Characterization of Cytotoxic Cells from Reovirus-Infected *SCID* Mice: Activated Cells Express Natural Killer- and Lymphokine-Activated Killer-Like Activity but Fail To Clear Infection†

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Severe combined immune deficient (*SCID*) mice infected orally with reovirus type 1/L die of hepatitis. Leukocytes bearing the cell surface antigens Thy-1.2 and asialoGM-1 (AsGM1) accumulate in the livers of infected animals. These cells display lytic activity toward natural killer-sensitive (YAC-1) and -resistant (P815) cell lines and murine hepatoma line Hepa 1/A1. Although these cells have the capacity to lyse infected hepatoma targets, they cannot clear the virus.

In normal adult mice, reovirus 1/L enters the host through M cells overlying the Peyer's patches (27) and causes localized, subclinical gastroenteritis (20). This infection results in production of virus-specific antibody, priming of specific B cells, and generation of virus-specific cytotoxic T-lymphocyte (CTL) precursors in the gut-associated lymphoid tissue (16, 24). CB.17 severe combined immunodeficient (*SCID*) mice (2) lack B and T cells but contain functional natural killer (NK) cells (7, 8, 13), enabling the study of innate cellular responses in the absence of specific, adaptive immunity. However, *SCID* mice are susceptible to persistent or lethal systemic infection by many microorganisms that cause subclinical infection or are non-pathogenic

in immunocompetent mice. For example, lethal infections by reovirus serotype 1/Lang (1/L) occur after oral inoculation of adult *SCID* mice (9). In adult *SCID* mice, oral inoculation of reovirus 1/L results in disseminated infection, with detectable virus in most tissues by 1 week postinoculation (p.i.). Although many tissues contain high concentrations of reovirus, pathological examination of infected mice reveals inflammatory lesions only in the liver (9). To elucidate the role of nonspecific immune responses in immunodeficient mice, we analyzed the phenotypic and functional characteristics of leukocytes responding to liver infection with reovirus type 1 in *SCID* mice.

Specific-pathogen-free CB.17 *SCID* breeding pairs (obtained from M. Bosma, Institute for Cancer Research, Fox Chase, Pa.) were bred and housed as previously described (9). BALB/c mice were obtained from the Jackson Laboratory (Bar Harbor, Maine). All mice were studied at ages 8 to 12 weeks.

Reovirus type 1/L stocks were prepared as previously described (11). Mice were orally inoculated with 10⁷ PFU of reovirus 1/L in 0.05 ml of gel saline (16, 23). For NK cell depletion, some mice were inoculated with rabbit anti-AsGM1 (Wako Chemicals USA, Richmond, Va.), 25 µg given intraperitoneally, a day before reovirus inoculation and then every 4 days. Control mice received normal saline or normal rabbit serum.

Liver nonparenchymal cells (NPC) and spleen cells were stained with rabbit anti-AsGM1 followed by fluorescein isothiocyanate (FITC)- or Texas red-conjugated goat anti-rabbit immunoglobulin G (IgG) (Southern Biotech), FITC-conjugated mouse anti-Thy-1.2 (Becton-Dickinson, Mountain View, Calif.), biotinylated anti-CD8 (53-6.72) (14) and anti-CD4 (GK1.5) (5) followed by Texas red-avidin, or culture supernatant from hamster hybridoma 145-2C11 (anti-CD3) (15) followed by FITC-conjugated rabbit anti-hamster IgG (Caltag, San Francisco, Calif.). Stained cells were analyzed on a Becton-Dickinson FACS IV apparatus equipped with argon and krypton lasers.

Target cells included the NK target cells Yac-1 lymphoma (26), P815 mouse mastocytoma cells resistant to NK cell lysis (26) (both from the American Type Culture Collection), and the murine hepatocarcinoma cell line Hepa 1/A (3, 23) (from Barbara Knowles, Wistar Institute, Philadelphia, Pa.). For some assays, target cells were infected with reovirus type 1 at a multiplicity of infection of 10 at 48 h before use as targets in cytotoxicity assays.

Primary cells were incubated on ice for 45 min with undiluted anti-Thy-1.2 (30H12) hybridoma culture supernatant or with a 1:25 dilution of rabbit anti-AsGM1, washed, resuspended in 10% rabbit complement (LO-TOX complement, Cedarlane) in RPMI for 30 min at 37°C, and washed twice in RPMI–5% fetal calf serum prior to use. Control cells were incubated with complement alone. Adherent cells were depleted by incubation on a nylon wool column in complete media at 37°C for 45 min, which was followed by elution of nonadherent cells, or by incubation in a plastic dish in complete media for 1 h at 37°C.

To determine the effect of reovirus infection on liver NPC, liver NPC were isolated from *SCID* mice 1 to 4 weeks after oral inoculation with reovirus serotype 1/L. Liver NPC were stained for expression of the NK surface antigen, AsGM1 (12), Thy-1.2, CD4, CD8, CD3, or surface immunoglobulin and then analyzed with flow cytometry. Noninfected *SCID* mice and *SCID* mice at 1 week p.i. yielded an average of 10⁶ NPC per liver, whereas infected *SCID* mice at weeks 2 to 4 p.i. yielded fivefold more NPC per liver. Flow cytometric analysis revealed that approximately 60% of the NPC in noninfected *SCID* livers bore AsGM1, and this increased to 70 to 80% in infected livers

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FIG. 1. We isolated liver NPC by using a modification of an in situ perfusion protocol described by McIntyre and Welsh (17). Single-cell suspensions of liver NPC had >95% viability by trypan blue exclusion. One-color flow cytometric analysis of liver NPC isolated from noninfected *SCID* mice and *SCID* mice 2 weeks after oral inoculation with reovirus type 1 was done. NPC were stained with rabbit anti-AsGM1 and FITC-conjugated goat anti-rabbit IgG. Noninfected NPC contained a large number of AsGM1^{dim} cells (A). After infection with reovirus, a population of AsGM1^{bright} cells comprising approximately 30% of all AsGM1⁺ cells appeared (B).

at 2 to 4 weeks p.i. Within the AsGM1⁺ population, there were two distinct cell populations, with the more intensely positive cells being found only in infected livers. The AsGM1^{bright} cell population increased markedly during infection (Fig. 1), whereas weakly positive cells did not increase (Fig. 1). Two-color staining for AsGM1 and Thy-1.2 revealed a marked in-

FIG. 2. Two-color flow cytometric analysis of liver NPC isolated from noninfected *SCID* mice and mice orally inoculated (INOC.) with reovirus 1, 2, and 4 weeks previously. NPC were stained with rabbit anti-AsGM1 and Texas red-conjugated goat anti-rabbit IgG plus FITC-conjugated anti-Thy-1. Starting at 2 weeks p.i., the percentage of cells bearing both AsGM1 and Thy-1 increased from 16% in noninfected mice to 40 to 50% at 2 to 4 weeks p.i., with the majority of Thy-1⁺ cells being AsGM1^{bright}. The horizontal and vertical lines represent gates set after analysis of control cells and demonstrate the upper limits of background fluorescence within each experiment.

TABLE 1. Lysis of Yac-1 targets by liver NPC and spleen cells from reovirus-infected and noninfected *SCID* mice^a

Effector cells and wk p.i.	% Specific lysis at E:T ratio of:			
	12.5:1	25:1	50:1	100:1
Liver NPC				
0	0	0	2	7
1	59	58	82	ND ^b
2	43	56	57	67
4	45	60	53	68
Spleen cells				
0	3	1	0	7
1	6	11	15	25
2	17	26	35	45

^a A standard ⁵¹Cr release assay was used to measure cytotoxic activity against various target cell lines. Target cells (10⁷) were labeled with 200 mCi of ⁵¹Cr (Amersham) added to 10 ml of complete media and incubated overnight at 37°C in 5% CO₂. Effector cells, at various E:T ratios, were suspended in 0.1 ml of complete media and then added to wells containing 2 to 5,000 target cells, and the 96-well plate was incubated for 4 to 6 h at 37°C in 5% CO₂. Datum points were assayed in triplicate. *SCID* mice were either noninfected (week 0) or orally inoculated with reovirus type 1 1, 2, or 4 weeks previously. Noninfected mice were used as controls at each time point tested. Each datum point reported is the mean of triplicate experiments. Spontaneous release of ⁵¹Cr was ≤10% in all experiments.

^b ND, not determined.

crease in Thy-1.2⁺ cells after infection (Fig. 2), of which the majority was also AsGM1^{bright}. There were no CD3⁺, surface immunoglobulin⁺, CD4⁺, or CD8⁺-bearing cells in noninfected livers or in NPC from livers at 2 weeks p.i. (data not shown). In contrast, liver NPC isolated from normal, noninfected BALB/c mice contained significant numbers of cells bearing Thy-1⁺CD4⁺ (12 to 30%), Thy1⁺CD8⁺ (0 to 6%), CD3⁺ (6 to 20%), and AsGM1⁺ (approximately 53%), showing that the failure to detect these markers in *SCID* liver NPC was not due to the method of isolation. Furthermore, conventional BALB/c mice did not contain the population of Thy-1.2⁺AsGM1^{bright} liver NPC seen in infected *SCID* mice.

Flow cytometric analysis of spleen cells isolated from noninfected *SCID* mice revealed approximately 10% of cells to be AsGM1⁺Thy-1.2⁺. The increase in double-positive cells was specific to infected livers, since an increase in Thy-1.2-bearing splenocytes did not occur following infection (data not shown).

To assess the functional cytotoxic activity of liver NPC, ⁵¹Cr release assays were performed by using the Yac-1, P815, and Hepa 1/1A cell lines as targets. NPC from noninfected *SCID* mouse livers exhibited virtually no lysis of the Yac-1 targets, while infected *SCID* mouse livers contained NPC that exhibited high levels of cytotoxicity by 1 week p.i. (Table 1). The release of label was not due to the presence of virus passaged with cells, as Yac-1 cells infected with reovirus did not affect spontaneous release of ⁵¹Cr (data not shown). Noninfected *SCID* mice had no significant splenic cell cytotoxic activity, while splenocytes from infected *SCID* mice at 1 and 2 weeks p.i. displayed an increase in lysis of Yac-1 cells (Table 1).

To determine if reovirus infection stimulated cytotoxic activity toward the P815 targets, liver NPC or spleen cells non-adherent to plastic or nylon wool were tested and found to exhibit lytic activity toward P815 targets only when from infected *SCID* mice (Fig. 3). Spleen cells isolated from noninfected *SCID* mice injected with 100 µg of the gamma interferon inducer poly(I·C) exhibited significant preferential lysis of Yac-1 targets compared with P815 targets (data not shown), as described previously (7).

We examined the capacity of liver NPC to lyse reovirus-

FIG. 3. Four-hour ^{51}Cr release assay with P815 targets and liver NPC effectors from noninfected *SCID* mice and *SCID* mice 4 weeks p.i. After isolation, adherent NPC were removed on a nylon wool column and then assayed for lytic activity toward the P815 targets. NPC from noninfected livers (●) had no lytic activity, while NPC from infected livers (○) exhibited significant lysis of these cells.

infected hepatoma targets in vitro. NPC from noninfected livers exhibited no lysis of noninfected targets, whereas NPC from infected livers had slight activity (2% at an effector-to-target cell [E:T] ratio of 10:1 to 9% at E:T ratio of 80:1). NPC from infected and noninfected livers were equally effective in lysing infected Hepa 1/1A cells (15% at an E:T ratio of 10:1 to 34% at an E:T ratio of 80:1). To determine if cytotoxicity depended on reovirus infection, NPC from infected *SCID* mouse

FIG. 4. Five-hour ^{51}Cr release unlabeled-target inhibition assay. Liver NPC effects were isolated from *SCID* mice 2 weeks p.i. and used at an E:T ratio of 50:1 in all wells. Effector cells were incubated with 2,000 ^{51}Cr -labeled Hepa 1/A1 targets that were noninfected (---) or infected (—) with reovirus 48 h prior to assay. Effector cells were assayed for specific cytotoxic activity against targets in the presence of nonradiolabeled, noninfected (○) or reovirus-infected (●) competitors at various ratios. Lysis of target cells by NPC was assayed by measuring the release of ^{51}Cr after 5 h of incubation as described in Materials and Methods. Infected targets were more sensitive to lysis by NPC than were noninfected targets. Lysis of targets was inhibited by both infected and noninfected competitors in an equivalent fashion.

FIG. 5. Two-color flow cytometric analysis of liver NPC isolated from *SCID* mice at 2 weeks p.i. NPC were stained with rabbit anti-AsGM1 and Texas red-conjugated goat anti-rabbit IgG plus FITC-conjugated anti-Thy-1. Mice were treated with intraperitoneal injections of saline or normal rabbit serum as controls or treated with injections of rabbit anti-AsGM1 antibodies. Treatment with anti-AsGM1 resulted in a decrease in the percentage of AsGM1^{bright}-Thy-1⁺ cells to 2%, compared with 41 to 47% of cells bearing both markers in control mice.

livers were tested for lysis of ^{51}Cr -labeled Hepa 1/1A targets in the presence of various numbers of unlabeled infected or noninfected competing Hepa 1/1A cells. Both competitors could similarly inhibit the lysis of labeled infected targets (Fig. 4). Moreover, addition of unlabeled infected targets did not increase the lysis of labeled, noninfected targets.

Liver NPC were treated with specific antibodies to AsGM1 or Thy-1.2 plus complement to detect which cells mediated cytotoxic activity toward the Yac-1 targets. Treatment of liver NPC with anti-AsGM1 plus complement completely abrogated lytic activity (data not shown). Previous reports suggested that splenic NK cell activity in *SCID* mice is mediated by AsGM1⁺-Thy-1.2⁺ NK cells (7). However, treatment of liver NPC with anti-Thy-1.2 plus complement reduced Yac-1 lysis by >50%, showing that the Thy-1.2⁺-AsGM1^{bright} cells in infected livers could lyse NK cell-sensitive targets.

To distinguish between Yac-1 lysis mediated by macrophages (4) and that mediated by NK cells, nonadherent liver NPC were assayed for activity against Yac-1 targets. Depletion of adherent cells had no effect on lysis of Yac-1 cells (data not shown).

Depletion of NK cells in *SCID* mice by treatment with anti-AsGM1 in vivo markedly inhibited the formation of the AsGM1^{bright}-Thy-1⁺ population compared with control mice (Fig. 5). Assays of NPC cytotoxic activity towards Yac-1 targets revealed that in vivo anti-AsGM1 treatment significantly abrogated the increase in lytic activity normally seen at 2 weeks p.i. (64% versus 21% specific lysis at an E:T ratio of 100:1). However, the histologic appearance of reovirus-infected livers at 2 weeks p.i. was not affected and no differences in reovirus titers in tissue between anti-AsGM1-treated and control mice were found (data not shown).

This report describes the phenotype and function of hepatic and splenic leukocytes in noninfected and reovirus-infected *SCID* mice. Systemic reovirus infection in these animals results in the accumulation of large numbers of NPC cells in the liver between 1 and 2 weeks p.i. Interestingly, reovirus infection stimulates a subset of the AsGM1^{bright} NK cells that express the Thy-1.2 antigen in vivo. Expression of Thy-1.2 on NK cells is associated with NK cells recently derived from bone marrow (10). Since the number and proportion of Thy-1.2⁺ cells increase concomitantly with the increase in NPC yield after reovirus infection in *SCID* mice, the Thy-1⁺ NK cells in infected livers most likely represent cells recruited from the bone marrow or thymus (10).

The ^{51}Cr release assay results indicate that NPC from in-

fectured livers are cytotoxic to both NK cell-sensitive and -resistant cell lines (26). We have shown that the population of NPC which bears the AsGM1 marker possesses cytotoxic activity toward the Yac-1 line, and this cytotoxic activity increases dramatically with reovirus infection of the liver. In addition, we found that cells that coexpress Thy-1.2 and AsGM1 antigens possess cytotoxic activity. These findings strongly suggest that activated NK cells accumulate primarily in the livers of reovirus-infected *SCID* mice.

On the basis of the observation that lysis of Yac-1 targets increases markedly before there is a significant increase in liver NPC yields or in the AsGM1⁺-Thy-1⁺ population, our data support the notion that the NPC in the liver become activated before either recruitment from the spleen or bone marrow or proliferation of resident liver NK-like cells.

The liver NPC in both noninfected and infected mice display an enhanced in vitro lytic capacity towards reovirus-infected targets in assays done in vitro. The phenomenon of selective NK cell lysis of virus-infected cells has been described with other viruses (6, 25) but not previously with reovirus. Our experiments using reovirus-infected and noninfected target cells to compete for cytotoxic activity of liver NPCs provides a model for NK-like activity. It is assumed that nonradiolabeled cells inhibit cytolysis of radiolabeled targets by forming couplets with effector cells. As both infected and noninfected, nonradiolabeled cells inhibit lysis of the radiolabeled infected targets, these data suggest that formation of effector-target couplets is not enhanced by reovirus infection of the target hepatoma cell. Therefore, virus-induced target cell sensitivity to lysis may be governed by mechanisms unrelated to those that regulate couplet formation. Finally, if enhanced lysis of infected targets were due to target cell secretion of soluble mediators which enhanced NK cell activity, then the addition of nonradiolabeled, infected targets to labeled, noninfected targets should increase lysis of the labeled, noninfected cells. This did not occur. Therefore, we favor a model in which couplet formation is independent of virus infection and in which the virus infection does not release a soluble mediator. The prolonged augmentation of liver NK cell number and activity observed over several weeks of infection makes this model of reovirus type 1-induced liver disease unique. Prior studies of murine hepatotropic viral infections in immunocompetent animals, using lymphocytic choriomeningitis virus and mouse hepatitis virus, have demonstrated an early rise in liver NK cell number and activity peaking at day 3 of infection and waning to almost undetectable levels by day 7 (17). *SCID* mice are unable to clear reovirus after enteric infection (9), and this virus persistence may be the stimulus for the ongoing high levels of NK cell activity in the liver and spleen.

Several mechanisms may cause hepatic injury in reovirus-infected *SCID* mice. The first relates to the capacity of reovirus to lyse or damage infected hepatocytes in vivo. This interpretation is consistent with a model of reovirus-induced myocarditis in *SCID* mice in which direct viral injury has been suggested to cause heart damage (21). However, reovirus grows in primary myocyte culture, whereas following reovirus infection, primary cultured hepatocytes from *SCID* mice do not show evidence of cytopathic changes or cellular death (1, 22). It is possible that hepatocytes from *SCID* mice cannot support infection unless they are stimulated by another cell type. Thus, the inflammatory response to infected hepatocytes may be necessary for continued recruitment of hepatocytes that support infection, similar to the effects of hepatotoxin exposures required for reovirus-induced hepatocellular infection (18, 19). The second places the inflammatory response, due to either hepatocellular infection or NPC infection with reovirus, as

pivotal in lethal disease. Here, the inflammatory response, through the release of mediators such as tumor necrosis factor or leukotrienes, would cause hepatocyte damage in infected mice. The third proposes that liver NPC cytotoxic towards virus-infected hepatocytes retard a fatal outcome of infection in *SCID* mice. The ability of liver NPCs to lyse reovirus-infected hepatoma targets in vitro and the close association of the mononuclear cell infiltrate with reovirus antigen-containing hepatocytes support this hypothesis (9). Furthermore, the capacity of NPC from noninfected livers to lyse reovirus-infected hepatoma targets but not noninfected hepatoma or classic NK targets suggested that this cell population has a sentinel role in conventional mice. Studies that used anti-AsGM1 treatment to deplete NK-like NPCs during reovirus infection failed to show any effect on liver histopathology, but this observation may result from the inability to totally eliminate all NK-like cells in vivo. Clarification of the pathogenic versus protective function of the liver NPC in this model may provide insight into mechanisms of hepatic injury in immunocompromised hosts by normally nonpathogenic viruses.

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