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Mechanisms of Lymphocytic Choriomeningitis Virus-Induced Hemopoietic Dysfunction

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Results of this study showed that lymphocytic choriomeningitis virus infection causes a marked activation of natural killer (NK) cells not only in the spleen but also in the bone marrow. This activity reached its peak at about day 3 of infection and declined after days 6 to 7. Enhanced NK cell activity was found to correlate with decreased receptivity for syngeneic stem cells in bone marrow and spleen, with the notable exception that decreased receptivity persisted longer in bone marrow. Treatment of infected recipients with anti-asialo GM_1 (ganglio-*N*-tetraosylceramide) significantly increased the receptivity for syngeneic hemopoietic cells. These findings are consistent with the hypothesis that NK cell activation causes rejection of syngeneic stem cells, thus resulting in hemopoietic depression. To understand the mechanisms behind the prolonged decrease in bone marrow receptivity (and bone marrow function in the intact mouse) mentioned above, we followed the changes in the number of pluripotential stem cells (CFU-S) circulating in the peripheral blood and in endogeneous spleen colonies in irradiated mice, the limbs of which were partially shielded. It was found that following a marked early decline, both parameters increased to normal or supranormal levels at about day 9 after infection. Because the bone marrow pool of CFU-S is only about 20% of normal at this time after infection, a marked tendency for CFU-S at this stage in the infection to migrate from the bone marrow to the spleen is suggested. It seems, therefore, that as NK cell activity declines, the spleen regains the ability to support growth of hemopoietic cells and the bone marrow resumes an elevated export of stem cells to the spleen. This diversion of hemopoiesis could explain both the long-standing deficiencies of the bone marrow compartment and the prolonged decrease in the receptivity of this organ.

Viral infections are believed to be a common cause of transiently disturbed function of hemopoiesis and the immune system (35, 36). Furthermore, relatively trivial infections may trigger severe aplastic crisis in predisposed individuals (15, 27). Previous experience has shown that the murine lymphocytic choriomeningitis virus (LCMV) infection is a fruitful model for studying virus-host interactions including virus-induced dysfunction of immunohemopoiesis (2). Adult mice infected intraperitoneally (i.p.) with this virus normally undergo a relatively mild disease followed by marked immunological and hematological dysfunction. Thus, during the first week of the infection there is profound suppression of pluripotential stem cell (CFU-S), granulocyte-macrophage progenitor (CFU-GM), and prothymocyte compartments (3, 29). Erythropoiesis, as measured by ^{59}Fe uptake into hemopoietic tissues, is also markedly suppressed (3).

After day 10 of infection there is a striking overshoot of CFU-S and erythropoiesis in the spleen (3). In the bone marrow, however, the CFU-S, CFU-GM, and prothymocyte compartments and erythropoiesis remain depressed for over 3 weeks (3, 29). These findings raise two questions. First, what mechanism causes the initial lesion to the immunohemopoietic system? Second, what is the cause of the marked discrepancy between early recovery of hemopoiesis in the spleen and prolonged suppression in the bone marrow compartments? The various mechanisms by which LCMV could affect the immunohemopoietic system have been discussed in detail elsewhere (2), and the hypothesis has been advanced that an interferon-dependent mechanism is involved. In other systems, natural killer (NK) cells have been implicated as regulators of hemopoiesis. NK-like cells mediate rejection of semisyngeneic or allogeneic bone marrow cells (5, 17, 30), and the nonviral interferon-inducer polyinosinic-polycytidilic acid enhances this process (24, 25). Moreover, interferon substantially increases NK cell-mediated cytotoxicity against certain syngeneic primary cells, including cells of hemopoietic origin (10–12, 25, 33).

Our working hypothesis, therefore, was that the interferon response induced by the LCMV infection causes NK cell activation in the hemopoietic tissues, leading to rejection of syngeneic hemopoietic stem cells. However, to make this hypothesis probable it is necessary first to demonstrate that changes in receptivity for hemopoietic stem cells in bone marrow and spleen correlate with hemopoietic depression and recovery in the intact mouse. Second, it must be shown that LCMV infection brings about NK cell activation not only in the spleen (31) but also in the bone marrow and that the changes in receptivity correlate with NK cell activity. Consequently, these points were studied.

MATERIALS AND METHODS

Virus. LCMV of the viscerotropic Traub strain was used throughout the study. The virus preparations employed were tissue culture supernatants obtained after passage of the virus in L cells. LCMV, lethal by intracerebral (i.c.) inocu-

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lation, was titrated by i.c. injection of serial 10-fold dilutions into young adult Swiss mice.

Mice. Essentially, the effect of LCMV infection was studied in locally bred C3H/Sscl mice. In a single experiment, *nu/nu* BALB/c mice were used. These animals were obtained from Bomholtgård, Ltd., Ry, Denmark. Acute infection was induced by i.p. inoculation of mice (age, 2 to 3 months) with 10^3 mean i.c. lethal doses (LD_{50}s) of the virus. Immunized mice were animals infected i.p. as described above 2 to 4 months before use in the study. Persistent carriers were produced by inoculating neonatal mice (≤18 h old) with 10^3 LD_{50}s of the virus.

X irradiation. X irradiation was administered with a Siemens Stabilipan therapy machine operated under the following conditions: 200 kV, 25 mA, 1.0-mm copper filtration. The dose rate was 47 R/min, and the half-value layer was 1.5-mm copper.

Exogenous CFU-S assay. For the study of exogenous spleen colony formation recipient mice were given 900 R, and within 3 h they were injected intravenously (i.v.) with 2.5×10^5 syngeneic bone marrow cells or 0.5 ml of pooled whole blood from either acutely infected mice or matched uninfected controls. A minimum of four donors were used for the preparation of each pool. Four or five recipients of the same sex as the donors were used per pool. At 6 or 7 days after irradiation, the recipients were killed and their spleens were fixed in Bouin solution; spleen colonies were scored with the naked eye by previously accepted criteria (7).

Bone marrow and spleen receptivity assays. Evaluation of the receptivity of bone marrow and spleen was based on the outgrowth of normal hemopoietic stem cells in lethally irradiated mice. Therefore, basically the same approach as described above was used here, except that in this case the variable to be tested was the status of the recipients and not that of the donor cells. Briefly, lethally irradiated (900 R) mice in different phases of the infection, or uninfected mice used as controls, received 2.5×10^5 normal syngeneic bone marrow cells by the i.v. route. Six days later their spleens and sternums were removed and fixed in Bouin solution. Colonies in spleen were scored as described above. The sternums were studied microscopically on longitudinal sections through the mid-zone of the bone. In each section several bone-lined compartments could be examined. If such a compartment contained 500 or more cells it was scored positive; the results are presented as the number of positive compartments over the total number examined in that group.

In a few experiments ^{125}I-labeled 5-iodo-2′-deoxyuridine (^{125}IUdR) was used to measure the engraftment (1). The basic approach was the same as that described above, except that 2.5×10^6 normal bone marrow cells were transferred i.v. to the irradiated recipients. Five days later the mice were given 10^{-7} mol of 5-fluoro-2′-deoxyuridine i.p., followed by an i.p. injection of 0.5 μCi of ^{125}IUdR 1 h later. Twenty hours later the splenic incorporation of ^{125}IUdR was measured. Transplanted mice with ^{125}IUdR incorporation that was not higher than that of untransplanted controls were excluded; this was the case with two mice.

CFU-S migration assay. The extent of CFU-S migration from an area of shielded bone marrow to the spleen was evaluated by techniques described previously (19). Briefly, mice were irradiated with 900 R while a portion of one hind limb was shielded with a lead ring (length, 10 mm; thickness, 1.5 mm). Mice were sacrificed 7 days later, and colonies in spleen were scored as described above, with the exception that only colonies on the back of the spleen were counted. This was done because of the greater variation in the size of colonies in spleen in this assay, combined with the greater ease with which colonies at this location could be evaluated.

TABLE 1. Growth of exogeneous hemopoietic stem cells in spleen and bone marrow of LCMV-infected mice

Recipient mice	No. of colonies in spleen[a]	P^b	Sternum repopulation[c]	P^d
Uninfected	45		8/16	
Infected				
Day 0	0	<0.02	0/14	<0.01
Day 3	0	<0.02	0/9	<0.05
Day 9	≥63	>0.1	0/14	<0.01
Day 12	≥62	<0.05	0/10	<0.05

[a] Values are medians of four to five recipients. In some groups the large number of colonies made counting difficult; this is shown as the symbol ≥.
[b] Statistical significance relative to controls (by the Mann-Whitney rank test).
[c] Number of repopulated bone-lined compartments out of the total number of compartments examined.
[d] Statistical significance relative to controls (by the Fisher exact test).

Cytotoxicity assay. Cytotoxic activity was measured in the ^{51}Cr release assay described previously (28). Pooled cells from at least four mice constituted effector cells. Assays were run for 6 to 8 h, and calculations were carried out as described previously (28). One lytic unit (LU_{50}) is defined as the number of cells required to obtain 50% lysis.

Target cells. Cells were propagated either in monolayers in Eagle minimal essential medium supplemented with 10% heat-inactivated (56°C, 30 min) fetal calf serum, L-glutamine, and antibiotics or in suspension in RPMI 1640 medium, which was supplemented similarly. Monolayer cell lines were L-929, a continuous mouse liver fibroblast line and Vero, a continuous African green monkey kidney cell line. Suspension cultures were EL-4, a mouse lymphoblastoid line; YAC-1, a lymphoma line known to be highly sensitive to NK cell cytotoxicity (18); and P815, a mouse mastocytoma.

Antiserum treatment. Single-cell suspensions of spleen or bone marrow cells were treated in vitro with either monoclonal anti-Thy-1.2 (Becton Dickinson & Co, Paramus, N.J.) or rabbit anti-asialo-GM_1 (Wako Pure Chemical Industries Ltd., Osaka, Japan). Guinea pig serum with low cytotoxicity to mouse lymphocytes was used as a complement source. To deplete NK cells in vivo anti-asialo-GM_1 was diluted 1/10 in sterile saline and given i.v. in a volume of 0.3 ml on days −1 and +2 relative to virus inoculation.

Interferon assay. Samples were titrated in serial threefold dilutions with L-929 cells as the target cells and vesicular stomatitis virus as the challenge virus. Titers are expressed as the highest reciprocal dilution resulting in a 50% reduction in the cytopathic effect.

RESULTS

Receptivity of bone marrow and spleen for syngeneic stem cells during the course of the LCMV infection. Acute LCMV infection is associated with profound depression of stem cell function, lasting about 1 week in the spleen and about 2 to 3 weeks in the bone marrow compartment (3). As this might reflect rejection of the relevant stem cells, we evaluated the ability of bone marrow and spleen to accept exogenous syngeneic stem cells at various times relative to virus inoculation. Groups of mice in different phases of the infection, as well as uninfected controls, were lethally irradiated and reconstituted with normal syngeneic bone marrow cells.

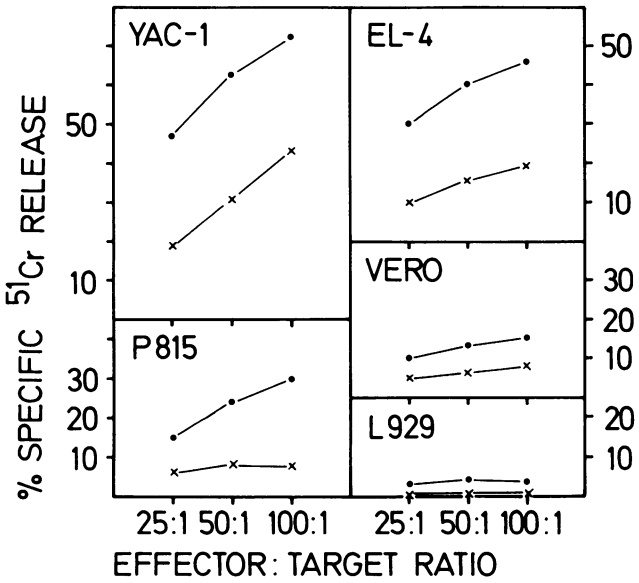

FIG. 1. Target cell specificity of LCMV-induced cytotoxic spleen (●) and bone marrow (×) cells on day 3 of infection.

Six days later, the take of the transplanted bone marrow cells in the bone marrow and spleen was evaluated (Table 1). In agreement with previous findings (5), no colonies could be detected in the spleens of mice infected with LCMV at the time of cell transfer. By 9 days after virus inoculation splenic receptivity had returned to normal, with a tendency toward an overshoot. In the bone marrow a decrease in receptivity also occurred very rapidly. However, in bone marrow the decrease persisted for a longer period, and on day 12 receptivity still was depressed. Results of additional experiments showed that at day 16 bone marrow receptivity was about normal (data not shown). Thus, decreased receptivity of spleen and bone marrow for exogenous syngeneic stem cells correlated with the depressed stem cell function previously demonstrated in the same compartment in the intact animal (3).

NK cell activity in bone marrow and spleen of LCMV-infected mice. NK cells are known to be of importance in rejecting allogeneic and semisyngeneic stem cells (6, 17, 30), and acute LCMV infection is an efficient activator of NK cell activity in the splenic compartment (31, 33). If, however, NK cells are critically involved in the apparent rejection of syngeneic stem cells during acute LCMV infection, activated NK cells should be found also in the bone marrow. A preliminary experiment therefore was carried out to look for NK cell activation in the bone marrow 3 days after virus inoculation, i.e., at the time when peak activity is reached in the spleen (31, 32). We found that bone marrow cells from the LCMV-infected mice killed NK-susceptible YAC-1 lymphoma cells much more efficiently (42% lysis at an effector:target cell ratio of 100:1) than did cells from control mice (11% lysis). Similar findings were made when bone marrow cells from athymic (nude) mice were tested (39% lysis on day 3 of infection compared with 11% lysis with cells from uninfected mice; effector:target cell ratio of 100:1). To establish that the LCMV-activated cytotoxic cells from bone marrow on day 3 were similar to the splenic NK cells on day 3, the cells were compared with regard to target selectivity and cell surface phenotype. The cytotoxic activity of bone marrow and spleen cells on day 3 showed a similar killing

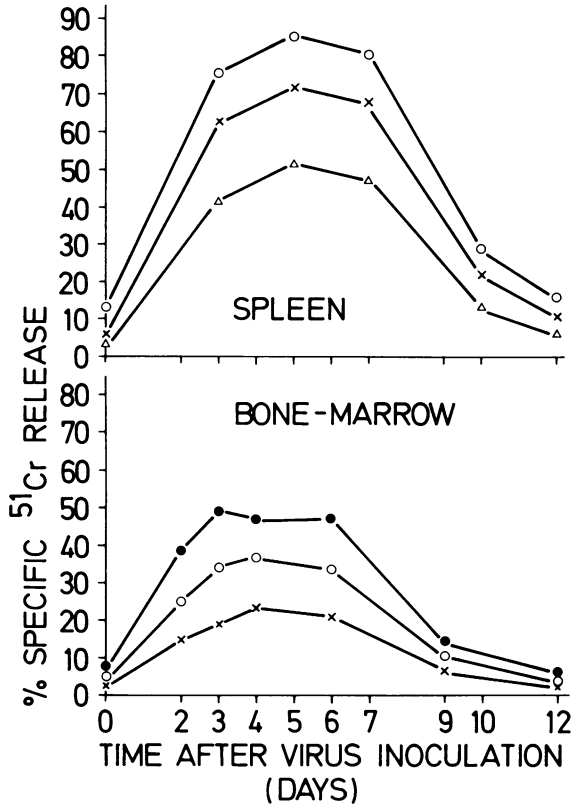

FIG. 2. Time course of NK cell activation in spleen and bone marrow of LCMV-infected mice. Symbols for effector:target ratios on YAC-1 targets are as follows: ●, 100:1; ○, 50:1; ×, 25:1; △, 12:1.

selectivity when compared on a panel of five different target cells (Fig. 1). It should also be noted that the bone marrow cells lysed YAC-1 lymphoma cells considerably more efficiently than they lysed P815 cells, which are known to be particularly sensitive to macrophage-mediated cytotoxicity (8). Pretreatment with anti-asialo-GM_1 antiserum and complement completely eliminated the cytotoxic activity of bone marrow (Table 2), as well as that of spleen cytotoxic cells on day 3 (data not shown). Pretreatment with monoclonal anti-*Thy-1.2* antibody and complement caused a reduction in bone marrow cell cytotoxicity on day 3 that was intermediate between that obtained following treatment of splenic cells on day 3 and LCMV-specific cytotoxic T cells on day 8 (Table 2). Because we have previously described enhanced expression of *Thy-1* antigen on splenic NK cells later in the

TABLE 2. Surface phenotype of LCMV-induced cytotoxic bone marrow cells on day 3 of infection

Expt	Treatment of effectors	% LU_{50} recovered[a]
A	Sham	100
	Complement alone	100
	Anti-asialo-GM_1 + complement	0
B	Sham	100
	Complement alone	91
	Anti-*Thy-1.2* + complement	39[b]

[a] Percentage of LU_{50} recovered compared with that from sham-treated cells; values are medians of two to three experiments.

[b] In the same experiments the median recovery of LU_{50} in spleen cells treated with anti-*Thy-1.2* + complement on day 3 was 65%. LCMV-specific, T-cell-mediated cytotoxicity was completely eliminated.

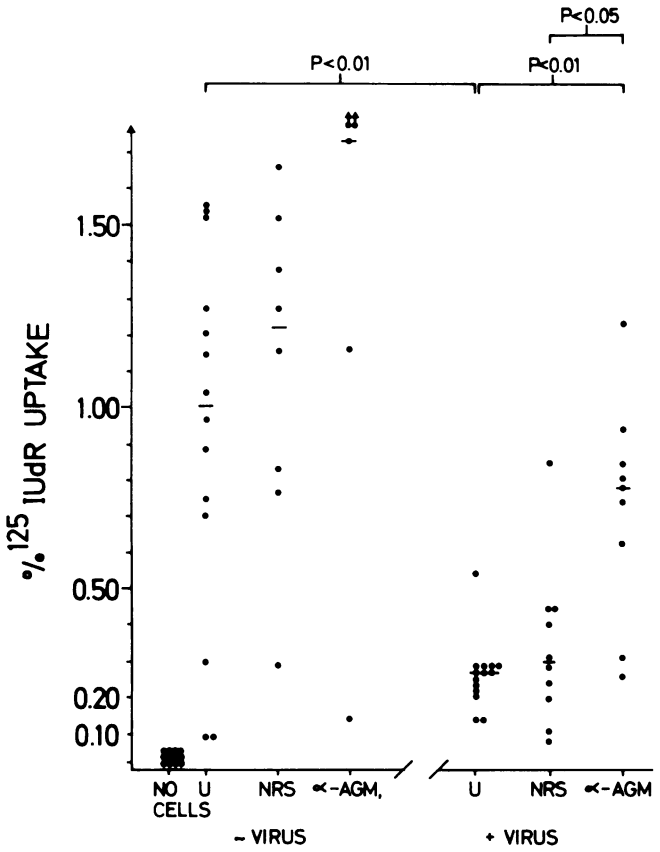

FIG. 3. Effect of NK cell depletion on hemopoietic reconstitution of LCMV-infected recipients. Groups of mice were treated with normal rabbit serum (NRS) or anti-asialo-GM_1 (α-AGM_1) or were left untreated (U). One day later the animals were lethally irradiated and reconstituted with normal bone marrow cells; some of the mice in each group were then infected. Two days later the serum treatment was repeated. Engraftment was assessed 5 days later by measuring splenic retention of ^{125}IUdR. Pooled results from three experiments are presented.

LCMV infection (16), we conclude that the bone marrow cells have the characteristics of activated NK cells on day 3.

Having established the presence of LCMV-activated NK cells in bone marrow, we investigated the time course of their appearance, comparing it with splenic NK cell activity. It was found (Fig. 2) that the kinetics of NK cell activation in bone marrow is virtually identical to that in the spleen, peaking on day 3 to 5 and declining to almost normal at about days 9 to 10. Thus, enhanced NK cell activity correlates with the initial decrease in the receptivity of the hemopoietic tissues, but it does not explain the prolonged depression of bone marrow receptivity.

Effect of in vivo NK cell depletion on receptivity in LCMV-infected recipients. To further investigate the relationship between NK cell activity and receptivity, we evaluated the effect of treatment with anti-asialo-GM_1 antibody on the hemopoietic take in infected recipients. Groups of mice were injected with either anti-asialo-GM_1 antibody or normal rabbit serum or were left untreated. One day later the animals were lethally irradiated and reconstituted with normal bone marrow cells; some of the mice in each group were then infected with LCMV. Two days later serum-treated mice received a second treatment.

Because colony formation takes at least 5 days (7), the short assay period required in these experiments, which was due to the changing status of the recipients, makes the ability to support colony formation an assay that is insensitive to anything but all-or-none differences in receptivity. Therefore, we used ^{125}IUdR uptake as a parameter for graft take in the following experiments, because this assay would be more sensitive to quantitative differences in hemopoietic outgrowth. As was also the case with colony formation, ^{125}IUdR incorporation was strongly inhibited by acute LCMV infection of the recipients (Fig. 3). However, when the infected recipients had been depleted of NK cells through treatment with anti-asialo-GM_1 antibody, a substantially higher ^{125}IUdR incorporation was obtained than in infected controls (untreated or treated with normal rabbit serum) (Fig. 3). Also in uninfected recipients treatment with anti-asialo-GM_1 tended to enhance the take; the effect, however, was not statistically significant ($P > 0.1$). Titration of interferon levels in serum and spleen homogenates obtained from normal rabbit serum-treated and anti-asialo-GM_1-treated mice on day 3 after infection (i.e., at the time of peak activity) showed only minor differences between the two groups (serum, 3^7 versus 3^8; spleen, 3^7 versus 3^6, for normal rabbit serum-treated and anti-asialo-GM_1-treated LCMV-infected recipients, respectively; median values for three mice).

Migratory pattern of CFU-S during the course of LCMV infection. Our results were completely consistent with the hypothesis that NK cells mediate LCMV-induced hemopoietic suppression, except for the prolonged depression in the bone marrow compartment. To find a mechanism that might explain this exception, we studied the traffic of CFU-S during the different phases of infection. Therefore, the effect of LCMV infection on the CFU-S content of the blood was evaluated by the exogenous spleen colony assay (7). The recipients in the assay were preimmunized or persistently infected mice. In these animals injection of normal stem cells gives rise to colony counts similar to those in normal recipients, but notably, contamination with LCMV does not affect colony formation (4). We found (Table 3) a decrease in the number of circulating CFU-S early (days 4 to 6) in infection. This was followed by an increase to normal or supranormal levels at about day 9 of infection, i.e., at the time when the number of CFU-S in the spleen begins to increase, although it is still low in the bone marrow compartment (3).

One explanation for this finding is that the production of CFU-S in bone marrow remains low at day 9 and has been taken over by the spleen. Alternatively, the CFU-S are produced at normal or supranormal levels in bone marrow but migrate to the spleen as soon as they are produced. To distinguish between these two possibilities, we investigated

TABLE 3. Number of CFU-S circulating in the blood of LCMV-infected mice

Donor mice	No. of colonies/0.5 ml of blood	P^a
Uninfected	7 (8)[b]	
Infected		
Days 4 to 6	0 (4)	<0.02
Days 9 to 12	14 (11)	<0.01
Days 14 to 16	1 (4)	<0.01

[a] Statistical significance relative to that of preceding group (by the Mann-Whitney rank test).

[b] Median number of colonies found. Values in parentheses represent number of pools tested.

the export of bone marrow-derived CFU-S to the spleen during the different phases of infection by use of the CFU-S migration assay (19). In this assay we evaluated colony formation in spleen in irradiated mice, one limb of which was partially shielded to allow production of endogeneous CFU-S. The results (Fig. 4) show that mice irradiated 5 days after infection had virtually no endogeneous colonies in spleen. In contrast, mice irradiated early in the recovery phase had an increased number of endogeneous colonies in spleen. Thus, this shows that there is no defect in the ability of the bone marrow to produce stem cells early in the recovery phase. However, the CFU-S appear to migrate to the spleen as soon as they are produced, instead of repopulating the bone marrow itself, an interpretation that is also consistent with the poor receptivity of the bone marrow for exogenous CFU-S in this phase of infection.

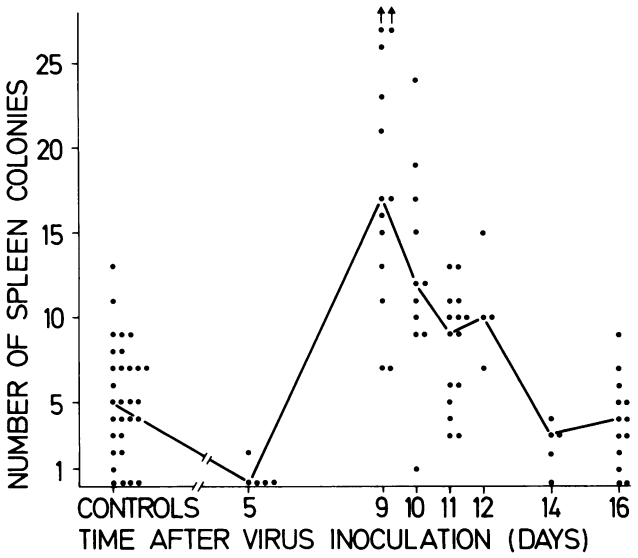

FIG. 4. Endogenous colony formation in spleens in partially limb-shielded mice irradiated (900 R) at various times after LCMV infection. Each point represents one mouse. Lines are drawn between the median numbers for each group.

DISCUSSION

In this study we used a murine model for studying various aspects of virus-induced hemopoietic dysfunction. Based on certain observations (6, 10, 11, 17, 24, 25, 30) we suggested that NK cell activation might be important in the virus-induced suppression of hemopoiesis. In agreement with the hypothesis we found a strong activation of cytotoxic cells in bone marrow. The effector cells do not seem to be mature T cells, because nude mice gave a marked cytotoxic response, and their target specificities were different from those reported for activated macrophages (8, 26). Characterization of the surface phenotype indicated that the bone marrow NK cells are not only asialo-GM_1^+ but also express moderate levels of *Thy-1*. According to recent findings (13) this classifies the cells as relatively immature NK cells and indicates that in situ activation of precursors constitutes the predominant basis for the enhanced cytotoxic activity.

With regard to the association between increased NK cell activity and hemopoietic depression, the following observations have been made. First, with the exception of a more long-lasting bone marrow defect (including low CFU-S content [3] and reduced receptivity), hemopoietic dysfunction was found to correlate with enhanced NK cell activity. In fact, looking at the CFU-S pool as a whole, one finds that recovery coincides with the decline in NK cell cytotoxicity. Although at first it is only from the spleen compartment that an increased number can be isolated (3), the results of this study reveal that CFU-S production, to a large extent, takes place in the bone marrow. Second, both virus-induced NK cell activation (31) and hemopoietic dysfunction (4) are expressed in heavily irradiated animals. Third, treatment of infected recipients with antibody to asialo-GM_1 almost restores normal receptivity. Because this antibody appears to deplete selectively mice of NK cells in vivo (14), we favor the interpretation that the effect produced by this treatment is a result of NK cell depletion. Taken together, the available data therefore strongly suggests that NK cells play an important role in the LCMV-induced hemopoietic dysfunction. Other mechanisms may be involved as well, however, because treatment with anti-asialo-GM_1 only partly restored the ability of infected animals to accept bone marrow. The direct effect of LCMV-induced interferon on bone marrow precursors thus needs to be evaluated further.

In the experiments described here we used an adaptive transfer model which, although it appears to reflect nicely the situation in the intact animal, may be too simple. Hemopoietic depression during acute LCMV infection in the intact animal thus may be a more complex event than realized from these studies. On the other hand, however, this assay may be very relevant in relation to the question of whether NK cells are involved in rejection of a foreign bone marrow graft. NK cells have been implicated as a possible mechanism behind bone marrow graft rejection and graft versus host disease in humans (9, 20), and infection with virus such as cytomegalovirus is a known risk factor for initiating graft versus host disease and graft rejection (23). Earlier results have demonstrated that NK cells probably are of major importance in rejecting allogeneic and semisyngeneic bone marrow grafts (6, 17, 30). Thus, a similar type of NK cell-related mechanism also may be active in rejection of syngeneic bone marrow grafts, as shown here after virus infection.

Whereas NK cell activation may explain the initial lesion to the hemopoietic tissues, the mechanism behind the long-lasting depression of several bone marrow functions, as well as receptivity, must be sought elsewhere. In this connection it is important to realize that the present data show that the depression of bone marrow function is not as uniform as previously believed (2). Our results indicate that there is increased migration of CFU-S from bone marrow to spleen early in the recovery phase. This observation, together with the picture of a hyperactive spleen in the mice at this time of the infection, leads us to believe that the relative inactivity of the bone marrow reflects a diversion of hemopoiesis rather than a direct virus-induced lesion. This view is supported by the fact that very similar findings have been made in polyinosinic-polycytidilic acid-treated mice (21).

If the CFU-S migrate to the spleen as soon as they are produced, instead of repopulating the bone marrow itself, the reconstitution of the bone marrow would be delayed. According to this interpretation, the prolonged depression of other bone marrow compartments (CFU-GM, prothymocyte, and erythropoiesis) (3, 29) would be secondary to a preferential increase in splenic hemopoiesis. Because stem cells in the spleen differentiate primarily in the erythroid direction (34), this would mean that there is rapid recovery of erythropoiesis at the expense of other immunohemopoietic precursor cell functions which are predominantly directly bone marrow dependent (22).

In conclusion we suggest that NK cells play an important role in the pronounced virus-associated suppression of hemopoiesis but that secondary regulatory phenomena may contribute to further immunohemopoietic disorder via prolonged depletion of certain compartments in the bone marrow.

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