3, IgG1) from Bioproducts for Science, Inc. (Indianapolis, IN). FITC- and CY5-conjugated $F(ab')_2$ fragments of anti-NKR-P1 (3.2.3, IgG1) were made in our laboratory, as described (23). The corresponding isotype controls were obtained from Becton Dickinson (San Jose, CA), PharMingen, and Sigma. Con A was purchased from Calbiochem Company (La Jolla, CA) and ^{3}[H]thymidine (^{3}[H]TdR, thymidine,[methyl-^{3}H]247.9GB q/mmol, 6.70 Ci/mmol) was obtained from DuPont NEN Products Co. (Boston, MA).

Isolation of hepatocytes

Viable hepatocytes were isolated from normal or partially hepatectomized rat livers after their perfusion with collagenase, using a method previously described by Seglen (39).

Isolation of lymphoid cells from spleen and peripheral blood

Peripheral blood was obtained by cardiac puncture using heparinized syringes. Spleens were removed under sterile conditions and single-cell suspensions prepared in RPMI 1640 medium containing 10% FBS (RPMI-FBS) as described (40). Peripheral blood and spleen mononuclear cells were obtained by centrifugation on Ficoll-Hypaque gradients (density = 1.077) at $300 \times g$ for 20 min at room temperature. The mononuclear cells were collected from the gradient interface, washed three times in RPMI-FBS, and immediately used in the experiments described.

Isolation of lymphoid cells from liver

MNL were isolated from rat livers by using a modification of the method of Richman et al. (41). Briefly, rats were killed by cervical dislocation. Livers were perfused with PBS, excised, and weighed. The livers were minced with scissors into 1- to 2-mm^3 fragments, which were washed five times in RPMI-FBS by $1 \times g$ sedimentation for 2 min each to eliminate blood cells and subcellular debris. The liver fragments were then digested for 3 h in a triple enzyme solution containing 0.4% collagenase (w/v), 0.02% DNAse (w/v), 30 U/ml hyaluronidase, and 20% FBS (v/v) in RPMI 1640 medium. The liver digest was then filtered through Nitex mesh filter (70-μm pore size; Lawshe Industrial Co., Bethesda, MD) and washed twice with RPMI-FBS. All of the above-described isolation steps were performed at room temperature. The liver cells were resuspended in 50% metrizamide (Sigma Chemical Co.) in PBS at a final ratio of 5:7 (v/v). Three milliliters of the mixture were transferred into 15-ml conical tubes (Corning Glass Works, Corning, NY) and overlaid with 1.5 ml of RPMI 1640. This gradient was then centrifuged at $400 \times g$ for 20 min at 4°C. The nonparenchymal cells were carefully removed from the metrizamide-RPMI 1640 interface. The cells were washed twice in RPMI 1640-FBS, centrifuged on Ficoll-Hypaque gradient (density = 1.077) at $300 \times g$ for 15 min to remove erythrocytes, and washed three times in RPMI 1640-FBS. Twenty-five microliters of the suspension of MNL in RPMI 1640-FBS was mixed with 25 μl of 0.4% (w/v) of trypan blue solution in PBS, and the cell number and viability were determined in a hemocytometer.

Hepatocytes in single-cell suspensions are extremely fragile large cells (80–100 μm). After 10 min of incubation in the presence of trypan blue on microscopic slides at room temperature, it was possible to discriminate between large blue cells with a very low nuclear/cytoplasmic ratio, which often had two nuclei, and small, unstained MNL. In addition, utilizing Giemsa-stained cytospins of the isolated liver-resident MNL, it was possible to determine the degree of contamination of separated liver-resident MNL with liver-parenchymal cells.

Immunofluorescent staining and flow cytometry

Lymphoid cells were adjusted to a concentration of 0.25×10^6/0.2 ml in PBS containing 0.1% (w/v) sodium azide and 1% FBS (v/v). For direct single-color immunofluorescent staining, the cells were incubated with FITC-labeled mAbs. For two-color flow cytometry, cells were incubated with both FITC- and PE- or CY5-labeled Abs, while for three-color flow cytometry, they were incubated with FITC-, PE-, and CY5-conjugated mAbs. Cells suspended in PBS-azide buffer alone or stained with FITC-conjugated IgG1 or IgG3 monoclonal Igs and/or PE- or CY5-labeled isotype control mAbs nonreactive with rat cells were used as negative controls. The cells were incubated with the Abs or reagents at 4°C for 30 min, washed two times with PBS-azide buffer, and resuspended in 1% (w/v) paraformaldehyde. The stained cells were examined by single- or two-color flow cytometry on a FACScan, as previously described (42), and by three-color flow cytometry, using FACStar Plus. Data were analyzed using the Reproman program (Flow Cytometry Analysis Software for Personal Computers, True Facts Software Inc., Seattle, WA).

Cell sorting

To obtain highly purified populations of liver-resident NK cells, MNL obtained from rat livers were separated on metrizamide and Ficoll-Hypaque gradients, stained with FITC-labeled $F(ab')_2$ fragments of anti-NKR-P1 mAb (10 μg/ml/10^7 MNL), and sorted in a flow cytometer. Brightly stained (NKR-P1^{bright}) and unstained (NKR-P1$^-$) cells were harvested, washed, and used for functional studies.

E:T cell binding assays

Binding of NK cells to target cells was performed as described before (42). Briefly, liver lymphocytes (effectors) and YAC-1 tumor cells (targets) were adjusted to a concentration of 1×10^6 cells/ml and mixed in RPMI-FBS at an E:T cell ratio of 1:1 in a volume of 200 μl. Control cell samples and E:T cell mixtures were pelleted by centrifugation at $100 \times g$ for 5 min, incubated at 37°C for 10 min, and resuspended in RPMI-FBS. The number of lymphocytes forming conjugates with tumor cells were counted per 500 lymphocytes. The frequency of effector (E) binding to target (T) was determined using the following formula:

$$\%\ \text{conjugate-forming cells/E population} = \frac{\text{number of E bound to T}}{\text{number of E}} \times 100$$

The proportion of conjugate-forming cells per NK cell population was calculated according to the following formula:

$$\frac{\%\ \text{conjugate-forming cells}}{\%\ \text{NKR-P1}^{bright}\ \text{NK cells}} \times 100$$

Cytotoxicity assays

NK activity was measured against YAC-1 tumor cells and normal or regenerating rat liver hepatocytes, and LAK activity was measured

against P815 target cells. ADCC was measured against Ab coated P815 target cells. Redirected killing via NKR-P1 was measured against P815 tumor cells using a standard 4-h ^{51}Cr-release microcytotoxicity assay as described earlier (42). In all cases, the target cells were labeled with 100 μCi of ^{51}Cr (specific activity, 5 μCi/mM, DuPont NEN) at 37°C for 1 h. Target cells were then washed, mixed, and incubated with effector cells at four different E:T ratios ranging from 100:1 to 12.5:1 in U-bottom, 96-well plates (Costar, Cambridge, MA). For the ADCC assay, after ^{51}Cr labeling, P815 target cells were sensitized with rat antiserum against P815 tumor cells at a subagglutinating concentration. In redirected killing assays, effector cells were sensitized with anti-NKR-P1 mAb and then tested against ^{51}Cr-labeled P815 target cells. P815 cells preincubated with a preimmune rat serum or with a nonreactive IgG1 mAb served for these assays as negative control-targets, respectively. Spontaneous release and maximum release were determined by incubating target cells without effectors in medium alone or in 5% Triton X-100, respectively. The spontaneous release was less than 10% for YAC-1 and P815 target cells and less than 25% for hepatocytes. The assay was performed in triplicate. Radioactivity was counted in a gamma counter, and the percentage of specific lysis determined according to the formula:

$$\% \text{ specific lysis} = \frac{\text{mean experimental cpm release} - \text{mean spontaneous cpm release}}{\text{mean maximal cpm release} - \text{mean spontaneous cpm release}} \times 100$$

LU were calculated using a computer program based on the formula developed by Pross at al (43). One lytic unit was defined as the number of effector cells needed to lyse 20% of 5×10^3 target cells. LU per 10^7 effector cells were calculated on the basis of the following formula:

$$\frac{LU_{20}/10^7 \text{ MNL}}{\%\ \text{NKR-P1}^{\text{bright}}\ \text{NK}} \times 100$$

Proliferation assays

^{3}[H]thymidine incorporation assays were performed as described previously (40). Briefly, lymphoid cells were plated at a concentration of 2.0×10^4 cells/well in 0.2 ml of RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine (Life Technologies), 5×10^{-5} M 2-ME, and antibiotics (streptomycin plus penicillin; hereafter referred to as complete tissue culture medium (TCM)) in U-bottom wells of 96-well plates (Costar). IL-2 and Con A were added at final concentrations ranging from 0.022 to 222 nM and from 1 to 20 μg/ml, respectively. Control wells contained cells in medium alone. The assays were performed in three to six replicates. The plates were incubated at 37°C in humidified atmosphere of 5% CO_2 in air for 5 days. On day 4 of the culture, the cells were pulsed with 1 μCi of ^{3}[H]thymidine/well, and on day 5 they were harvested by semiautomatic Skatron Cell Harvester (Skatron Instruments, Inc., Sterling, VA), and their radioactivity was determined in a scintillation counter.

IL-2-induced stimulation of NK activity

Lymphoid cells were cultured in horizontally positioned T25 flasks (Corning Glass Works), at an optimal density of 2×10^6 viable cells/ml (5 ml/flask) in TCM containing 22 nM of rIL-2, at 37°C in a humidified atmosphere of 5% CO_2 in air for 24 h.

Generation of adherent NK (A-NK) cells

A-NK cells were generated using a modification of a previously described technique (44). Briefly, 10×10^6 MNL were incubated for 2 h in 5 ml of TCM in horizontally positioned T25 flasks at 37°C to eliminate plastic adherent macrophages and B cells. The suspension of nonadherent lymphocytes was then transferred into new T25 flasks, supplemented with 22 nM of rIL2, and cultured for 24 h at 37°C in humidified atmosphere of 5% CO_2 in air. After this incubation, the nonadherent lymphocytes were decanted and the adherent (A-NK) cells were washed three times with warm (37°C) TCM to completely eliminate nonadherent cells. The A-NK cells were counted per surface area (grid) under an inverted microscope (magnification 200x), supplemented with conditioned medium, and additionally cultured for 6 days. Expansion of A-NK cells was determined on the basis of the number of A-NK cells at the beginning and the end of culture, as described previously (44).

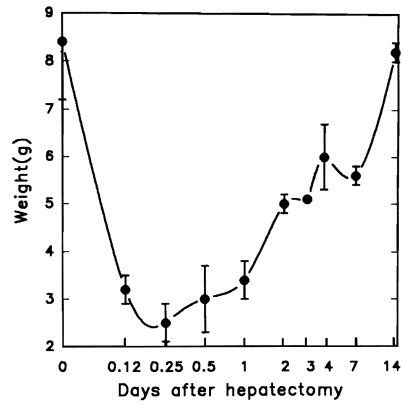

FIGURE 1. Kinetics of the liver mass reconstitution after partial hepatectomy in rats. Livers from 70% partially hepatectomized F344 rats at various time points after the operations, as well as from the sham-operated animals, were weighed as described in *Materials and Methods*. The data are mean ± SEM of at least five livers per each point. The weight of livers from 48 h to 14 days after partial hepatectomy was significantly different ($p < 0.001$) from that at 3 h after the operation.

In vivo depletion of NK cells

Fisher rats, either sham-operated or partially hepatectomized on day 0, were injected i.p. on day −1 and on day +4 with anti-NKR-P1 mAb or with the isotype control mAb (100 μg/rat/injection). The animals were killed on day 7 after the operation, and their body- and liver-weights as well as phenotype and function of splenocytes were determined.

Statistical analysis

Statistical significance of results was calculated using Wilcoxon's signed-rank pair and Mann-Whitney *U* tests. Differences were considered significant when the *p* value was <0.05.

Results

Reconstitution of liver mass and of the pool of liver-resident lymphoid cells after partial hepatectomy

After 70% partial hepatectomy in rats, both liver weight (Fig. 1) and liver volume (data not shown) rapidly increased and doubled during the first 2 to 4 days. After this initial acute phase of growth, liver continued to increase in size more slowly and reached the size of a normal liver after 14 days. In addition, hepatocyte mitotic activity, ploidy, and expression on the cell surface of the class I MHC Ags were dramatically changed during the first few days following partial hepatectomy and returned to baseline values by day 14 (data not shown). Thus, in rats, after 70% partial hepatectomy, liver regeneration was completed following a relatively short period of 14 days. Next, using metrizamide and Ficoll-Hypaque density gradients, we separated liver-resident MNL from normal and regenerating rat livers and determined, by both morphologic and flow cytometric (definition of cell size and CD45 expression) analyses, their purity and changes in absolute number following partial hepatectomy. We found that preparations of separated liver-resident MNL were not

contaminated with hepatocytes, which was easily recognizable by both light microscopy and flow cytometry. In fact, MNL isolated from liver were homogenous populations of cells with a typical size (~10 μm) and morphology of MNL (data not shown). During the period of liver regeneration, the number of liver-resident lymphoid cells was found to increase, both per organ and per gram of the liver (Fig. 2, *A* and *B*). However, in contrast to the steady increase in liver weight and volume, the increase in the number of MNL occurred in two distinct waves, the first between 6 and 12 h and the second between 48 and 72 h after hepatectomy. During the first wave, the number of MNL, as compared with the number at 3 h after hepatectomy, was increased 2.7-fold per liver and 2.9-fold per g of liver tissue. During the second wave, the number of recovered MNL increased 6.2-fold per liver and 3.7-fold per g of liver tissue. These values exceeded the number of liver-resident MNL in normal liver both per organ (+27%, Fig. 2*A*) and per g of tissue (+150%, Fig. 2*B*). In addition, the data shown in Figure 2 indicate that the increase in the number of liver MNL was transient and was followed by decreases in their number between 12 h and 24 h and between day 4 and day 7 after partial hepatectomy. The number of liver-resident MNL returned to normal values by day 14 after partial hepatectomy. Although the mechanisms responsible for these changes in the number of liver-resident MNL during liver regeneration remain unclear at this time, it is possible that augmentation of the number of liver-resident lymphocytes in the regenerating liver is mainly a consequence of the influx of PBL into the liver, because of the magnitude and rapidity of the event and because of a simultaneous two- to threefold decrease in the absolute number of PBL (data not shown). Since an increase in spontaneous proliferation of resident lymphocytes in the regenerating liver was observed between 24 and 72 h after partial hepatectomy (Fig. 2*C*), it is also likely that in situ proliferation contributed to the observed increase in number of MNL.

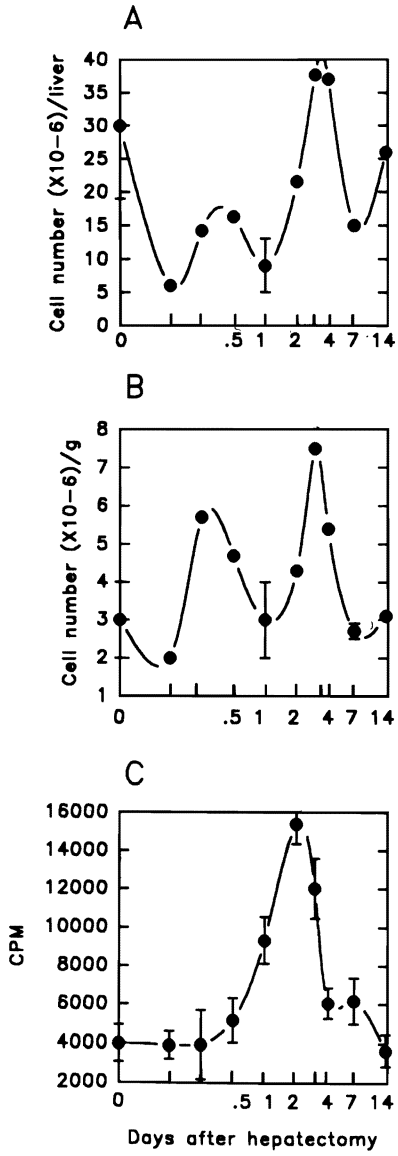

FIGURE 2. Changes in the number and spontaneous proliferation of liver-resident MNL after partial hepatectomy. Liver-resident MNL were separated from sham-operated or partially hepatectomized F344 rats at various time points after surgery using metrizamide and Ficoll-Hypaque density gradients. The number of separated MNL was determined per organ (*A*) and per gram of liver tissue (*B*), using morphologic and flow cytometric analyses as described in *Materials and Methods*. The spontaneous proliferation of liver-resident MNL was assessed by a ^{3}[H]TdR incorporation assay after 5-day culture in TCM alone (*C*), as detailed in *Materials and Methods*. The data are mean ± SEM of three to eight experiments (SEM was not shown when <5%). Results in *C* are from one experiment and represent mean ± SEM of triplicates.

Phenotype of normal and regenerating liver lymphoid cells

To determine whether the increase in the number of liver-resident lymphoid cells observed during liver regeneration was accompanied by changes in the composition of liver-resident lymphocytes, flow cytometry analyses of normal and regenerating liver-resident lymphocytes, stained with FITC-conjugated Abs specific for T cells (anti-CD3, anti-TCR-αβ, and anti-CD5) and NK cells (anti-NKR-P1), as well as B cells and macrophages (anti-Ia), were performed. In humans and mice, some T cells are $CD5^{-}$, and a small proportion of B cells are $CD5^{+}$. However, in rats, lymphocytes with these phenotypes have not been defined. Therefore, we analyzed by two-color flow cytometry cells co-expressing CD5 and the B cell marker (CD45RA or A/B, OX-33), or CD5 and CD3 or TCR-αβ, using splenocytes of Fisher and nude rats and Fisher rat liver-resident MNL. The data showed that only Fisher rat splenocytes contained a small subset (4–7%) of lymphocytes which co-expressed CD5 and CD45RA (data not shown). On the

Table I. *Phenotype of liver, spleen, and peripheral blood mononuclear leukocytes of normal and partially hepatectomized rats*

Markers	Source of MNL	Sham-Operated	Partially Hepatectomized
CD5	Liver	29 ± 10	24 ± 7
Ia		25 ± 6	19 ± 5
NKR-P1^{bright}		36 ± 8	32 ± 9
CD5	Spleen	54 ± 6	51 ± 2
Ia		51 ± 4	50 ± 2
NKR-P1^{bright}		7 ± 1	9 ± 1
CD5	Blood	83 ± 1	73 ± 6
Ia		57 ± 13	17 ± 1
NKR-P1^{bright}		6 ± 2	14 ± 8

[a] Lymphoid cells were obtained from normal (sham-operated) and partially hepatectomized rats 24 h after surgery. Liver-resident MNL, spleen, and peripheral blood lymphoid cells were separated on gradients, as described in *Materials and Methods*. The cells were stained with FITC-conjugated OX-19 (anti-CD5), OX-6 (anti-Ia), and 3.2.3 (anti-NKR-P1) mAbs and analyzed on FACScan. The data are mean percentages ± SEM of positive cells from eight experiments with liver-resident lymphoid cells and from two experiments with spleen and blood MNC. Two normal and five partially hepatectomized rats were donors of the lymphoid cells for each experiment.

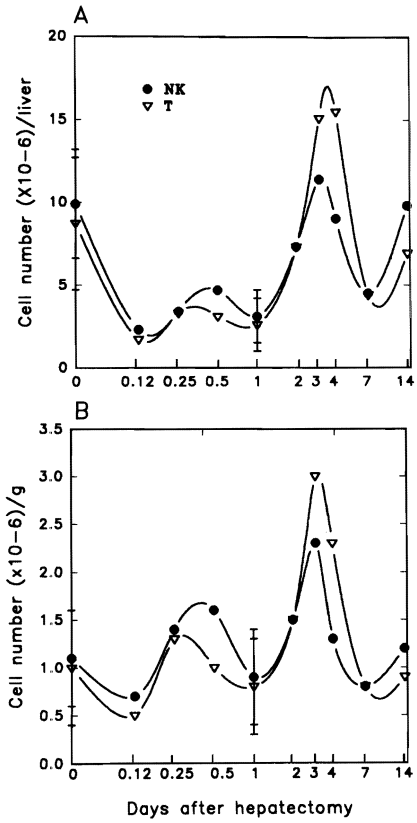

FIGURE 3. Changes in the number of liver-resident NK and T cells during liver regeneration. The experiments were performed as described in Figure 2. Separated liver-resident MNL were stained with FITC-conjugated anti-CD5 or anti-NKR-P1 mAbs and analyzed on FACScan. The data represent absolute numbers of NK cells or T cells calculated on the basis of the absolute number of liver-resident MNL and the relative number of CD5$^+$ or NKR-P1^{bright} cells. The results are presented per organ (*A*) and per gram of liver tissue (*B*).

other hand, two-color flow cytometry of Fisher rat splenocytes or liver-resident MNL demonstrated that CD5 and CD3 were co-expressed on the same lymphocytes. Also, there was no detectable population of CD5$^-$CD3$^+$ T cells (data not shown). These results indicate that in rats CD5 is as good a marker of T cells as is CD3. We have previously reported that NKR-P1$^+$ lymphocytes include NKR-P1^{bright} TCR$^-$ (i.e., NK) and NKR-P1^{dim} TCR$^+$ (i.e., T) cell populations (29). Here, we confirm and extend these findings by showing that <2% of NKR-P1^{bright} lymphocytes in either splenocyte or liver-resident MNL populations co-express T cell markers in normal as well as in partially hepatectomized rats (data not shown). Thus, expression of NKR-P1 at a high density (NKR-P1^{bright}) on the cell surface represents a specific marker for rat NK cells both in the spleen and the liver.

Having defined the specificity of mAbs we had available for rat T cells and NK cells, and using anti-Ia mAb as a marker for both B cells and monocytes/macrophages, we next studied phenotypic changes in MNL populations in the peripheral blood, spleen, and liver of rats following partial hepatectomy. In these flow cytometry analyses, the gate was set on MNL, and parenchymal liver cells were excluded. In a normal rat liver, T cells and NK cells were found to be present in the ratio of 29 (T):36 (NK), which substantially differed from that in normal rat peripheral blood (83:6) or spleen (54:7) (Table I). In partially hepatectomized rats at 24 h after surgery, significant changes in the proportion of the lymphocyte populations were observed in the peripheral blood, as compared with sham operated rats. Specifically, a decrease in the proportion of Ia$^+$ lymphocytes and an increase of NKR-P1^{bright} NK cells (Table I) were observed. However, only the decrease of Ia$^+$ lymphocytes, but not the increase of NK cells, was significant ($p < 0.05$), because the absolute number of circulating lymphoid cells was found to be two- to threefold lower than in normal rats 24 h after partial hepatectomy (data not shown).

At all time points assessed between 3 h and 14 days after partial hepatectomy, the proportions of liver-resident T cells and NK cells were found to be similar to those in normal rats. A minor change in the ratio of two lymphoid cell populations was seen at 72 h and 96 h after partial hepatectomy, when the proportion of T cells was slightly increased in comparison to that of NK cells (Fig. 3). However, the absolute number of both resident T and NK cells, determined either per organ or per gram of liver, was found to increase in two waves, between 6 h and 12 h and between 48 h and 72 h after partial hepatectomy (Fig. 3, *A* and *B*). This time course and pattern were similar to those described above for the liver-resident MNL (Fig. 2, *A* and *B*). Again, the absolute numbers of both liver-resident T and NK cells, calculated both per organ or per gram of tissue, returned to normal values on day 14 after partial

Table II. Co-expression of CD45 with T cell, NK cell, or B cell/macrophage markers on normal and partially hepatectomized rat splenocytes and liver-resident MNL[a]

Cells	Markers	Days after Hepatectomy			
		Day 0	Day 1	Day 4	Day 7
Splenocytes	$CD45^+$	99	99	99	98
	$CD5^+$	39	35	33	38
	$CD5^-$NKR-P1^{bright}	11	17	12	12
	$CD5^+$NKR-P1^{bright}	0.2	0.2	0.1	0.2
	$CD3^+$	40	33	34	37
	$CD3^-$NKR-P1^{bright}	10	15	10	11
	$CD3^+$NKR-P1^{bright}	0.2	0.1	0.2	0.2
	TCR-$\alpha\beta^+$	36	34	32	35
	TCR-$\alpha\beta^-$NKR-P1^{bright}	10	15	10	12
	TCR-$\alpha\beta^+$NKR-P1^{bright}	0.2	0.1	0.2	0.2
	Ia^+	41	39	41	39
Liver MNL	$CD45^+$	96	94	95	91
	$CD5^+$	19	16	15	11
	$CD5^-$NKR-P1^{bright}	57	30	30	38
	$CD5^+$NKR-P1^{bright}	1.2	0.5	0.3	0.6
	$CD3^+$	14	17	19	11
	$CD3^-$NKR-P1^{bright}	57	30	28	40
	$CD3^+$NKR-P1^{bright}	1.1	0.4	0.2	0.3
	TCR-$\alpha\beta^+$	13	13	11	9
	TCR-$\alpha\beta^-$NKR-P1^{bright}	57	28	30	41
	TCR-$\alpha\beta^+$NKR-P1^{bright}	1.0	0.2	0.4	0.3
	Ia^+	24	28	25	20

[a] Spleen and liver-resident MNL were separated from sham-operated or partially hepatectomized Fisher rats and purified on gradients, as described in *Materials and Methods*. The cells were stained with PE-conjugated anti-CD45 and simultaneously with CY5-labeled anti-NKR-P1 and either FITC-tagged anti-CD5, anti-CD3, anti-TCR-$\alpha\beta$, or anti-Ia mAbs. Three-color flow cytometry was then performed. The data are percentages of positive cells from one representative experiment of two performed.

hepatectomy. A similar pattern of changes was also observed for liver-resident Ia^+ lymphoid cells (B cells and macrophages; data not shown).

As indicated in Table I, a significant proportion of MNL in normal and regenerating liver populations were found to be negative for either NK cell, T cell, or B cell/macrophage markers. Since no contamination with liver parenchymal cells was detected in these preparations, we hypothesized that normal or regenerating livers contained MNL which were immature and thus did not express the usual markers of mature lymphocytes. To test this hypothesis, time course experiments were performed, using three-color flow cytometry with PE-conjugated anti-CD45 (OX-1, leukocyte common Ag), vs CY5-labeled anti-NKR-P1 and vs FITC-conjugated anti-CD5, anti-CD3, anti-TCR-$\alpha\beta$, or anti-Ia mAbs. In these experiments, by gating on $CD45^+$ cells, it was possible to determine the exact proportions of MNL ($CD45^+$ cells) in populations of splenic and liver MNL. Furthermore, the exact proportions of mature NK cells, T cells, and B cells/macrophages were determined among splenic and liver-resident populations of MNL at various time points after partial hepatectomy (Table II). We found that $\geq$99% of splenocytes and between 91 and 96% of liver-resident MNL separated either from normal or partially hepatectomized rats were $CD45^+$. However, only 86 to 91% of $CD45^+$ splenocytes and 72 to 100% of $CD45^+$ liver-resident MNL expressed markers of mature NK cells, T cells, or B cells/macrophages. It is important to note that the proportion of MNL lacking the markers of mature lymphocytes was found to be increased in the liver after partial hepatectomy. These findings support our hypothesis that liver-resident MNL contain a significant proportion of immature lymphoid cells, which are particularly evident during the period of liver regeneration. The data presented in Table II confirm that NKR-P1 expressed at high density is a specific marker of rat NK cells. In addition, the data presented in Table II confirm that no significant fluctuation occurs in the proportions of NK cells:T cells:B cells/macrophages during liver regeneration.

Overall, the flow cytometry studies demonstrated that liver regeneration in rats was accompanied by a dramatic increase in the number of all liver-resident MNL. In addition, the viability, cell size, and mean fluorescent intensity of all tested surface Ags on liver-resident lymphoid cells were similar in sham-operated rats and partially hepatectomized animals at all tested time points after treatment (data not shown). Thus, despite the significant changes in the number of liver-resident MNL, the main phenotypic characteristics of the liver MNL did not significantly change during liver regeneration.

Functional characteristics of normal and regenerating liver NK cells

To determine whether liver regeneration is accompanied by functional changes of liver-resident NK cells, various spontaneous and IL-2-induced functions of liver-resident NK cells were compared in sham operated rats and in partially hepatectomized animals 24 h after partial hepatectomy, at the time when major humoral and cellular events in liver regeneration have been shown to occur (2, 3). Both spontaneous and IL-2-induced functions of liver-resident NK cells were found to be profoundly and consistently suppressed 24 h after partial hepatectomy. Thus, as shown in Table III, lymphocytes from regenerating rat livers were suppressed in spontaneous killing of YAC-1 target cells (NK activity: 85% decrease), P815 target cells (spontaneous LAK activity: 98% decrease), Ab-coated P815 target cells (ADCC, triggering of NK cells through FcγRIII: 68% decrease), P815 targets in the presence of anti-NKR-P1 mAb (redirected killing, triggering of NK cells through NKR-P1 receptor: 90% decrease) and regenerating liver hepatocytes (100% suppression). In contrast, binding of resident NK cells to target cells (YAC-1) was not different in regenerating liver from that in normal liver (data not shown), indicating that the described suppression of spontaneous NK cytotoxicity in regenerating liver was at the level of effector cell triggering rather than that of target cell recognition. Similar to the spontaneous NK cell

Table III. *Functions of liver-resident NK cells in sham-operated and partially hepatectomized rats*[a]

Functions	Sham-Operated	Partially Hepatectomized	% Suppression
Spontaneous			
YAC-1 lysis (n = 7)	410 ± 139	61 ± 24	85
P815 lysis (n = 3)	199 ± 119	4 ± 2	98
ADCC (n = 3)	2,838 ± 1,268	900 ± 380	68
Redirected lysis (n = 2)	716 ± 438	70 ± 66	90
Lysis of regenerating hepatocytes (n = 2)	98 ± 57	0 ± 0	100
IL-2-induced, 24 h			
YAC-1 lysis (n = 3)	979 ± 399	307 ± 193	69
P815 lysis (n =2)	427 ± 187	40 ± 13	91
Adherence (n = 7)	5.4 ± 0.3	2.2 ± 0.2	59
Day 5			
Proliferation (n = 6)	64,278 ± 9,651	19,412 ± 3,535	70
Day 7			
YAC-1 lysis (n = 2)	595 ± 205	263 ± 11	56
P815 lysis (n = 2)	331 ± 189	110 ± 48	67

[a] Liver-resident MNL were isolated from sham-operated and partially hepatectomized rats 24 h after the operation and assayed either fresh, for spontaneous activities, or after 24 h, 5 days, or 7 days of in vitro stimulation with 22 nM of IL-2, for IL-2-induced activities, as described in *Materials and Methods*. Cytotoxicity data are presented as $LU_{20}/10^7$ of NK (NKR-P1^{bright}) cells; adherence is measured by determining the number of A-NK cells/grid (200× magnification) and proliferation is shown in cpm after ^{3}[H]TdR incorporation into cell nuclei. Results are mean ± SEM of two to seven experiments. Numbers (n) of experiments performed are in parentheses. All differences between functions of liver-resident NK cells in normal and partially hepatectomized rats were statistically significant ($p < 0.001$).

functions, IL-2-induced NK cell functions were also suppressed in liver-resident MNL 24 h after partial hepatectomy (Table III). Thus, after 24 h of in vitro IL-2 stimulation, inhibition of IL-2-induced augmentation of YAC-1 killing (69%), suppression of IL-2-induced generation of LAK activity (killing of P815, 91%) and decrease of NK cell plastic adherence (59%) were found consistently (Table III). Similarly, inhibition of the proliferation of liver-resident MNL (70%) and a decrease (56–67%) in the generation of LAK activity from highly purified NK cells (≥98% NKR-P1^{bright}), obtained by IL-2-induced plastic adherence, were observed after 5 or 7 days, respectively, of culture in the presence of IL-2 (Table III). In addition, 24 h after partial hepatectomy, the generation of IL-2-induced LAK activity in 7-day bulk cultures of liver-resident lymphocytes was also decreased (data not shown). These experiments demonstrated that, 24 h after partial hepatectomy, liver-resident NK cells were profoundly suppressed in a variety of functional responses that are normally triggered in NK cells following interactions between various cell surface receptors and their ligands.

Functional characteristics of NK cells purified from regenerating liver-resident MNL

In a mixed population of liver-resident MNL separated from regenerating liver, the described suppression of NK cell functions could be viewed as a result of either in vivo functional changes of NK cells or in vitro interactions of NK cells with other MNL. To examine these possibilities, functions of highly purified (>95% NKR-P1^{bright}) liver-resident NK cells, which were positively sorted from sham-operated or partially hepatectomized rat livers, were tested 24 h after the operation (Fig. 4). The results of this experiment (Figs. 4 and 5, Table IV) showed that the suppression of NK cell functions seen with the unseparated populations of liver-resident MNL obtained from regenerating liver was also present when highly purified liver-resident NK cells were tested for cytotoxicity and IL-2-induced proliferation. Therefore, functional impairments of liver-resident NK cells after partial hepatectomy could not be induced in vitro by other MNL but rather were a consequence of in vivo events following hepatectomy. In contrast to the positively sorted (NKR-P1^{bright}) population, NKR-P1$^-$ liver-resident MNL did not mediate NK-cell activity (data not shown).

Kinetics of immunosuppression of liver-resident NK cells during liver regeneration

To correlate more closely the observed suppression of liver-resident NK cell functions to the process of liver regeneration, kinetic experiments were performed between 3 h and 14 days after partial hepatectomy. As shown in Figure 6, suppression of spontaneous NK activities, including NK cytotoxicity, LAK activity, ADCC, and redirected killing by liver-resident NK cells was evident as early as 3 h after partial hepatectomy, reached the maximal level after 12 h, slightly recovered after 24 h and remained significantly ($p < 0.001$) suppressed until day 14. Similar kinetics of suppression during liver regeneration were also observed with certain IL-2-dependent functions of liver-resident NK cells, such as in vitro 24 h IL-2-induced augmentation of LAK activity (killing of P815) and development of plastic adherence (Fig. 7, *B* and *C*). In contrast, IL-2-induced augmentation of NK cytotoxicity (killing of YAC-1 targets, Fig. 7*A*), as well as 5-day IL-2-induced proliferation of liver-resident NK cells (Fig. 7*D*), was suppressed during only the first 24 h after partial hepatectomy, increased between 2 and 7 days, and returned to normal by day 14 of liver regeneration. In addition, during liver regeneration, changes of liver-resident T cells in proliferative response to Con A were similar to those of liver-resident NK cells in response to IL-2 (Table V, Fig. 7*D*).

Local immunosuppression of NK cells during liver regeneration

To determine whether NK cell suppression during liver regeneration is local or systemic, NK cell functions were compared in the liver, peripheral blood, and spleen of normal or partially hepatectomized rats. It was found that, 24 h after partial hepatectomy, only spontaneous NK cytotoxicity was slightly decreased in the peripheral blood and spleen, but to a lesser extent ($p < 0.05$) than in the liver ($p < 0.001$) (Table VI). The other NK cell functions

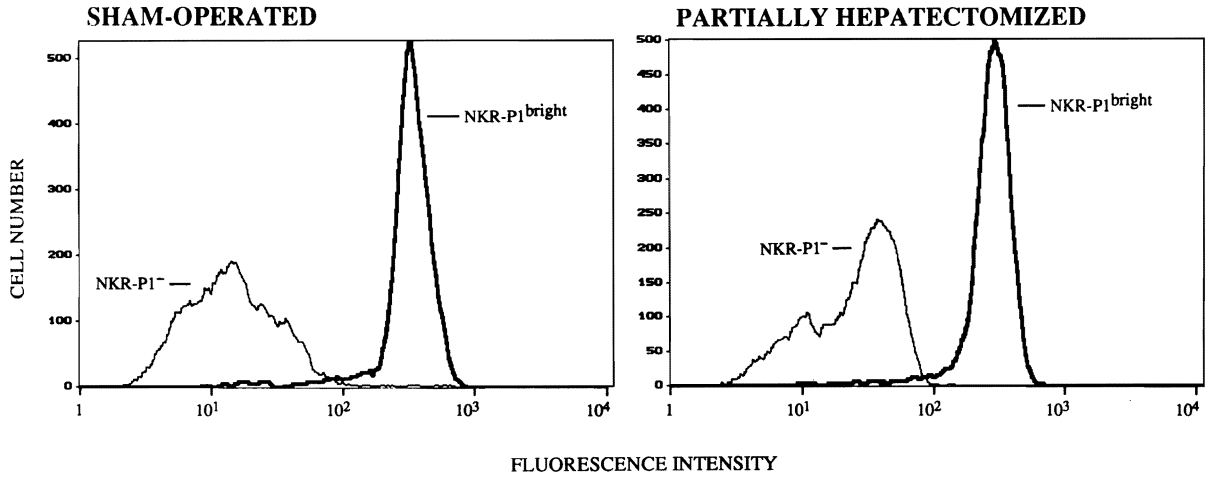

FIGURE 4. Histograms of sorted liver-resident NK cells from sham-operated or partially hepatectomized rats. Fisher rats were operated 24 h before the experiment. Liver-resident MNL were isolated, stained with FITC-labeled $F(ab')_2$ fragments of anti-NKR-P1 mAb, and sorted into NKR-P1^{bright} and NKR-P1$^-$ populations. Positively sorted cells were >95% NKR-P1^{bright}, while negatively-sorted cells were 99% NKR-P1$^-$.

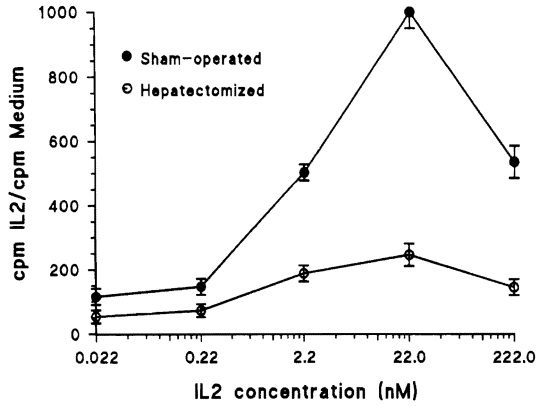

FIGURE 5. IL-2-induced proliferation of sorted liver-resident NK cells from normal and partially hepatectomized rats. Sorting of liver-resident NK cells into NKR-P1^{bright} and NKR-P1$^-$ was performed as described in the legend to Figure 4. Positively sorted, liver-resident NK cells were plated in 96-well, round-bottom plates (2×10^4 cells/well) at various concentrations of IL-2 each in six replicates. The cells were cultured for 5 days. The data are ratios of cpm of ^{3}[H]TdR incorporated into NK cells in the presence (cpm IL-2) and in the absence (cpm medium) of IL-2. The differences between liver-resident NK cells from normal and partially hepatectomized rats were statistically significant at IL-2 concentrations between 2.2 and 222 nM ($p < 0.005$).

tested, including ADCC and IL-2-induced adherence and proliferation, were either unchanged or slightly increased in the spleen or peripheral blood of partially hepatectomized rats (Table VI). These data indicated that suppression of NK cell functions during the course of liver regeneration occurred predominantly in the liver.

Table IV. *Cytotoxic activity of highly purified liver-resident NK cells from normal and partially hepatectomized rats*[a]

	Sham-Operated		Partially Hepatectomized	
Target Lysis	Unsorted	Sorted	Unsorted	Sorted
YAC-1 (NK)	426	411	93	122
P815 (LAK)	107	128	5	9
P815 (ADCC)	990	1580	336	661
P815 (redirected)	286	153	62	45

[a] Liver-resident MNL were separated from sham-operated (normal) and partially hepatectomized rats. Surgery was performed 24 h before the experiment. Freshly isolated liver-resident MNL were stained with FITC-tagged $F(ab')_2$ fragments of anti-NKR-P1 mAb, and NKR-P1^{bright} cells were separated by sorting from NKR-P1$^-$ cells, as described in *Materials and Methods*. The results are $LU_{20}/10^7$ of NK cells. All differences between liver-resident NK cells from sham-operated and from partially hepatectomized rats were statistically significant ($p < 0.003$).

Cytotoxicity of liver-resident NK cells against regenerating hepatocytes

To test the possibility that liver-resident NK cells are involved in the control of liver regeneration, experiments were performed to correlate hepatocyte alterations after partial hepatectomy and changes in cytotoxic activity of liver-resident NK cells against normal or regenerating liver hepatocytes. On days 2, 3, or 4 after partial hepatectomy, hepatocytes significantly differed from those obtained from normal liver or from liver 14 days after hepatectomy. Hepatocytes obtained from the regenerating livers had ~threefold higher proportion of cells in G2-M phases of the cell cycle, four- to sixfold lower number of diploid cells, and ~twofold higher density (mean fluorescent intensity) of MHC class I Ag expression on the cell surface (data not shown). In contrast, normal rat liver hepatocytes and hepatocytes from 14-day-regenerating livers were similar in terms of the cell cycle, ploidy, or MHC class I Ag

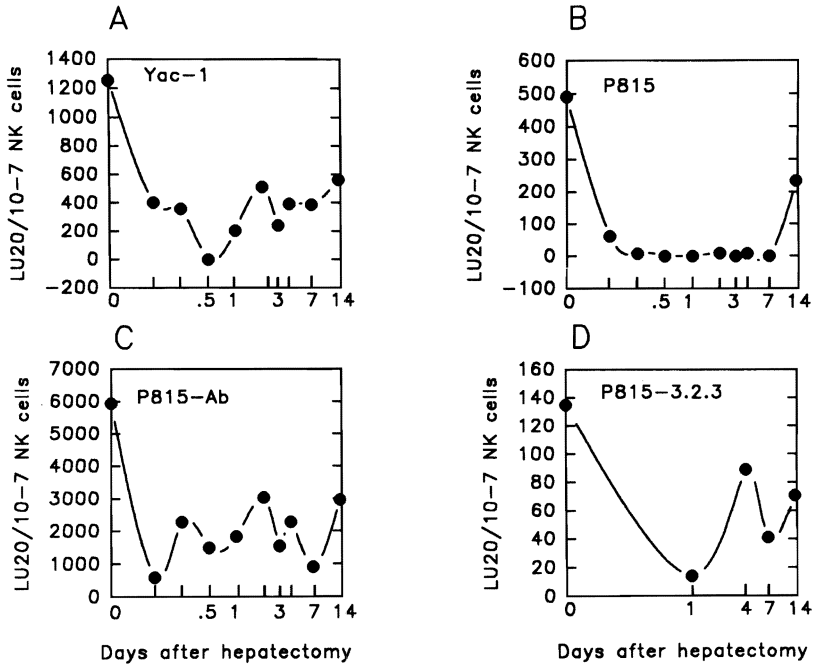

FIGURE 6. Suppression of spontaneous cytotoxic activity of liver-resident NK cells during liver regeneration. Liver-resident MNL were separated from sham-operated or partially hepatectomized rats at various time points after surgery and assayed for cytotoxicity against the following ^{51}Cr-labeled target cells, as detailed in *Materials and Methods*: YAC-1 (NK activity, in *A*); P815 in the absence or presence of preimmune rat serum or IgG1 isotype control mAb (spontaneous LAK activity, in *B*); P815 in the presence of rat anti-P815 Abs (ADCC, in *C*); and P815 in the presence of anti-NKR-P1 mAb (redirected killing, in *D*). The data are $LU_{20}/10^7$ NK (NKR-P1^{bright}) cells, obtained from four different E:T cell ratios, each measured in triplicate. All differences between the functions of liver-resident NK cells from normal and partially hepatectomized rats were significant ($p < 0.001$).

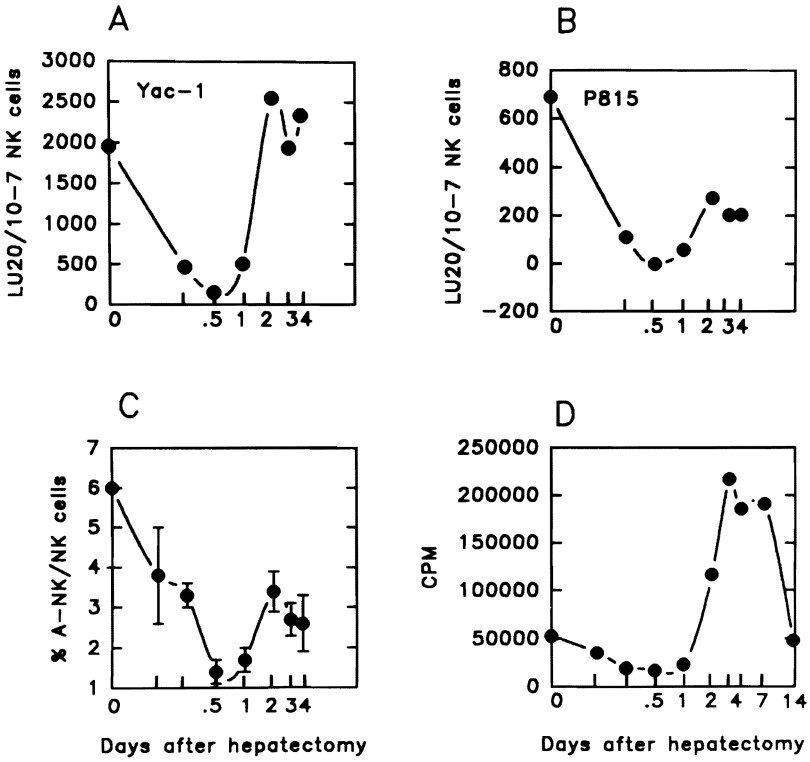

FIGURE 7. Changes in IL-2-induced functions of liver-resident NK cells during liver regeneration. Liver-resident MNL were obtained from sham-operated or partially hepatectomized rats, as indicated in Figure 4, induced with 22 nM of IL-2 for 24 h, and assayed for cytotoxicity against YAC-1 (*A*) and P815 (*B*) target cells, for the capacity to adhere to a plastic surface (*C*) and for proliferation in 5-day cultures (*D*), as described in *Materials and Methods*. The data are $LU_{20}/10^7$ NK cells in *A* and *B*, as defined in *Materials and Methods*. The results shown in *C* and *D* represent mean ± SEM of seven and three replicates, respectively. All differences between normal and regenerating livers were significant ($p < 0.001$).

expression (data not shown). These observations indicated that hepatocytes significantly changed during liver regeneration both phenotypically and functionally.

Next, normal or regenerating liver-resident NK cells were tested for cytotoxic activity against normal or regenerating liver hepatocytes. Normal liver hepatocytes and hepatocytes harvested from livers 14 days after partial hepatectomy, when liver regeneration was finished, were found to be completely resistant to lysis by liver-resident NK cells obtained either from normal rats or from rats at any period after partial hepatectomy (Fig. 8). Thus, under normal physiologic condition, in adult rats, liver-resident NK cells did not significantly kill autologous hepatocytes. In contrast, NK cells isolated from normal liver or those

Table V. *Con A-induced proliferation of liver-resident T cells after partial hepatectomy*[a]

Time After Hepatectomy (days)	cpm
Sham-operated	59,100 ± 5,122
0.125	49,166 ± 5,220
0.25	28,095 ± 9,846
0.5	3,665 ± 1,406
1	31,053 ± 4,698
2	76,700 ± 11,686
4	94,400 ± 4,535
7	56,135 ± 4,535
14	59,000 ± 5,500

[a] Liver-resident MNL were obtained from sham-operated or partially hepatectomized rats, stimulated in vitro with Con A (5 μg/ml) for 5 days and their proliferation assessed using ^{3}[H]TdR incorporation assay, as described in *Materials and Methods*. The results are mean ± SEM of three to six replicates. Similar results were obtained after stimulation of MNL with 2.5 or 10 μg/ml of Con A. The findings were repeated in five experiments for day 1 after the operation and in three experiments for days 4, 7, and 14. The differences between sham-operated and partially hepatectomized rats on 0.25, 0.5, 1, and 4 days after the operation were statistically significant ($p < 0.001$).

Table VI. *Functional characteristics of liver, spleen, and peripheral blood NK cells in normal and partially hepatectomized rats*[a]

Functions	Source of MNL	Sham-Operated	Partially Hepatectomized
Spontaneous	Liver		
YAC-1 lysis		429 ± 10	175 ± 3
ADCC		1,157 ± 26	936 ± 34
IL-2 Induced			
24-h adherence		8 ± 2	3 ± 0.3
5-day proliferation		107,930 ± 7,778	2,493 ± 300
Spontaneous	Spleen		
YAC-1 lysis		644 ± 26	490 ± 15
ADCC		826 ± 40	740 ± 41
IL-2-induced			
24-h adherence		17 ± 4	16 ± 4
5-day proliferation		90,772 ± 14,816	117,229 ± 6,446
Spontaneous	Blood		
YAC-1 lysis		113 ± 6	80 ± 4
ADCC		1,170 ± 56	1,669 ± 90
IL-2-induced			
24-h adherence		7 ± 1	19 ± 1
5-day proliferation		50,231 ± 3,207	86,576 ± 9,173

[a] Liver, spleen, and blood MNL were isolated from sham-operated and partially hepatectomized rats 24 h after the operation and assayed either fresh or after 24 h and 5 days of in vitro co-incubation with 22 nM of IL-2, as described in *Materials and Methods*. The data are mean ± SEM of triplicates of $LU_{20}/10^7$ NK cells (lysis of YAC-1 or ADCC); of the number of A-NK cells counted in seven different microscopic fields (24-h adherence); and of triplicates of cpm after ^{3}[H]TdR incorporation into cell nuclei (5-day proliferation). The differences between sham-operated and partially hepatectomized rats were significant ($p < 0.001$) only for liver-resident MNL in all assays performed.

obtained from liver after the first phase of regeneration (i.e., between day 7 and 14) had significant cytotoxicity in 4-h ^{51}Cr-release assays against hepatocytes from day 2 (Fig. 8) or day 3 (data not shown) regenerating livers. These observations indicate that hepatocytes during the acute stage of liver regeneration became sensitive to lysis by liver-resident NK cells obtained from normal liver. However, NK cells isolated from day 1 or day 4 regenerating liver were not cytotoxic against hepatocytes obtained

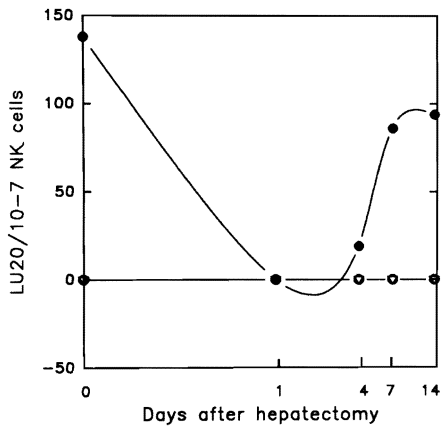

FIGURE 8. Correlations of changes in spontaneous cytotoxicity of liver-resident MNL against regenerating liver hepatocytes with liver regeneration. Liver-resident MNL (effectors) and hepatocytes (targets) were separated from sham-operated and partially hepatectomized rats at various time points after surgery and tested in 4-h ^{51}Cr-release assays. The data are $LU_{20}/10^7$ NK cells, as defined in *Materials and Methods*. Lysis of day 2 regenerating liver hepatocytes (●) by liver-resident MNL on day 1 and 4 after partial hepatectomy was significantly suppressed ($p < 0.001$) in comparison to that of MNL from normal or day 7 and 14 regenerating livers. Lysis of hepatocytes from normal livers (○) or those from livers 14 days after hepatectomy (▽) could not be detected using MNL from either sham-operated or partially-hepatectomized rats.

from either day 2 (Fig. 8) or day 3 (data not shown) regenerating liver. These NK cells were also not cytotoxic against hepatocytes from normal or day 14 regenerating livers (Fig. 8). Hence, NK cells obtained from liver at the time of acute regeneration are suppressed and are unable to kill NK-sensitive regenerating hepatocytes. Exactly the same pattern of cytotoxicity mediated by normal or regenerating liver-resident MNL against regenerating liver hepatocytes was seen in the cytotoxicity assays performed in the presence of IL-2. However, in comparison with the assays without IL-2, the IL-2-induced cytotoxic activity of liver-resident NK cells against hepatocytes was ~threefold higher (data not shown). These data demonstrated that development of both sensitivity of hepatocytes to killing by liver-resident lymphocytes and complete suppression of the ability of NK cells to mediate cytotoxicity against regenerating liver hepatocytes occurred during the acute phase of liver regeneration. Since NK-cell-mediated cytotoxicity might be potentially damaging to the sensitive, regenerating liver hepatocytes, its suppression might be important for protection of regenerating hepatocytes and thus for promotion of the process of liver regeneration. After the acute phase of liver regeneration, both hepatocyte resistance to killing by autologous NK cells and cytotoxic activity of liver-resident lymphoid cells were restored in parallel.

Liver regeneration in rats depleted of NK or T cells

To assess directly whether liver-resident NK cells and/or T cells participate in regulation of the liver regeneration process, we compared liver regeneration following 70% partial hepatectomy in normal Fisher rats, Fisher rats depleted of NK cells, and T cell-deficient nude rats. Fisher rats were injected i.p. with either anti-NKR-P1 mAb or IgG1 isotype control mAb (100 μg/rat) on day −1, partially hepatectomized on day 0, reinjected with mAbs on day +4, and evaluated for body and liver weights on day +7 after hepatectomy. In parallel, nude rats were partially hepatectomized and tested as described above. Preliminary experiments showed that a single i.p. injection of anti-NKR-P1 mAb in doses between 50 and 200 μg/rat resulted 1 day later in a complete elimination of NKR-P1^{bright}CD3^{-} cells in both the spleen and liver of Fisher rats. It also resulted in suppression of NK cell activities, including killing of YAC-1 targets, ADCC, redirected killing of P815 targets in the presence of anti-NKR-P1 mAb, and generation of A-NK cells in the presence of IL-2 (data not shown). In addition, in partially hepatectomized animals on day 4 after the first or second injection of anti-NKR-P1 mAb, a complete depletion of NK cells was achieved, as verified by both phenotypic and functional testing of splenocytes (data not shown). The number and functions of T cells, B cells, and macrophages were normal in the NK cell-depleted animals (data not shown), confirming previous findings (45). These initial experiments demonstrated that by using anti-NKR-P1 mAb it was possible to achieve in vivo a prolonged depletion of NK cells in various tissues of Fisher rats, including the spleen and liver. In contrast, injection of IgG1 isotype control mAb did not have any effect on either the number or functions of NK cells, T cells, B cells, or macrophages in Fisher rats (data not shown). As expected, nude rats showed an almost complete absence of T cells in the spleen but, in comparison with normal Fisher rats, had ~twofold higher proportion of NK cells, B cells, and macrophages. These phenotypic characteristics, as well as NK cytotoxicity of splenocytes, were quite stable and did not significantly change for 7 days after partial hepatectomy of nude rats (data not shown). However, we found that under these experimental conditions, the weight of the liver was significantly augmented in Fisher rats depleted of NK cells on day 7 after partial hepatectomy in comparison with the control animals injected with IgG1 isotype control mAb (Fig. 9). In contrast, on day 7 after partial hepatectomy, liver weight in nude rats was similar to that in partially hepatectomized Fisher rats treated with IgG1 isotype control mAb (Fig. 9). In aggregate, these data strongly support the hypothesis that NK cells are involved in control of liver regeneration.

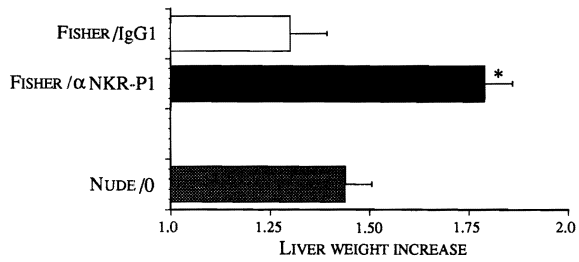

FIGURE 9. Liver regeneration in the absence of NK or T cells. Fisher rats were injected i.p. 1 day before and 3 days after partial hepatectomy with either IgG1 isotype control mAb or with anti-NKR-P1 mAb (100 μg/rat per injection). The animals were killed on day 7 after the operation and their body and liver weights determined. Nude rats were partially hepatectomized and killed on day 7 after the operation, as were Fisher rats. The data are mean ± SEM of the liver weight increase determined in five animals from each group, using the following formula: experimental liver weight − 30% control liver weight/30% control liver weight. The liver weight increase after partial hepatectomy was significantly greater in Fisher rats treated with anti-NKR-P1 mAb than in Fisher rats injected with isotype control mAb ($p < 0.004$) or in nude rats ($p < 0.02$)

Discussion

In this report, evidence was presented in support of the hypothesis that liver-resident NK cells may influence the process of liver growth and regeneration. Partial hepatectomy in rats was found to be followed by two major changes: a rapid augmentation in the number of liver-resident NK cells, as well as other MNL; and a concomitant profound suppression of major functions of NK cells, including the ability to spontaneously kill regenerating liver hepatocytes and other NK sensitive targets and to mediate ADCC and redirected killing through the NKR-P1 receptor. In addition, IL-2-induced NK-cell functions, such as augmentation of NK cytotoxicity, generation of A-NK cells, and LAK activity, as well as proliferation, were also suppressed in MNL obtained from livers in early stages of regeneration. In contrast to the majority of spontaneous NK-cell activities, which were found to be suppressed throughout the process of liver regeneration, killing of regenerating liver hepatocytes was suppressed only during the relatively early phase (days 2–4), at the time when hepatocytes were susceptible to NK cell lysis and liver growth was most rapid. At the later stages of liver regeneration, when growth of the liver was significantly slowed or had ceased, both cytotoxicity of liver-resident NK cells for regenerating hepatocytes and resistance of hepatocytes to this NK cell activity were restored. These findings could be interpreted to mean that, at the time liver regeneration is completed, functionally recovered liver-resident NK cells kill only proliferating (i.e., NK-sensitive) hepatocytes, sparing nonproliferating (i.e., NK-resistant) hepatocytes and thus controlling further growth of the liver. If this interpretation is correct, it indicates that liver-resident NK cells might be involved in regulation of liver regeneration. In direct support of this hypothesis, we found in partially hepatectomized Fisher rats that in vivo depletion

of NK cells, by using anti-NKR-P1 mAb, resulted in a significant augmentation of liver regeneration when cytotoxicity of liver-resident NK cells for regenerating hepatocytes was recovered.

Increases in the number of liver-resident NK cells have been observed in mice after partial hepatectomy (36), but also after various treatments that damage liver tissue and/or stimulate the immune system, including infection with hepatotropic viruses or treatment with various biologic response modifiers (33, 37, 46). Wiltrout et al. have shown that a rise in the number of NK cells in murine livers after treatment with biologic response modifiers was mainly a consequence of an influx from the peripheral blood of NK cells newly produced in the bone marrow (47). It is possible that a similar mechanism takes place during the acute phase of liver regeneration following partial hepatectomy in the rat. In support of this possibility, we observed a significant increase in the number of liver-resident NK cells in the early phase of liver regeneration. At the same time, only low spontaneous proliferation of liver-resident MNL was found. The presence in the normal as well as regenerating liver of a high proportion of NK cells (32–36%) and their low proportion in peripheral blood (6–14%) suggest that accumulation of NK cells in both normal and regenerating liver occurs as a consequence of a specific mechanism of selection. Our more recent finding of phenotypic and functional differences between circulating and liver-resident NK cells (Vujanovic et al., unpublished results) support this hypothesis. The immature endothelial cells and hepatocytes (which are rare in the normal adult liver, but numerous in growing young or partially hepatectomized liver), as well as cytokines produced or accumulated in the liver, might play an important role in the mechanism(s) of specific selection and accumulation of NK cells in the liver.

A local defect of NK cell functions in the liver at the time of liver regeneration is a new and significant finding. This inhibition of NK cell functions was dramatic and detectable in the first 3 to 12 h after partial hepatectomy. It was largely restricted to liver-resident NK cells, as only minor changes occurred in circulating and spleen NK cells. It comprised all major NK-cell functions, including the ability to spontaneously lyse autologous regenerating liver hepatocytes. NK cells freshly isolated from regenerating liver tissues and purified to >95% of $CD3^{-}NKR\text{-}P1^{bright}$ cells by sorting were also found to be functionally suppressed. Therefore, the observed NK-cell defects were most likely induced in vivo. While the suppression of major IL-2-induced NK-cell functions was reversed between 24 and 48 h after partial hepatectomy, the majority of spontaneous NK cell functions remained profoundly suppressed during the entire period of liver regeneration. Spontaneous killing of regenerating liver hepatocytes was evident only when the effector cells were obtained from the normal liver or the already regenerated liver, and not from the liver in the acute phase of liver regeneration. Therefore, we concluded that such selective inhibition of liver-resident NK cells probably represents an important regulatory mechanism, allowing liver regeneration to take place in the presence of NK cells that are potentially lethal for regenerating hepatocytes. In addition, we have observed increases in the number of liver-resident NK cells during the acute phase of liver regeneration. It is possible that these cells participate in liver regeneration, serving as a source of hepatocyte growth factors. At the same time, because the observed inhibition of the ability of liver-resident NK cells to lyse regenerating hepatocytes was reversed before the process of liver regeneration was completed, it is likely that liver-resident NK cells participate in the termination of liver regeneration. This latter hypothesis is supported by our finding that in vivo depletion of NK cells by anti-NKR-P1 mAb resulted in augmentation of liver regeneration following partial hepatectomy. The mechanisms that suppress functions of liver-resident NK cells during liver regeneration as well as the potential involvement of liver-resident NK cells in control of the process of liver regeneration are completely unknown and are under current investigation in our laboratory. Inasmuch as the decrease of NK cell functions during liver regeneration was predominantly local, it is reasonable to assume that it was caused by a factor(s) produced by and/or accumulated at a high concentration in the regenerating liver. The observed differences in a decrease of spontaneous (3 h–14 days) vs IL-2-induced (24 h) functions of liver-resident NK cells indicate that distinct factors might be involved or that different mechanisms of inhibition are operative at various time points after partial hepatectomy. In a parallel study performed in our laboratory (Francavilla et al., manuscript in preparation), we have observed that at least two factors might participate in the liver regeneration-related inhibition of liver-resident NK cells; i.e., TGF-β and a recently discovered and biochemically characterized and cloned factor named the augmenter of liver regeneration (12, 14, 48).

The described regeneration-related immunosuppression in the liver was not restricted to NK cells but also involved liver-resident T cells, suggesting a more generalized nature of this phenomenon. The mechanisms of regeneration-related immunosuppression are likely to be biologically important and applicable not only to regenerating liver but also to other normal or pathologically altered tissues, where active growth is present or where regeneration is induced. For example, local immunosuppression known to occur in growing or developing tissues (49–52), decidua during pregnancy (53), malignant tumors during their destructive expansion to normal surrounding tissues (54), or various virus-infected (55–58) and physically or chemically injured (59–63) tissues could be, at least partially, a consequence of the development of local regeneration-related immunosuppressive mechanisms. The nature of these mechanisms is unknown, but, in view of their possible general biologic importance, efforts are being made in our laboratory to investigate this phenomenon.

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