MYELOID AND LYMPHOID DISORDERS occur in many acute human and animal virus infections. Conspicuous changes are seen in the numbers of circulating leukocytes, and in recent years it has become clear that the host’s defense and immunologic responsiveness are markedly affected by several viruses. Obviously, these virus-induced immunohemopoietic disorders may have significant pathogenetic and clinical implications; the latter have prompted further investigation of the phenomena in different virus infections in man and in experimental animals. Lymphocytic choriomeningitis (LCM) virus infection in mice has provided one appropriate model system for the study of these phenomena. Intraperitoneal (i.p.) inoculation of adult mice with LCM virus produces a transient viremic infection, which is associated with no overt clinical signs, but which nevertheless profoundly affects immunohemopoietic functions. Thus the postirradiation recovery of hemopoiesis in LCM virus-infected animals is severely impaired; the host’s defense against unrelated viruses and against transplanted tumors is decreased; and the infection is associated with prolonged depression of humoral and cell-mediated immune responsiveness. In the LCM virus-infected mouse, we have observed that the postirradiation formation of hemopoietic spleen colonies from endogenous or exogenous stem cells (CFU) is completely suppressed. This rather striking
observation has led us to assume that immunohemopoietic disorders induced by this virus might be attributable to a profound inhibition of the myeloid and lymphoid precursor cells, rather than to any direct cytotoxic action on the mature cells.

The aim of the present work was to throw further light on a number of questions related to the LCM virus-induced inhibition of the pluripotential CFU. First, it was important to decide whether this inhibition also occurred in non-irradiated infected mice, and to investigate possible changes in the CFU compartment during the course of the infection. Second, the in vitro colony-forming cell (CFC), which is considered to be a precursor of granulocytes and macrophages, and the function of cells committed for erythropoiesis were examined. Third, the activity of humoral hemopoietic stimulators (colony-stimulating factor (CSF) and erythropoietin) in the infected animals was explored. Finally, in the light of recent reports describing inhibitory effects of interferon preparations and interferon inducers on CFU and CFC, we decided also to reinvestigate the question of interferon production in LCM virus-infected mice.

MATERIALS AND METHODS

Virus. The LCM virus employed was the Traub strain, and the stock virus used was culture fluid obtained after passage of the virus in L cells. The stock virus was examined for possible contamination by Dr. M. J. Collins, Jr., Microbiological Associates, Bethesda, Md. Except for LCM virus, none of the 11 murine viral contaminants described by Collins and Parker was found, nor ectromelia or mouse cytomegalovirus. LCM virus, lethal by intracranial inoculation, was titrated by i.e. injections of serial dilutions in groups of white Swiss mice.

Mice. The experimental animals were strictly inbred C3H/Svel mice obtained from Statens Seruminstutit, Copenhagen, Denmark. Acute LCM virus infection was induced by i.p. inoculation of mice age 2-3 mo (18-23 g) with 10^5 mean i.e. lethal doses (LD50) of the virus. Mice with persistent LCM virus infection or mice that had previously been immunized to the virus were used as recipients of CFU. Persistent virus infection was produced by inoculating neonatal mice with 10^6 LD50 of the virus within the first 18 hr of life. This treatment resulted in a state of immunologic tolerance to the LCM virus, and, throughout life, the mice carried infectious virus in their blood and organs in titers much higher than ever seen during the acute course of the infection. The immunized recipients were mothers of the infected babies. They had a transient infection, and were highly immune when their babies, at the age of 4 wk, were taken from them.

Preparation and histologic examination of cells. Cell suspensions were prepared by passing spleens through stainless steel meshes, or by dispersing femoral bone marrows with the successive use of 18- and 25-gauge needles. Following this procedure, washing and adjusting of the cells were carried out with Hank’s balanced salt solution. Total nucleated cell counting was done with a hemocytometer, and differential counts were obtained by scoring of at least 200 cells in smears stained with May-Grünwald-Giemsa.

CFU. For the assay of CFU, recipients were given 850 R of X-rays, and within the subsequent 2 hr they were injected intravenously (i.v.) with 1.5 x 10^5 bone marrow cells, 2-2.5 x 10^6 spleen cells, or 0.5 ml of whole blood from infected mice or normal controls of the same sex. Since hemopoietic colony formation was completely suppressed in normal recipients of LCM virus-contaminated cells, such mice could not be used for the assay. Preimmunized or persistently infected mice were therefore used as recipients. In these animals, injections of normal bone marrow cells gave rise to spleen colony counts that were similar to those produced in normal recipients, but significantly, colony formation was not affected by contaminating LCM virus. Recipient spleens were removed and fixed in Bouin’s solution on day 7 after irradiation. Spleen colonies were scored by the naked eye and by histologic study of a longitudinal midsection through the hilus. Although the latter gave lower figures, the two methods produced parallel results, and only the counts per midsection obtained by microscopy were therefore reported.
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CFC and CSF. CFC were estimated by an in vitro two-layer culture method.18 10^5 bone marrow cells or 10^5 spleen cells were cultured in an upper layer with McCoy's 5A medium (Gibco), 0.3% agar, and 10% human serum, on top of a leukocyte feeder layer containing 10^6 normal human leukocytes in medium with 0.5% agar and 15% human serum. Each layer contained 1 ml, and 35-mm petri dishes were used. Triplicate or quadruplicate cultures were made in all experiments. The petri dishes were placed in a humidified incubator at 37°C constantly flushed with 7.5% CO_2 in air. After 7 days of incubation, the number of colonies (> 50 cells) was counted, using a dissecting microscope.

The level of serum CSF was studied by using a single-layer assay with no feeder layer. Serum, 10 μl, was added to triplicate cultures containing 10^5 bone marrow cells from C57Bl mice in medium with 0.3% agar and 10% human serum. The number of colonies was scored after 7 days of incubation.

Erythropoietic activity. For the measurement of ^59Fe uptake, amounts of 1.0 μCi ^59Fe (as ferric citrate, sp act about 10 μCi/μg Fe, Amersham, England) were injected iv. After 24 hr, 100-μl samples of blood were collected by cardiac puncture, and the spleen and the two intact femurs of each mouse were removed. The radioactivity of these samples was determined using a well-type crystal gamma scintillation counter. The percentages of injected iron present in peripheral blood, spleen, and femurs were calculated, assuming the total blood volume to be 5% of the individual body weight.

For hypertransfusion heparinized blood was collected from C3H donors, and the red cells were washed three times. The packed red cells were injected i.p. in amounts of 0.5 ml on days -8, -7, -6, and -4 relative to the ^59Fe injection. The resulting packed cell volumes (PCVs), as determined on day +1, were in the neighborhood of 0.70 (a few mice with PCVs of 0.60 or lower were discarded). Erythropoietin, when administered, was given subcutaneously in doses of 1 unit (Step I Erythropoietin, Connaught Laboratories, Canada) on days -3, -2, -1, and 0.

Interferon assay. Interferon activity was determined by a plaque inhibition assay that was performed largely as described by Wagner et al.19 Serum or 20% suspensions of homogenized spleens were acidified to pH 2 by overnight dialysis against saline adjusted with HCl; after redialysis to neutrality, bovine serum albumin was added to make a final concentration of 0.5%, and the samples were stored at −70°C. Two-milliliter aliquots of serial dilutions were added to duplicate monolayers of L cells grown in Eagle's minimum essential medium in 50-mm petri dishes. After incubation for 4 hr at 37°C, the drained monolayers were first infected by vesicular stomatitis virus (VSV), adjusted to produce about 50 plaques, and then covered with agar medium. Plaque numbers were recorded 2 days later after being stained with neutral red. The reciprocal of the dilution at which the plaque number was reduced to 50% of the control plaque count was considered to be the titer of interferon.

RESULTS

Cytological changes. In peripheral blood (Fig. 1) there was some decrease of the PCV during the LCM virus infection, with minimum figures near 80% of the normal values appearing on day 10 post infection (p.i.). Depression of the leukocytes was more rapid and was most distinct in the case of the lymphocytes, which reached about 30% of the control values on days 4–7 p.i. After this initial depression, there was a transitory rise in circulating leukocytes, and, later, a gradual increase from subnormal to normal levels.

In bone marrow, there was a marked reduction in the number of nucleated red cells and lymphocytes (Table 1). Granulocytic cell counts showed less of a decrease with an intermediate rise of mature granulocytes on day 11 p.i.

Changes in CFU. The CFU contents in bone marrow and spleen were studied at different stages of the acute LCM virus infection. Figure 2 shows that similar results were obtained using preimmunized and persistently infected recipients. In the bone marrow there was, shortly after the virus inoculation, a fall in the CFU content to values near 10% of the normal level, and, still by
Fig. 1. Changes in PCV, lymphocytes, and granulocytes in peripheral blood during acute LCM virus infection. Means ± SD from separate groups of ten mice. Mean values of uninfected controls: PCV, 0.48; lymphocytes, 4800; granulocytes 1300/μL.

Table 1. Changes in Femoral Bone Marrow after LCM Virus Infection*

<table>
<thead>
<tr>
<th></th>
<th>Nucleated Red Cells</th>
<th>Immature Granulocytes</th>
<th>Mature Granulocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>No LCM</td>
<td>119</td>
<td>119</td>
<td>176</td>
</tr>
<tr>
<td>Day 6 p.i.</td>
<td>4</td>
<td>12</td>
<td>131</td>
</tr>
<tr>
<td>Day 11 p.i.</td>
<td>15</td>
<td>48</td>
<td>130</td>
</tr>
<tr>
<td>Day 18 p.i.</td>
<td>171</td>
<td>106</td>
<td>130</td>
</tr>
</tbody>
</table>

*Femoral shaft counts (×10⁴) obtained from pools prepared from groups of four mice.
†Myeloblasts, promyelocytes, and myelocytes.

Fig. 2. Changes in content of CFU in bone marrow and spleen during acute LCM virus infection. CFU were determined in pools of femoral bone marrows (circles) and spleens (triangles) obtained from groups of four mice on various days after virus inoculation. Separate experiments were performed. Closed points indicate mean CFU numbers as determined in four to five preimmunized recipients, and open points indicate mean CFU numbers as determined in six persistently infected recipients. SD calculated from compiled data from same day p.i. Mean values of uninfected control donors: 880 CFU per femoral bone marrow and 640 CFU per spleen.
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Changes in CFC and in CSF activity. The content of CFC in femoral bone marrow was measured in groups of mice that had been infected with the LCM virus on various days before the assay. Figure 3 shows that the number of CFC in bone marrow changed almost parallel with that of CFU. After day 1 p.i., there was a rapid decrease of the CFC, and low levels of about 10% of the normal values were maintained at least until day 18 p.i. The CFC assay of spleen cells revealed about 400 CFC per spleen in normal mice, and this small number had not changed significantly in mice examined on day 6 and 12 p.i.

For the study of CSF activity, 10 μl of serum, collected from normal controls and groups of mice that had previously been infected with the LCM virus on various days, were added to the test cultures of normal bone marrow cells. The number of in vitro colonies that developed are recorded in Fig. 3. While serum from uninfected mice and serum obtained on day 1 p.i. had no detectable CSF activity, that obtained on days 3 and 6 p.i. showed a pronounced activity that was still present on day 12 p.i. Repetition of the entire experiment gave parallel results; only the colony counts were generally lower.

Changes in erythropoietic activity and in responsiveness to hypertransfusion and erythropoietin treatment. For measurement of erythropoietic activity, ^59^Fe was injected into groups of mice that had previously been inoculated with LCM virus on different days. From Fig. 4A it can be seen that the ^59^Fe incorporation in blood decreased rapidly after day 1 p.i. From day 3 to day 9, the up-takes were between 6% and 11% of the normal values. Thereafter, an increase occurred, and the incorporation in blood on day 14 p.i. reached almost 200% of that in the controls. Deposition of ^59^Fe in spleen and bone marrow (Fig. 4B) also diminished after day 1 p.i., although the declines were less pronounced. After day 9 p.i., however, there was a marked discrepancy between the findings...
in the two types of tissue. While splenic uptakes increased to about 400% of the control values, the uptakes in the bone marrow still remained subnormal for a considerable time. The $^{59}$Fe incorporation in mice with persistent LCM virus infection (not shown) was found to be virtually the same as that in uninfected controls.

Further investigation of erythropoiesis was carried out in groups of mice infected with LCM virus either 4 or 14 days before the $^{59}$Fe injection, i.e., in animals which showed either maximum suppression or maximum enhancement of $^{59}$Fe uptake in blood in the experiment mentioned above. The animals were pretreated with repeated injections of packed red cells, or with hypertransfusion plus exogenous erythropoietin as described in Materials and Methods. In addition, the experiment comprised corresponding uninfected control groups, as well as infected but untreated mice. Table 2 indicates that the injections of erythropoietin were completely unable to restore the suppressed $^{59}$Fe uptake in blood, spleen, and bone marrow of those mice that had been infected with LCM virus either 4 or 14 days prior to the $^{59}$Fe injection.

Table 2. Effect of Hypertransfusion and Erythropoietin Treatment on 24-hr $^{59}$Fe Uptakes in Mice Infected With 1.0 $\mu$Ci $^{59}$Fe 4 or 14 Days After LCM Virus Inoculation

<table>
<thead>
<tr>
<th>$^{59}$Fe Injection Relative to Virus Inoculation</th>
<th>Mean $^{59}$Fe Uptake ± SD (per cent of uptakes in uninfected, untreated controls)</th>
<th>Blood</th>
<th>Bone Marrow</th>
<th>Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Control HT + HT + EP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No LCM</td>
<td>100 ± 10 2.1 ± 1.2 98 ± 38</td>
<td>100 ± 8 21 ± 4 57 ± 16 100 ± 23 24 ± 3 363 ± 44</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 4 p.i.</td>
<td>6.1 ± 2.8 1.3 ± 0.6 2.2 ± 1.1</td>
<td>40 ± 19 19 ± 2 24 ± 3 56 ± 28 38 ± 4 61 ± 16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 14 p.i.</td>
<td>167 ± 31 1.7 ± 0.7 79 ± 54</td>
<td>60 ± 17 20 ± 4 33 ± 11 350 ± 59 30 ± 7 333 ± 141</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Minimum five mice per group.

*Mean uptake in controls: 22.9% in blood, 1.6% in two femurs, and 1.6% in spleen.
	HT, hypertransfusion—0.5 ml packed red cells days 8, 7, 6, and 4 before $^{59}$Fe injection.
	EP, erythropoietin treatment—1 unit days 3, 2, 1, and 0 before $^{59}$Fe injection.
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Fig. 5. Interferon activity in serum (circles) and spleen extracts (triangles) during acute LCM virus infection. Pools were obtained from groups of five to eight mice on various days after virus inoculation. Closed and open points indicate interferon titers found in separate experiments.

Interferon production in LCM virus infection. The interfering activity of serum and of splenic extracts obtained from mice representing various stages of acute LCM virus infection was examined. Figure 5 shows that appreciable titers of interferon appeared shortly after virus inoculation, and that the activity persisted for the first week of infection. In the later stages of the acute infection (and in persistently infected mice), no measurable activity was found.

All the samples assayed for interferon had been temporarily acidified to pH 2.0 as described in Materials and Methods. The results of further attempts to characterize the interfering factor in the serum of the acutely infected mice are recorded in Table 3. Like interferon, the factor was nonsedimentable at

| Table 3. Characterization of the Interfering Factor in Serum of Mice with Acute LCM Virus Infection |
|-------------------------------------------------|---------------------------------|-----------------|----------------|
| Serum Preparation* | Indicator Virus | Cell Culture | Interfering Activity |
| LCM serum† | VSV | L cells | $2^{7.9}$ |
| LCM serum centrifuged (105,000 g for 2 hr) | VSV | L cells | $2^{8.3}$ |
| LCM serum | EMCV | L cells | $2^{7.9}$ |
| LCM serum | VSV | Chick embryo fibroblasts | $2^{5.7}$ |
| Normal serum + LCM virus§ | VSV | L cells | No trace |
| LCM serum from X-irradiated mice§§ | VSV | L cells | $2^{6.2}$ |

*Sera treated as described under Materials and Methods including exposure to pH 2.
†Serum pool from 14 mice inoculated with LCM virus 40 hr previously.
§$10^3$ or $10^6$ LD₅₀ of LCM virus were added to each monolayer of L cells.
§§Serum pool from six mice pretreated with 850 R of X-rays and then inoculated with LCM virus 40 hr previously.
105,000 g for 2 hr. It suppressed encephalomyocarditis virus (EMCV), as well as VSV, and showed very slight activity in chick embryo fibroblasts. Moreover, the artificial addition of LCM virus to the interferon assay system did not interfere with the numbers of VSV plaques obtained. As the LCM virus-induced suppression of hemopoiesis was repeatedly demonstrated in x-irradiated mice, we also examined the radiosensitivity of interferon production by this virus. It can be seen that the pretreatment of mice with 850 R of x-rays before the inoculation of LCM virus did not interfere with the amount of interferon produced.

DISCUSSION

Previous investigations have demonstrated complete and fatal suppression of postirradiation hemopoietic colony formation in mice with acute LCM virus infection. The present studies on the incorporation and of the compartments of CFU and CFC indicate that profound hemopoietic disorders exist even in nonirradiated LCM virus-infected mice presenting no overt symptoms. Obviously, the suppressive mechanism(s) starts to function very soon after virus inoculation and is operating for at least the first week of the infection. As the CFU compartment is normally a self-maintaining population, the CFU must constitute a direct target of this suppressive action. Indeed, it can be imagined that the CFU population may be the only immediate target, and that changes in erythropoiesis and CFC are secondary phenomena consequent to the suppression of the pluripotent stem cells. This assumption, however, is difficult to reconcile with the very prompt and steep decrease in erythropoiesis and CFC, and the evidence that committed progenitor cells of at least the erythroid series have also a certain measure of self-generating capacity. Therefore it seems more likely that the early suppression directly affects CFU as well as committed progenitors of erythrocytes and granulocytes-macrophages.

The first evidence of recovery from suppression was apparent about day 10 p.i., and led to a significant overshoot above normal of total erythropoiesis, splenic incorporation, and splenic CFU. In the bone marrow, however, incorporation remained low for an additional period of time. A preferential increase of incorporation and CFU in spleen, rather than in bone marrow, has been described in conditions of increased demand for erythropoiesis. Moreover, an erythropoietic demand was reported to direct CFU into erythropoiesis at the expense of CFC. It was therefore tempting to imagine that there was a predominance of the erythropoietic demand in the recovering animals around days 10–18 p.i., and that this might contribute to the further delay in the restoration of the bone marrow. Alternatively, the preferential increase of splenic erythropoiesis and CFU might be due to an endotoxin effect from a possible intestinal invasion by bacteria or bacterial products occurring during the course of the viral infection. The elevated erythropoiesis at the recovery stage of the acute infection was readily abrogated by hypertransfusion. In fact, this treatment seemed to prolong the virus-induced erythropoietic hyporeactivity, as indicated by the subnormal rather than supranormal responsiveness to erythropoietin on day 14 after infection. The hypertransfusion data therefore
indicated that the elevated erythropoiesis after LCM virus infection was clearly distinct from that caused by the polycythemia-inducing Friend virus, which was not affected by hypertransfusion plethora.27

The mechanism(s) by which the LCM virus exerted its early comprehensive suppression in the acutely infected mice could involve a killing off of the target cells and/or some inhibition of their proliferation. The noncytopathic nature of the LCM virus,28 as well as the fact that the entire process of hemopoietic colony formation is unimpaired in the heavily infected persistent LCM virus carriers,10 make it extremely improbable that the virus by itself directly destroys (or affects) the hemopoietic cells. Moreover, a mere killing off of the CFU would hardly account for the completeness of the suppression. Thus the extent of the decrease in CFU (Fig. 2) would not alone explain the observation that previous studies8,10 never revealed the development of one single spleen colony within any mouse during the first week of the infection. It seems most probable, therefore, that some inhibition of hemopoietic cell proliferation occurs during this period. Such inhibition could readily explain the complete annulment of observable hemopoietic colony formation in the infected mice. Possibly, it could account also for at least some of the reduction in the contents of hemopoietic precursor cells. Thus there is evidence of a considerable turnover rate within the different compartments of hemopoietic precursors,20,29 implicating an appreciable daily removal of such cells by differentiation or other loss. If this removal continues even in nonproliferating conditions,20 a steep depletion could be expected.

The proliferation of hemopoietic precursor cells is to a pronounced degree directed by various humoral stimuli,30 and it is conceivable that such stimuli could be upset as a result of the viral infection. CSF and erythropoietin are powerful stimulators of CFC and of erythroid committed precursor cells. The present demonstration of increased levels of CSF activity in the infected mice, and of the failure of erythropoietin to restore their erythropoiesis, however, precludes the possibility that virus-induced decreases of these two promoters could play a role in the hemopoietic disorders. The idea that the changes might be attributable to a stress-induced hypercorticism is contradicted by recent studies on the function of CFU in ACTH-treated mice.31 The effects of the ACTH-induced hypercorticism bear no resemblance to the changes seen in the LCM virus-infected mice.

Among other humoral regulators, we focused particularly on the possible role of interferon. Preparations of interferon have been reported to depress the growth of hemopoietic cells grafted into irradiated animals,12 and also to act as CSF antagonists.13 Furthermore, experiments with nonviral interferon inducers caused hemopoietic changes that were very similar to the present findings. Thus repeated injections of polyriboinosinic–polyribocytidylic acid in mice produced transient leukocytopenia and reticulocytopenia, transient decrease of splenic CFU, lasting decrease of bone marrow CFU, and elevated CFU in blood, suggestive of increased traffic from the bone marrow towards the spleen.14 Most of the search for interferon induction by LCM virus has been in persistently infected mice and has given negative results (cf. Ref. 28). In two previous reports,12,33 the search for interferon in the acute LCM virus infection
was also negative, but very few experimental details were given. However, in a more recent study, there was a description of interferon production during the course of acute infection with the Molomut-Padnos (M-P) virus, which has now been identified as a LCM virus strain. In the present work, interferon kinetics were similar to those observed in the M-P virus study. Moreover, there was a striking correspondence between the temporal presence of interferon and the early, transient, comprehensive hemopoietic suppression. The ensuing assumption that interferon could be the relevant suppressive mediator searched for was supported by the concordant absence of interferon and of any suppressive activity in the heavily infected persistent carriers of the LCM virus.

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REFERENCES

18. Robinson WA, Pike BL: Colony growth of human bone marrow cells in vitro, in Stohl...
HEMOPOIESIS CHANGES WITH LCM


32. Wagner RR, Snyder RM: Viral interference induced in mice by acute or persistent infection with the virus of lymphocytic choriomeningitis. Nature 196:393-394, 1962


Changes in hemopoiesis during the course of acute LCM virus infection in mice

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