Distinct Host-Dependent Pathogenic Mechanisms Leading to a Similar Clinical Anemia After Infection with Lymphocytic Choriomeningitis Virus

MOHAMMED EL-AZAMI-EL-IDRISSI,*,[2] STÉPHANIE FRANQUIN,*,[3] MICHAEL J. DAY,‡ GRAZIELLA MAZZA,‡ CHRISTOPHER J. ELSON,‡ VÉRONIQUE PRÉAT,† CHARLES J. PFAU,§ AND JEAN-PAUL COUTELIER*,[1]

Unit of Experimental Medicine, Institute for Cellular and Molecular Pathology, and †Unit of Pharmaceutical Technology, Université Catholique de Louvain, 1200 Bruxelles, Belgium; ‡Department of Pathology & Microbiology, University of Bristol, Bristol BS18 7DU, United Kingdom; and §Department of Biology, Rensselaer Polytechnic Institute, Troy, NY 12181

The Docile strain of lymphocytic choriomeningitis virus (LCMV) induces anemia in a number of inbred strains of mice, including C3HeB/FeJ and CBA/Ht animals. A difference in the kinetics of anemia and in compensatory reticulocytosis suggested that impaired erythropoiesis was the major pathogenic mechanism involved in CBA/Ht mice, but not in C3HeB/FeJ mice. In both mouse strains an antierythrocyte autoantibody production that depended on the presence of functional CD4+ T lymphocytes was observed. Although depletion of T helper lymphocytes prevented anemia in C3HeB/FeJ mice, this treatment largely failed to inhibit the development of the disease in CBA/Ht animals. This observation indicated that the antierythrocyte autoimmune response induced by the infection was at least partly responsible for the anemia of C3HeB/FeJ mice, but not of CBA/Ht mice. Erythrophagocytosis was enhanced in both mouse strains after LCMV infection, but did not appear to be a major cause of anemia. These data clearly indicate that similar disease profiles induced by the same virus in two different host strains can be the result of distinctly different mechanisms. Exp Biol Med 230:865–871, 2005

Key words: anemia; lymphocytic choriomeningitis virus; antierythrocyte autoantibody; phagocytosis; reticulocytosis

Introduction

A wide range of pathologies, including choriomeningitis, hepatitis, generalized immunosuppression, and endocrine alterations, may follow infection of mice with lymphocytic choriomeningitis virus (LCMV) (reviewed in Ref. 1). The development of anemia has also been reported after infection with the virus (2–4), but different mechanisms may be involved in the pathogenesis of this disease. On the one hand, hematopoietic dysfunction occurs early after infection of different inbred mice and could involve natural killer (NK) cells or production of cytokines, including interferons (4–7). As a consequence, infection with LCMV of the WE strain leads, in immunocompetent mice, to mild aplastic anemia that resolves quickly after infection (4). In perforin-deficient mice chronically infected with the same WE strain of LCMV, a lethal pancytopenia with aplastic anemia develops through tumor necrosis factor and gamma-interferon secretion by CD8+ T cells (4). In addition, late onset of anemia in immunocompetent C3HeB/FeJ mice infected with the Docile strain of LCMV correlates with an autoimmune response (2, 3, 8) characterized by the production of antierythrocyte autoantibodies (9, 10).

To further analyze the pathogenesis of this anemia, the consequence of infection with the Docile strain of LCMV was compared in C3HeB/FeJ animals and in other mouse strains. Our results indicated that whereas the virus may trigger anemia in several mouse strains, an autoimmune etiology of the disease appears dominant only in C3HeB/FeJ

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[1] To whom correspondence should be addressed at Unit of Experimental Medicine, UCL-MEXP 7430, Avenue Hippocrate 74, B-1200 Bruxelles, Belgium. E-mail: coutelier@mexp.ucl.ac.be
[2] Current address: Pfizer Maroc, Casablanca, Morocco.
[3] Current address: GlaxoSmithKline Biologicals, Rixensart, Belgium.

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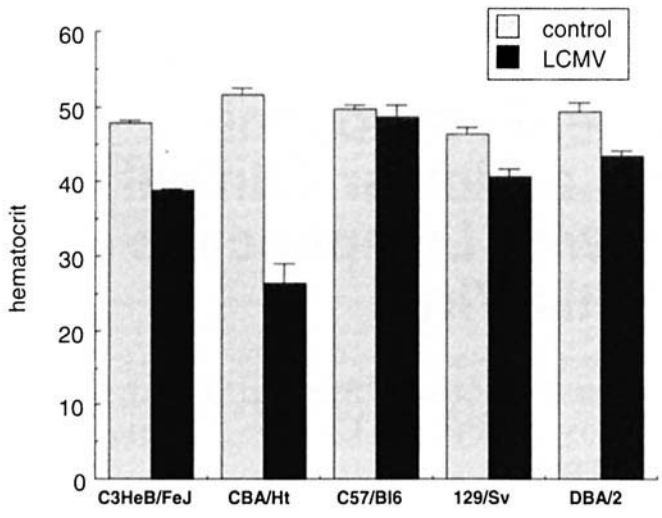

Figure 1. LCMV-induced anemia in different mouse strains. Hematocrits were measured in groups of five animals either uninfected (open columns) or 14 days after infection with LCMV (solid columns). Results are expressed as mean ± SEM.

mice. Therefore, the Docile strain of LCMV provides a useful model of how an apparently similar disease induced in different hosts by the same infectious pathogen may rely on distinct pathogenic mechanisms.

Materials and Methods

Mice and Viruses. C3HeB/FeJ, CBA/Ht, C57/Bl6, 129/Sv, and DBA/2 mice were purchased from the Jackson Laboratory (Bar Harbor, ME), from Iffa Credo (L'Arbresle, France), or were bred by Dr. G. Warnier at the Ludwig Institute for Cancer Research (Brussels, Belgium) and were used at the age of 6–8 weeks. The project was approved by the local commission for animal care.

For infection, a triple plaque-purified Docile substrain of LMCV was used (11). Virus stocks were grown in a canine kidney cell line (MDCK) for no more than three passages after plaque purification. Mice were injected intravenously (iv) with 300 plaque-forming units of virus.

Antibody Treatment. Anti-CD4 monoclonal antibody GK1.5 was made available by F.W. Fitch, and obtained through the courtesy of H.R. MacDonald (12). It was used as ammonium sulfate–precipitated antibody and injected intraperitoneally (ip) at a dose of 1 mg/mouse 5 hrs before, and 5 days after infection. As reported previously (3, 13) anti-CD4 treatment resulted in almost complete ablation of splenic CD4+ cells.

Hematocrit. Hematocrit was measured after centrifugation of heparinized blood in a Hettich-Haematokrit centrifuge (Hettich; Tuttlingen, Germany).

Reticulocyte Count. Reticulocytes were counted after staining of blood cells with New Methylene Blue (5 g/liter) for 5 mins at 37°C.

Analysis of Hematopoiesis in Spleen and Bone Marrow. The spleen was removed from each mouse immediately after death and fixed in 10% neutral buffered formalin. The entire spleen was sectioned sagitally, and paraffin wax–embedded for the preparation of 4-μm sections that were stained by standard hematoxylin-eosin methodology.

Smears of femoral bone marrow were fixed in 70% ethanol, then stained by Giemsa. The proportion of erythroblasts was determined for 200 cells per mouse.

Analysis of Antierythrocyte Autoantibodies by Radioimmunoassay (RIA). Antierythrocyte autoantibodies were detected on red cells with a direct RIA adapted from an indirect assay described previously (10). Briefly, 5 μl of washed and packed red cells were diluted in 100 μl of phosphate-buffered saline (PBS), then incubated for 3 hrs at 4°C with ^{125}I-labeled sheep polyclonal anti-mouse Ig antibody (Amersham, Rosendaal, The Netherlands). After appropriate washing, radioactivity bound to red cells was counted in a gamma-counter (Kontron AG, Munich, Germany).

Immunoglobulin Determination. Total IgG levels were assayed by enzyme-linked immunosorbent assay (ELISA) as described previously (3).

***Ex Vivo* Erythrophagocytosis.** The ability of macrophages to phagocytose red cells was measured as described previously (14). Briefly, macrophages were selected from the total peritoneal cell population on the basis of adhesion to plastic tissue culture dishes for 3 hrs, followed by overnight incubation. They were then incubated for 3 hrs with 20 μl of washed red cells in 2 ml of supplemented Dulbecco's medium containing 10% fetal calf serum, washed with PBS, and stained with *o*-toluidine. Phagocytosis was expressed as the percentage of cells having internalized at least five erythrocytes.

Liposome Preparation. Clodronate-containing liposomes were prepared following a method adapted from Van Rooijen (see Refs. 15, 16). Briefly, clodronate (dichloromethylene-bisphosphonate, kindly supplied by Boehringer-Mannheim GmbH, now Roche Diagnostics GmbH; Mannheim, Germany) was incorporated in a phosphatidylcholine and cholesterol lipidic film. The preparations contained both 150- to 500-nm and 1500- to 3000-nm liposomes. Mice were treated by iv injection of 200 μl of resulting liposomes. These clodronate-containing liposome preparations were found to decrease the number of peritoneal phagocytosing cells by 50% to 75% (data not shown), and to functionally prevent the development of autoantibody-mediated, phagocytosis-dependent thrombocytopenia (16, 17), When administered alone, they did not modify the hematocrits (data not shown).

Statistical Analysis. Statistical analysis was performed using a nonparametric unpaired Mann-Whitney test.

Results

LCMV-Induced Anemia in Different Mouse Strains. The effect of infection with the Docile strain of LCMV on induction of anemia in different strains of mouse was examined (Fig. 1). At the second week after infection,

anemia was apparent in four of the five mouse strains tested. It was most severe in CBA/Ht mice (highly significant difference between control and infected mice, $P = 0.0079$). A milder but also significant decrease in hematocrit was detected in C3HeB/FeJ mice ($P = 0.0079$) and, to a lesser extent, in 129/Sv and DBA/2 mice ($P = 0.0079$ and 0.0159, respectively). No significant anemia was found in C57/Bl6 animals ($P = 0.6905$). Because CBA/Ht mice had the lowest hematocrit of all at 2 weeks after infection, we decided to focus our study on this strain and compare it to the more well-characterized C3HeB/FeJ model. Kinetic analysis (Fig. 2) indicated that after reaching its lowest levels at 2 weeks after viral inoculation, anemia progressively regressed in CBA/Ht mice (significant difference between animals infected for 2 and 3 weeks, $P = 0.0159$).

In contrast, as reported previously (2, 3, 10), although anemia was initially milder than in CBA animals, it was similar in C3HeB/FeJ animals at 3 weeks as at 2 weeks after infection. These differences in kinetics, which were observed in three independent experiments, suggested that the mechanisms leading to anemia in the two mouse strains could be different.

Transient Decrease of Hematopoiesis in CBA/Ht and C3HeB/FeJ Mice Infected with the Docile Strain of LCMV. Infection of a number of different inbred mice with various LCMV strains has been reported to result in depressed hematopoiesis (2, 4–7). To evaluate the role of the Docile strain of LCMV in inhibiting red cell precursor production in our two mouse models, we counted circulating reticulocytes at different times after viral inoculation of both CBA/Ht and C3HeB/FeJ mice. As shown in Figure 3, the virus induced an early drop in reticulocyte count, which decreased in both mouse strains below the level of detection during the first week postinfection (p.i.), as expected from results reported by others (7). However, at later times, we observed a difference in the kinetics of reticulocyte recovery between CBA/Ht and C3HeB/FeJ mice. Indeed, in C3HeB/FeJ mice, reticulocyte count started to increase by Day 9 p.i. and reached a maximum on Day 15. At this time, it was significantly greater than in CBA/Ht animals ($P = 0.0079$), in which reticulocyte recovery started only at Day 12 p.i. and was slower, as it increased up to Day 21 p.i. A similar increase in reticulocytosis at 2 weeks after LCMV infection in C3HeB/FeJ animals when compared to CBA/Ht mice was found in two independent experiments.

Moreover, analysis of spleen sections indicated a difference in extramedullary hematopoiesis between CBA/Ht and C3HeB/FeJ mice infected with the Docile strain of LCMV (Fig. 4, shown for typical mice). In both strains, moderate hematopoiesis was observed in the spleen of uninfected animals (data not shown). In C3HeB/FeJ mice, the frequency of erythrocyte precursors was markedly increased 12 days after infection (Fig. 4A). In contrast, only sparse hematopoietic cells could be detected in spleens from CBA/Ht mice obtained 14 days after LCMV inoculation (Fig. 4B). Numerous macrophages were observed, interestingly, in spleens from CBA/Ht animals obtained 2 weeks after infection. Similar results were obtained from liver sections where, 2 weeks after LCMV infection, hematopoiesis could be found in C3HeB/FeJ, but not in CBA/Ht animals (data not shown). This was also confirmed by analysis of hematopoiesis in bone marrow smears (Table 1). At 2 weeks after LCMV infection, the proportion of erythroblasts was increased in C3HeB/FeJ mice compared with uninfected animals. In contrast, it remained the same in control and infected CBA animals, although the proportion of erythroblasts in uninfected

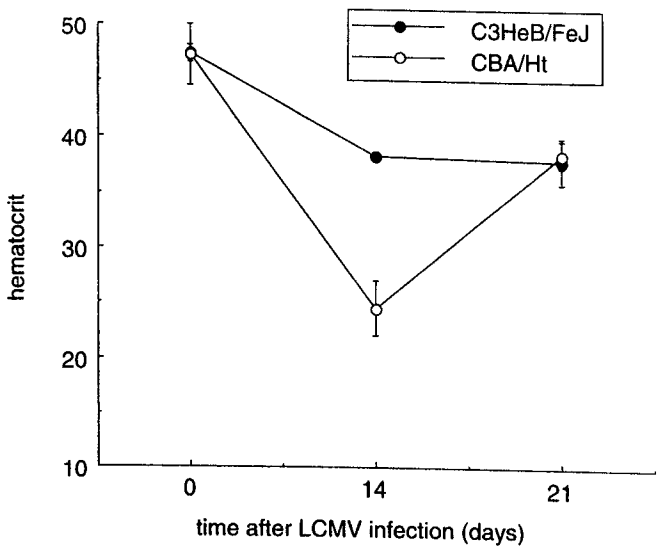

Figure 2. Kinetics of LCMV-induced anemia in CBA/Ht and C3HeB/FeJ mice. Hematocrits were measured in groups of three to five CBA/Ht (open symbols) and C3HeB/FeJ animals (closed symbols) either uninfected (0 day), or 14 or 21 days after infection with LCMV. Results are expressed as mean ± SEM.

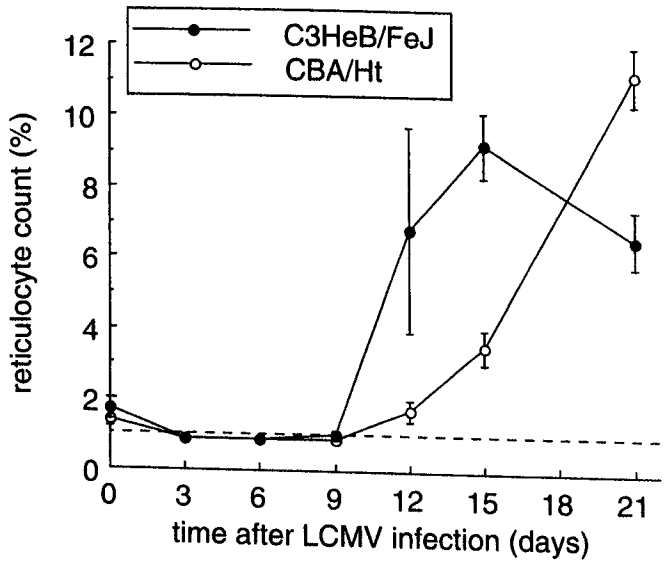

Figure 3. Reticulocyte count in CBA/Ht and C3HeB/FeJ mice after LCMV infection. Reticulocytes were counted in groups of five CBA/Ht and C3HeB/FeJ mice before (Time 0) or at different times after LCMV inoculation. Results are expressed as mean ± SEM. The dashed line represents the limit of detection.

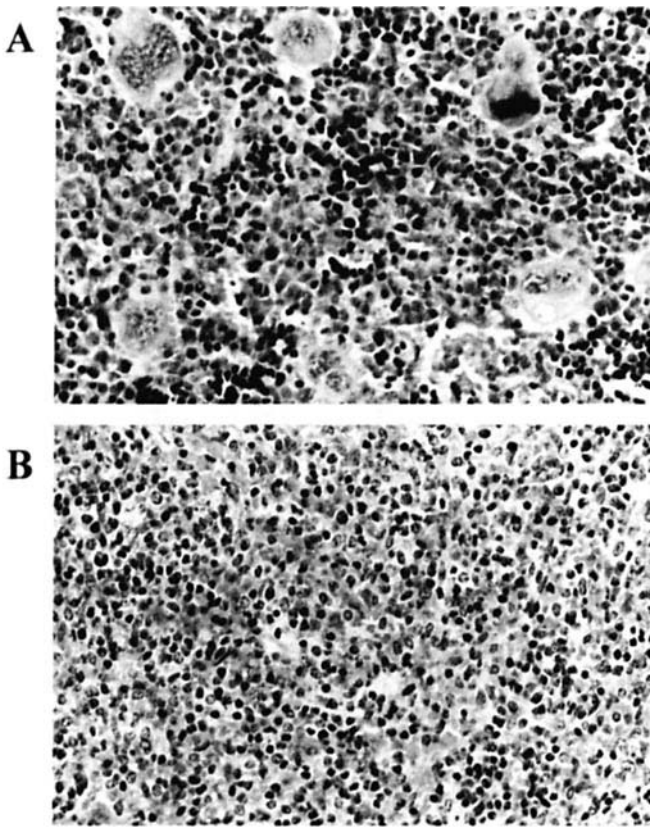

Figure 4. Splenic hematopoiesis after LCMV infection. (A) Splenic red pulp from a C3HeB/FeJ mouse 12 days p.i. with LCMV. There is evidence of marked hemopoietic activity with prominent megakaryocytes and numerous nucleated erythrocytes. (B) Splenic red pulp from a CBA/Ht mouse 14 days p.i. with LCMV. There are large numbers of macrophages with an absence of megakaryocytes and sparse nucleated erythrocytes. Hematoxylin and eosin, original magnification: ×200.

animals was higher in this strain than in C3HeB/FeJ mice. Together, these results suggested that anemia in CBA/Ht mice correlated strictly with a central depression of hematopoiesis, while in C3HeB/FeJ mice, other causes most likely were also involved.

Antierythrocyte Autoantibody and Total IgG Production Induced by LCMV Infection. Because involvement of anti–red cell autoantibodies has been correlated in C3HeB/FeJ mice with the development of

Table 1. Erythropoiesis in Bone Marrow of C3HeB/FeJ and CBA/Ht Mice Infected with LCMV

Mouse strain[a]	LCMV[b]	Bone marrow erythroblasts (%)[c]
C3HeB/FeJ	–	3.7 ± 0.8
	+	9.5 ± 0.8
CBA/Ht	–	8.5 ± 0.5
	+	6.0 ± 0.6

[a] Groups of four animals.
[b] Controls were uninfected animals; analysis was performed at Day 14 p.i.
[c] Mean ± SEM.

Table 2. Antierythrocyte Autoantibody and Total IgG After LCMV Infection

Mouse strain[a]	LCMV[b]	Antierythrocyte autoantibody (cpm)[c]	Total IgG μg/ml[c]
C3HeB/FeJ	–	1939 ± 209	209 ± 20
	+	6360 ± 1151	8280 ± 400
CBA/Ht	–	2196 ± 193	348 ± 26
	+	4599 ± 542	4183 ± 210

[a] Groups of five animals.
[b] Controls were uninfected animals; analysis was performed at Day 14 p.i.
[c] Mean ± SEM.

Docile LCMV–triggered anemia, these autoantibodies were assayed in the different mouse strains examined (Table 2). Both CBA/Ht and C3HeB/FeJ mice produced anti–red cell autoantibodies after LCMV infection, with significant differences between control and infected animals ($P = 0.0079$). A similar antierythrocyte autoantibody production was observed in 129/Sv and DBA/2 mice and, to a lesser extent, in C57/Bl6 mice (data not shown). Antibodies eluted from erythrocytes of LCMV-infected animals were predominantly of the IgG2a isotype in all mouse strains (data not shown). Finally, to determine whether LCMV induced a similar modulation of B lymphocyte responses in CBA/Ht and C3HeB/FeJ mice, total IgG levels were measured in the plasma of mice after 2 weeks of infection. Enhanced IgG production was observed in both mouse strains as a result of LCMV infection (Table 2).

$CD4^+$ Cell Dependence of Anemia Induced in CBA/Ht and C3HeB/FeJ Mice by Infection with the Docile Strain of LCMV. To further analyze the mechanisms leading to disease in C3HeB/FeJ and CBA/Ht mice infected with the Docile strain of LCMV, we next determined the relationship between antierythrocyte autoantibodies and the anemia by treating mice with an anti-CD4 monoclonal antibody. This treatment has been shown to prevent anemia in C3HeB/FeJ mice (3). We once again confirmed those results, and also demonstrated that this treatment largely abolished red cell–bound autoantibody (Fig. 5). Similarly, in CBA/Ht animals an almost complete elimination of antierythrocyte autoantibodies was observed after *in vivo* anti-CD4 treatment. The same conclusion was reached when autoantibodies were assayed in the eluates obtained from red blood cells (data not shown). In marked contrast, however, was the observation that in CBA/Ht mice, elimination of CD4+ cells had only a marginal effect on LCMV-induced anemia (Fig. 5). This result indicated that in CBA/Ht animals, but not in C3HeB/FeJ mice, LCMV-triggered anemia developed independently from the autoimmune antierythrocyte response.

Enhanced *Ex Vivo* Erythrophagocytosis by Macrophages from Mice Infected with the Docile Strain of LCMV. Because it has been suggested that erythrophagocytosis of opsonized red cells could be

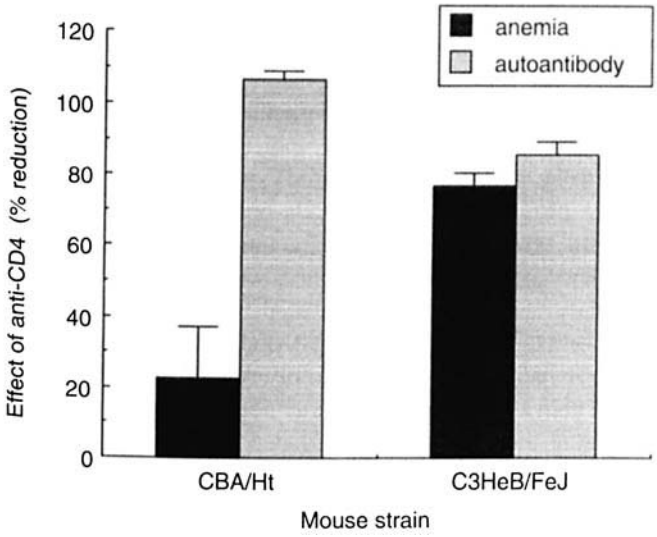

Figure 5. Effect of anti-CD4 treatment on LCMV-induced anemia and antierythrocyte autoantibody production in CBA/Ht and C3HeB/FeJ mice. Hematocrit was measured and antierythrocyte autoantibodies were assayed by RIA, in independent experiments, in groups of four to five CBA/Ht and C3HeB/FeJ mice that were either uninfected or infected with LCMV, with or without anti-CD4 treatment. Analysis was performed at 2 weeks p.i. in CBA/Ht mice and at 3 weeks p.i. in C3HeB/FeJ animals. The inhibitory effect of the anti-CD4 treatment is expressed as a percentage of reduction in anemia or autoantibody production (mean ± SEM), calculated as follows[a]: % of anemia reduction = 100 × (hematocrit of infected mouse treated with GK1.5 – mean hematocrit of infected mice)/(mean hematocrit of control mice – mean hematocrit of infected mice); % of autoantibody reduction = 100 × (mean autoantibody of infected mice – autoantibody of infected mouse treated with GK1.5)/(mean autoantibody of infected mice – mean autoantibody of control mice). ([a]For instance, individual data for anemia reduction in a C3HeB/FeJ mouse was calculated as follows: mean of hematocrit for control mice: 43.6; mean of hematocrit for LCMV-infected mice: 33.3; individual hematocrit of a mouse infected with LCMV and treated with anti-CD4: 41.5; individual % of anemia reduction = 100 × (41.5–33.3)/(43.6–33.3) = 79.6%. Data shown in the figure are the means ± SEM of these individual data.)

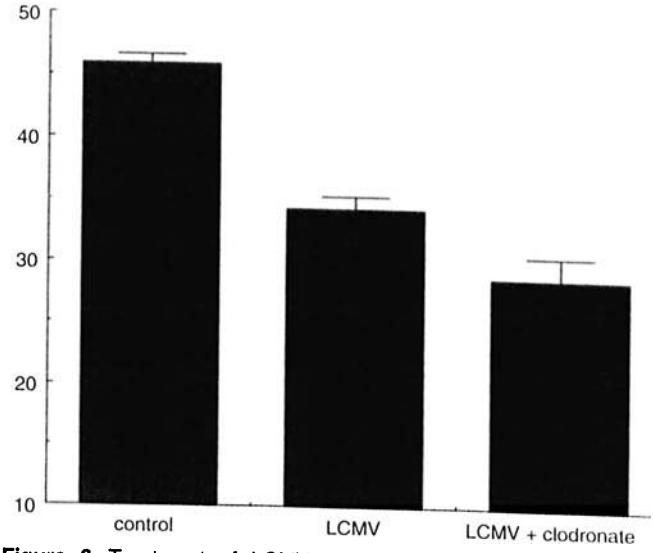

Figure 6. Treatment of LCMV-induced anemia with clodronate-containing liposomes. Hematocrits were determined in groups of four to six C3HeB/FeJ mice 20 days after infection with the docile strain of LCMV. Where indicated, clodronate-containing liposomes were administered on Days 5, 10, 14, and 18 after infection. Results are shown as mean ± SEM.

involved in LCMV-induced anemia (8), this capability was tested *ex vivo* using macrophages from uninfected and LCMV-infected C3HeB/FeJ mice. Our results showed an increased phagocytosis by macrophages from the latter mice of red cells from both control and LCMV-infected mice (Table 3). Note that red blood cells taken from infected mice and those devoid of autoantibody from uninfected mice were phagocytosed with equal ease by macrophages obtained from infected animals, whereas they were slightly more readily ingested than normal erythrocytes by macrophages derived from control animals. Similar results showing enhancement of macrophage phagocytic activity after LCMV infection were found with CBA/Ht mice (data not shown).

Table 3. *Ex Vivo* Erythrophagocytosis by Macrophages from LCMV-Infected C3HeB/FeJ Mice

Origin of macrophages[a]	Erythrophagocytosis (%)[b] of red cells from mice[c]	
	Control	LCMV
Control	6	12
LCMV	38	37

[a] Peritoneal macrophages were pooled from groups of five C3HeB/FeJ mice that were either uninfected (control) or infected for 3 weeks with LCMV.
[b] Erythrophagocytosis was expressed as a percentage of macrophages that had internalized at least five red cells.
[c] Red blood cells were obtained from the same mice as the macrophages.

Whereas the development of anemia was correlated in C3HeB/FeJ mice with antierythrocyte autoantibody production (Fig. 5), the similar uptake of normal and opsonized red cells by macrophages from LCMV-infected mice suggested that, in these animals, enhancement of phagocytosis was not the major mechanism explaining erythrocyte destruction. To confirm that increased phagocytosis of opsonized red cells was not the primary pathway leading to anemia, LCMV-infected C3HeB/FeJ mice were treated with clodronate-containing liposomes. Such a treatment was previously found to inhibit the development of virally exacerbated autoantibody-mediated thrombocytopenia (16, 17) and hemolytic anemia (data not shown). In contrast, no improvement of LCMV-triggered anemia was observed in C3HeB/FeJ mice after administration of clodronate-containing liposomes (Fig. 6).

Discussion

Different LCMV strains have been previously shown to depress erythropoiesis, through either NK cell activation or cytokine production by CD8 T lymphocytes (4–7). Variations in the severity of this inhibition of erythropoiesis have been linked to the genetic background of the infected host and to the viral dose rather than to the strain of LCMV

that was inoculated (7). With the exception of immunodeficient mice that cannot control viral replication (4), severe clinical anemia has not been reported, and reticulocyte production recovered quickly in the experimental conditions used so far (7). In this paper, we focused on the anemia that is induced after administration of a low dose of the Docile strain of LCMV. Our results indicated that whereas mice of several strains develop only mild anemia, LCMV-Docile triggers in CBA/Ht animals a more substantial anemia that reaches a maximum at 2 weeks after infection before a progressive return to higher hematocrit values. In contrast, anemia induced in C3HeB/FeJ mice, which is initially milder, does not regress as quickly as in CBA/Ht animals. This difference in the kinetics of anemia in these two mouse strains suggests that distinct pathological mechanisms are responsible for the development of the disease in C3HeB/FeJ and CBA/Ht mice. An initial drop in the reticulocytosis in all the mouse strains tested suggests that the virus triggered an inhibition of the erythropoiesis, as reported previously by others (7). In CBA/Ht mice, recovery of a compensatory erythropoiesis was delayed, as indicated by the low counts of blood reticulocytes and sparse splenic hematopoietic cells found at 2 weeks after infection. This could explain the enhanced anemia observed at this time in these animals.

In contrast, whereas erythropoiesis recovery was faster in C3HeB/FeJ mice, anemia, although milder, persisted paradoxically for a longer period of time in these animals. Such a prolonged anemia may be explained by pathogenic mechanisms other than impaired erythropoiesis, such as peripheral hemolysis. Antierythrocyte autoantibodies that may potentially induce red cell destruction were found in the different mouse strains analyzed in this study. However, the consequences of this autoimmune response were dissimilar, as indicated by anti-CD4 treatment that always resulted in a suppression of the autoantibody secretion. In CBA/Ht mice, this inhibition of antierythrocyte autoantibody production did not modify the disease, indicating that these autoantibodies played little if any role in the profound anemia that developed in these animals. In contrast, in C3HeB/FeJ mice, a similar effect of anti-CD4 treatment on the autoimmune response and on the late anemia induced in this mouse strain by LCMV showed the pathogenic role of these antierythrocyte autoantibodies, as already suggested previously (3, 8–10). Thus, the faster and more severe anemia in CBA/Ht mice could be entirely attributed to depressed erythropoiesis, whereas the delayed disease induced in C3HeB/FeJ mice also involved peripheral red cell destruction as a result of the LCMV-induced autoimmune response.

Erythrophagocytosis by cells of the reticuloendothelial system is a major mechanism, although not the sole mechanism, that may explain destruction of autoantibody-opsonized red cells (18). Viral infections may lead to an enhancement of phagocytosis of autoantibody-coated target cells that results in major anemia or thrombocytopenia (16, 19). Macrophages from LCMV-infected C3HeB/FeJ and CBA/Ht mice displayed an enhanced phagocytic activity. However, in contrast to macrophages derived from lactate dehydrogenase-elevating virus-infected mice that could ingest only erythrocytes coated with IgG2a autoantibodies (19), cells obtained from LCMV-infected mice could equally phagocytose autoantibody-opsonized and normal red cells. Moreover, *in vivo* destruction of phagocytic cells by clodronate-containing liposomes, a treatment that very efficiently prevents anemia mediated by erythrophagocytosis (20), had no effect on LCMV-induced anemia. These results suggest that enhancement of erythrophagocytosis by virally activated macrophages was not a major mechanism explaining the autoantibody-mediated anemia triggered by LCMV in C3HeB/FeJ mice.

Nevertheless, our observations indicate that the mechanisms responsible for LCMV-induced anemia in C3HeB/FeJ mice are clearly different from those involved in other mouse strains such as CBA/Ht animals. Therefore, infection with the Docile strain of LCMV is a useful model for the study of how a given microorganism induces a similar disease in animals of different genetic background by distinct mechanisms.

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