Suppression of Rat Bone Marrow Cells by Friend Murine Leukemia Virus Envelope Proteins

Stefan Mazgareanu,* Justus G. Müller,* Stefanie Czub,* Simone Schimmer,* Martin Bredt,† and Markus Czub*,†,‡

*Institut für Virologie und Immunbiologie, Universität Würzburg, Versbacherstrasse 7, D-97078 Würzburg; and †Pathologisches Institut, Universität Würzburg, Josef-Schneider-Strasse 2, D-97080 Würzburg, Germany

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INTRODUCTION

Hematological abnormalities are frequently associated with viral infections (Young, 1993). Of the retroviridae, Human Immunodeficiency Virus (HIV) (Re et al., 1994), Simian Immunodeficiency Virus (SIV) (Mandell et al., 1995; Watanabe et al., 1990), and Feline Immunodeficiency Virus (FIV) (Linnenberger and Abkowitz, 1995) cause bone marrow disorders with closely resembling characteristics, i.e., peripheral blood cytopenias affecting one or more hematopoietic lineages. This suggests that the pathogenesis of the bone marrow suppression associated with these lentiviruses is similar. Infections with other retroviruses, e.g., Feline Leukemia Virus (Lin- enberger and Abkowitz, 1995) or Murine Leukemia Virus (MuLV) (Belli and Fan, 1994; Kabat, 1989), often result in proliferative hematological disorders, although other defects originating in the bone marrow have been described (Linnenberger and Abkowitz, 1995).

We recently described pathogenic effects of a Friend MuLV, clone FB29, and of a variant, NT40, derived from FB29, in Fisher rats (Czub et al., 1995). Both viruses as well as the related clone PVC-211 (Masuda et al., 1992) cause a progressive neurodegenerative disease in neonatally inoculated Fisher rats. However, neither Friend virus nor the variants induced erythroleukemias in rats, as they do in mice (Masuda et al., 1992; Sitbon et al., 1986). As many MuLV-induced leukemias in mice depend on infection of one or more hematopoietic lineages, the bone marrow is a prime target for leukemogenic MuLV (Dexter et al., 1977). It was therefore of interest to determine whether in rats MuLV failed to infect the bone marrow.

In our previous study we observed a marked affinity of both viruses, FB29 and NT40, for cells of the monocytic lineage in rats (Czub et al., 1995). However, neither our group nor another group was able to efficiently infect macrophages with MuLV in vitro (Wong et al., 1989). For the sheep lentivirus, visna, it has been suggested that infection of macrophage precursors in the bone marrow played an important role for dissemination of virus by means of migration of infected monocytes into various tissues (Gendelman et al., 1985). A similar mechanism could have taken place in rats infected with FB29 or its derivative NT40. Infection of rat macrophage precursors before a postmitotic fixation occurred would provide a suitable explanation for our previous findings of macrophage susceptibility for MuLV (Czub et al., 1995). Therefore, we sought to identify whether rat macrophage precursors were susceptible for infection with MuLV.

Our results revealed that bone marrow cells (BM) were early and frequent targets for MuLV FB29 after inoculation of both neonatal and adult Fisher rats. Within the bone marrow, infection was clearly detected in Thy 1+ cells among which are early hematopoietic progenitor cells (van den Brink et al., 1991) as well as in...
macrophages. After exposure to MuLV FB29, a Thy $1^+$ subpopulation of rat BMC diminished irreversibly. As FB29-envelope proteins alone were also sufficient to reduce numbers of Thy $1^+$ cells, suppression of Thy $1^+$ BMC was not dependent on the addition of infectious virus, excluding direct infection as an obligatory cause for decreased Thy $1^+$ cell numbers.

RESULTS

Rat BMC are early targets for MuLV FB29

Inoculation of neonatal Fisher rats with MuLV FB29 has been shown to result in persistent infection of several organs such as blood, spleen, thymus, and central nervous system (Czub et al., 1995). Here, we show by means of a kinetic analysis that upon neonatal inoculation of rats BMC became productively infected at around 6±8 days postinoculation (dpi). The number of productively infected BMC increased to levels of 1±10% of all BMC producing virus [log 4.8 ± 0.1 focus forming units (FFU)/10$^6$ BMC; $n = 34$; means ± SE]. This occurred at around 12±14 dpi and this proportion of infected BMC was lifelong (Fig. 1).

Inoculation of adult Fisher rats with FB29 has previously been shown to result in an effective antiviral humoral and cellular immune response (Hein et al., 1995). In those animals, neither viremia nor productive infection of spleen or thymus has been detected when analyzed at or after 28 dpi (Hein et al., 1995). However, early after inoculation of adult rats, BMC became massively infected and in the first instance, i.e. up to 12 dpi, the kinetics of BMC infection closely resembled that of neonatally inoculated rats (Fig. 1). At no time was viremia detected (data not shown). After 12 dpi, a substantial reduction of virally infected cells appeared to have occurred, as productively infected cells could not be found either in the bone marrow (Fig. 1) or in other organs of animals inoculated as adults (Hein et al., 1995). It is likely that the decline of virus-infected cells was due to the emerging antiviral immune response. Despite the viral clearance in this latter group of animals, rat BMC were an early as well as a frequent target for MuLV FB29 in both neonatal and adult Fisher rats.

FB29-infected OX7$^+$ as well as OX7$^-$/CD11b/c$^+$ BMC

To identify and characterize BMC interacting with FB29, flow cytometric analyses using cell-specific and virus-specific immunolabeling were performed. In neonatally inoculated rats, around 12±31% ($n = 9$) of all OX7$^+$ BMC was found to express viral antigens (Fig. 2A). The number of double positive BMC remained at this level throughout the lifetimes. In rats inoculated as adults, a considerable proportion of OX7$^+$ BMC were also positive for antiviral staining (4±12%, $n = 4$; Fig. 2B) when analyzed after a substantial antiviral immune response had developed. Of infected rats inoculated either as neonates or adults, a considerable fraction of cells of the monocytic lineage which were defined by expression of CD11b/c (Robinson et al., 1986) could also be stained with antiviral antibodies (10±45% in neonatally inoculated animals, $n = 7$; Fig. 2D; 4±18% in rats inoculated as adults, $n = 4$; Fig. 2E), indicating viral infection of bone marrow-associated monocytes/macrophages. Viral tropism for monocytes/macrophages of other organs has been observed before in this animal model (Czub et al., 1995). After 14 dpi, BMC expressing viral antigens could not be found any longer in rats inoculated as adults. Altogether, these findings correlated well with the results of the infectious center assays (Fig. 1).

Decreased numbers of OX7$^+$ BMC in rats exposed to MuLV FB29

To investigate possible consequences of the massive bone marrow infection a flow cytometric analysis using monoclonal antibody OX7 was performed. This antibody stains many rat BMC including relatively primitive hematopoietic precursor cells (van den Brink et al., 1991). In neonatally inoculated rats we observed a decline of OX7$^+$ BMC, paralleling the spread of retroviral infection in this organ (Figs. 1 and 3A). In these persistently infected animals, numbers of OX7$^+$ BMC never recovered to levels of uninfected age-matched control animals and remained 22% below normal values (Fig. 3A). In rats inoculated as adults, numbers of OX7$^+$ BMC fell below normal values at the time when BMC became infected with FB29 and levelled out at 10% below numbers of OX7$^+$ BMC from uninfected controls (Fig. 3B). Numbers of OX7$^+$ BMC in rats inoculated as adults never reached levels of control animals again although D after
an antiviral immune response had been inaugurated; virus-infected cells could not be detected in these rats (Fig. 1). These results show that the cellular composition of rat bone marrow was significantly modified upon retroviral infection. Furthermore, it appears that the virus-induced modification(s) on the cellular arrangement of the bone marrow was irreversible, as viral clearance from the bone marrow and from other organs did not lead to restoration of OX7⁺ BMC numbers to normal levels.

**Histological analyses**

Histological analyses of total bone marrow by means of differential staining procedures revealed equal cellu-
larity, normal architecture, and maturation of the erythroid, myeloid, and thromboid lineage in infected bone marrow compared to that of normal animals (data not shown). There was no increase in either storage iron or fibrosis in infected bone marrow (data not shown). This indicated that the actual process of Thy 1^+ cell number reduction was difficult to visualize by histopathological techniques and that apparent reactions of the host in response to the decline of OX7^+ BMC did not occur.

Rapid decline of OX7^+ BMC after exposure to MuLV FB29 in vitro

To further explore virus-induced bone marrow alterations, BMC from uninfected adult rats (>5 weeks) were incubated in vitro with FB29 (MOI 1) or with Mock-in-

FIG. 4. A,B: Time course of numbers of OX7^+ rat BMC in vitro. BMC were incubated with MuLV FB29 (A) or with FB29-envelope proteins (B). Numbers of OX7^+ cells were determined by flow cytometry. Each symbol represents the ratio of numbers of OX7^+ rat BMC exposed to viral proteins to numbers of OX7^+ mock-treated cells (shown is a mean of nine experiments).

FB29 envelope protein(s) induced reduction of OX7^+ BMC both in vivo and in vitro

As BMC hardly became infected in vitro, however, numbers of OX7^+ BMC decreased upon viral exposure, and we studied the effects of viral proteins alone. Previous investigations on the pathogenesis of MuLV-induced diseases have pointed to viral envelope protein(s) playing a major role in the disease processes (Hasenkrug et al., 1996; Portis et al., 1990; Sitbon et al., 1986). We constructed a FB29-envelope protein expression vector (Fig. 5A) to test whether viral surface protein(s) alone would be sufficient to reduce numbers of OX7^+ BMC. Expression and secretion of FB29-envelope proteins into supernatants of transfected 3T3 cells was controlled by flowcytometric (Fig. 5B) and immunoblot (Fig. 5C) analyses. Supernatants of transfected cells containing FB29 envelope proteins were administered intraperitoneally (ip) into neonatal rats. Animals treated with envelope proteins exhibited a reduced number of OX7^+ BMC com-

FIG. 5. (A–C) Schematic diagram of a FB29-envelope gene expression vector, LTR-env-LTR(pBK) (A), and control of expression of FB29-envelope proteins in 3T3 cells. Expression of FB29-envelope proteins on NIH3T3 cells transfected with LTR-env-LTR(pBK) was analyzed by flow cytometry. Transfected (plain line) and nontransfected (dotted line) cells were stained with mAB 48 specific for gp70 of FB29 (B). Secretion of FB29-envelope proteins from NIH3T3 cells either transfected with LTR-env-LTR(pBK) (env) or with a control vector LTR-GFP-LTR(pBK) (GFP) and of supernatants from nontransfected cells was analyzed by immunoblotting (C).
pared to numbers of BMC from animals treated with control supernatants from untransfected cells (Fig. 3C). The decline of OX7+ BMC after treatment with envelope proteins was not as strong as after inoculation of replication-competent virus into neonates; however, just like in infected rats, numbers of OX7+ BMC from rats treated with envelope proteins remained below numbers of OX7+ BMC from control animals for life. Infectious virus bearing an envelope protein reactive with mAb 48 was never recovered from rats inoculated with viral proteins (data not shown), excluding the possibilities of laboratory contamination or generation of new viruses. In in vitro experiments, FB29-envelope proteins also reduced numbers of OX7+ BMC by up to 24% (Fig. 4B). These results demonstrate that infection was not obligatory for the induction of bone marrow alterations and that viral envelope proteins were sufficient to suppress numbers of OX7+ BMC.

Decline of OX7+ BMC was due to cell death

If the decline of OX7+ BMC was due to downregulation of Thy 1 molecules from the cell surface, other cellular surface markers on these cells might not be affected after exposure to viral proteins. Therefore, we doubly labeled BMC with mAb OX7 and with mAb OX22, the latter staining 95% of OX7+ BMC (data not shown). In neonatally inoculated animals, numbers of OX7+ and of OX22+ BMC declined in a similar manner, when stained in parallel at various timepoints pi (Figs. 6A and 6B). This suggested a loss of cells rather than a loss of cellular surface marker Thy 1.

To determine whether OX7+ BMC died upon exposure to FB29-envelope proteins we analyzed BMC in our in vitro system by means of propidium iodide (PI) incorporation. Thirty hours post initial exposure, OX7+ BMC exposed to viral envelope proteins incorporated PI more frequently than OX7+ BMC treated with control supernatants (Fig. 7). This tendency increased with time (Fig. 7). These results demonstrate that retroviral envelope proteins exhibited cytotoxic effects on Thy 1+ BMC from rats.

DISCUSSION

Many retroviruses can cause hematopoietic abnormalities, including HIV in humans (Maciejewski et al., 1994; Re et al., 1994), SIV in monkeys (Mandell et al., 1995; Watanabe et al., 1990), FIV in cats (Linenberger and Abkowitz, 1995), and MuLV in mice (Li and Fan, 1990; Tse et al., 1994). Here we report that retroviral infection of rats also caused bone marrow alterations. In all of our experimental approaches using MuLV FB29, we observed a reduction of numbers of Thy 1+ BMC, following very closely viral replication and/or the presence of viral envelope proteins.

Both Thy 1+ cells and monocytes/macrophages were identified as major cellular targets within the bone marrow for FB29. The Thy 1 antigen, a glycoprotein of 25 kDa (Letarte-Muirhead et al., 1975), is present on 25–40% of rat BMC (Williams, 1976). Cells bearing this antigen represent immature hematopoietic cells, including pluripotent stem cells (van den Brink et al., 1991). Both infection and decline of Thy 1+ BMC targeted only fractions of these cells but not the entire population, indicating that a subpopulation(s) with high susceptibility for infection and/or alteration existed. Once Thy 1+ subpopulations receptive for infection, alteration, or both are defined more precisely, it will be possible to show whether the decline of Thy 1+ BMC is due to direct interaction with viral envelope proteins or to indirect mechanisms.

In addition to cells bearing the Thy 1 antigen, mono-

FIG. 6. (A,B) Time course of numbers of OX7+ (A) and of OX22+ (B) rat BMC after inoculation of neonates with MuLV FB29. Numbers of OX7+ and of OX22+ rat BMC were determined by flow cytometry. Each symbol represents the ratio of numbers of OX7+ rat BMC from two individual animals: from one infected animal to numbers from a second, age-matched noninfected littermate. Of OX7+ BMC 95% were also positive for mAb OX22 (not shown).

FIG. 7. Effects of FB29-envelope proteins on the viability of Thy 1+ BMC in vitro. BMC were incubated with FB29-envelope proteins (▲) or with tissue culture supernatants from NIH3T3 cells transfected with LTR-GFP-LTR (○). Thy 1+ BMC were analyzed for their capacity to incorporate propidium iodide (PI) at different timepoints. Each symbol represents the ratio of Thy 1+/PI+ BMC to Thy 1−/PI− BMC. With time, Thy 1+ BMC exposed to viral envelope proteins incorporated PI more frequently than OX7+ BMC treated with control supernatants. Shown is a representative result of a total of three experiments.
cytes/macrophages were found to be susceptible for infection with FB29 within the bone marrow. Infection of bone marrow resident monocytes/macrophages went along without apparent modification of these cells. We did not observe either macrophage hyperplasia or myeloid hyperplasia as described in bone marrows of monkeys early after SIV infection (Mandell et al., 1995). However, cells of the monocytic lineage often become activated upon retroviral infection, with the possible consequence of damaging neighboring cells. For example, HIV-induced bone marrow failure appears to be related to TNF-α secretion by macrophages (Maciejewski et al., 1994). Just like after infection with FB29, macrophages are a major cellular target for HIV (McElrath et al., 1989) as well as for SIV (Mandell et al., 1995). Enhanced TNF-α production occurs upon exposure of macrophages to HIV envelope proteins gp120/160 (Clouse et al., 1991a; Macejewski et al., 1994; Riekmann et al., 1991). Effects of enhanced TNF-α levels on BMC could be of an inhibitory (Jacobsen et al., 1994) or a cytotoxic nature (Bigda et al., 1994) or both. Similarly, numbers of Thy 1+ BMC might have been reduced by an indirect mode of actions mediated through monocytes/macrophages activated by FB29-envelope proteins.

We would also expect pathological long-term consequences of high levels of TNF-α. As such, differentiation of the bone marrow could shift toward monocytes and macrophages, as observed in vitro (Jacobsen et al., 1994; Murphy et al., 1988). It would be interesting to find a retroviral-induced TNF-α-mediated shift of hematopoietic differentiation toward monocytes and macrophages in vivo, too, because macrophagotropic viral variants might take advantage of such a situation for enhanced viral spread and subsequent further tissue damage, including the bone marrow, in later stages of infection. This hypothesis could also explain why we observed an even more dramatic decline of Thy 1+ BMC beginning at 40±50 days after FB29 infection or administration of FB29-envelope proteins into neonatal animals. Both in vivo and in vitro data revealed that FB29-envelope proteins alone were sufficient to reduce numbers of Thy 1+ BMC. Analyses based on PI incorporation demonstrated that cell death was responsible for the decline of numbers of OX7+ BMC. It is thus likely that FB29-envelope proteins are directly cytotoxic. Apart from bone marrow suppression, cytotoxic effects of retroviral envelope proteins might also be involved in other pathological alterations, such as hemolytic anemia of mice (Sitbon et al., 1986), neurodegeneration of both humans and rodents (Hasenkugl et al., 1996; Lipton et al., 1991; Portis et al., 1990; Toggas et al., 1994), and T-cell loss during AIDS (Siliciano, 1996).

It has been suggested that antibodies directed against HIV-envelope proteins are capable of inhibiting colony formation by bone marrow progenitor cells derived from AIDS patients (Donahue et al., 1987). An immune-mediated mechanism might also play an important role in Parvovirus-induced myeloid depression (Segovia et al., 1995). We found no additional depressive effects on numbers of Thy 1+ BMC after clearance of viral infection from rats inoculated as adults. This indicates that elimination of virus-infected BMC by an antiviral immune response was compensated well in these animals. Furthermore, as the reduction of Thy 1+ BMC was irreversible, clearance of viral infection did not have a beneficial influence on the numbers of Thy 1+ BMC. Therefore, long-term consequences of reduced numbers of Thy 1+ BMC might be equal in all animals exposed to virus or viral envelope proteins independently of their state of infection and the antiviral immune response.

In summary, we describe a new bone marrow abnormality induced by envelope proteins of Friend MuLV in rats. As in susceptible mice, the rat bone marrow was shown to be a major target organ for MuLV; however, it does not give rise to leukemias. We further demonstrated lifelong susceptibility of the rat bone marrow for at least transient FB29 infection and associated alterations. This contrasts with the situation for MuLV-induced leukogenesis in AKR mice (Buckheit et al., 1987, 1988) and in mice inoculated with Moloney MuLV (Belli and Fan, 1994), respectively. In both cases, early postnatal infection of the bone marrow with MuLV has been proposed to be crucial for the development of leukemia in mice. It also appeared that the reduction of numbers of Thy 1+ BMC in rats very closely followed viral infection and/or exposure to viral proteins but that the pathogenesis of MuLV-induced leukemia in mice is composed of a rather slow multistep process (Belli and Fan, 1994; Kabat, 1989; Li and Fan, 1990). Thus, in the course of MuLV-induced rat bone suppression quite different virus-host interactions appear to be recruited than in the pathogenesis of MuLV-induced leukemias in mice.

MATERIALS AND METHODS

Animals, inoculation, viruses, and infectious center assay

Fisher rats were initially obtained from the Institut für Versuchstierzucht (Hannover, Germany) and were bred at our institute. Neonatal rats (0±24 hours postpartum) were inoculated ip with 100–105 FFU; adult rats (>5 weeks) were inoculated with 500–105 FFU. For some experiments, neonatal rats were inoculated with 100 μl of NIH3T3-cell culture supernatant transfected with FB29-envelope gene expression vector LTR-env-LTR(pBK) and with LTR-GFP-LTR(pBK), respectively.

FB29 is a molecular clone of the I– strain of Friend MuLV (Sitbon et al., 1986). Neonatal inoculation of rats with FB29 leads to infection of the brain, resulting in spongiform neurodegeneration and mild neurological signs (Hein et al., 1995). Infectivity of cell culture and of...
BMC was determined by means of a focal immunoassay using monoclonal antibody (mAb) 48 specific for FB29 (Robertson et al., 1991), peroxidase-conjugated rabbit anti-mouse immunoglobulin (Dako), and 3-amino-9-ethylcarbazol (Sigma) as described previously (Czub et al., 1991).

Construction of a FB29-envelope gene expression vector, LTR-env-LTR(pBK)

An envelope-LTR fragment (XbaI–PstI) from molecular clone FB29 (Genbank Accession No. Z11128) encompassing nucleotides 5634–561 was cloned into pBKCMV (Stratagene) from which the CMV promoter (Spel–Nhel) had been excised before, A 5’ LTR (ClaI–PstI) encompassing nucleotides 7702–561 from FB29 was placed upstream of the envelope-LTR sequence in pBK. A neo-resistance gene outside retroviral vector sequences is driven by a SV40 promoter (Fig. 5A). A similar procedure was chosen for construction of control vector LTR-GFP-LTR(pBK) in which the envelope gene was replaced by the green fluorescent protein, GFP (Heim et al., 1995). Plasmid DNA was transfected into NIH3T3 cells using the calcium phosphate procedure (Graham and van der Eb, 1973). Cells were cultivated in 10% FCS–RPMI 1640 containing 72 mg/l penicillin, 120 mg/l streptomycin, and 0.0028 M glutamine. Cells were incubated for 24 h at 37°C in a humidified atmosphere containing 5 vol.% CO2. After 24 h, MuLV stock or cell supernatants of transfected or nontransfected cells were precipitated with trichloroacetic acid (final concentration 5%, 10 min on ice), pelleted (10 min, 5000 g), washed twice in PBS–Tween for 10 min at room temperature, permeabilized with Triton X-100 (0.3%) (Sigma) for 30 min at room temperature, and stained intracellularly with goat polyclonal antisera directed against MuLV gp70 (kindly donated by R. Friedrich, Gießen) and FITC-conjugated rabbit anti-goat immunoglobulin G (Dako). Control staining was performed with normal goat serum.

Propidium iodide staining

In some experiments, BMC were incubated with PI (20 µg/ml) for 10 min at 4°C and analyzed by flow cytometry to distinguish dead from live cells (Radosевич et al., 1990). Prior to PI incubation cells were labeled with mAb OX7.

Immunoblot analysis

NIH3T3 cells were lysed in lysis buffer [4% sodium-dodecyl-sulfate, 20% glycerin, 16.6 mM Tris (pH 6.8), and 4% β-mercaptoethanol] and boiled for 10 min. Supernatants of transfected or nontransfected cells were precipitated with trichloroacetic acid (final concentration 5%, 10 min on ice), pelleted (10 min, 5000 g), washed with methanol, dried, resuspended in equal volumes of PBS and 2× lysis buffer, and boiled for 10 min. A total of 10 µl of the samples were subjected to electrophoresis in SDS–polyacrylamide gel and subsequently transferred onto a nitrocellulose filter. Nitrocellulose filters were blocked for 1 h at room temperature with PBS–Tween (0.1% bovine serum albumin, 3% nonfat powdered milk, 0.5% Tween 20) and incubated overnight at 4°C with goat polyclonal antiserum directed against MuLV gp70 (1/3000) and peroxidase-coupled rabbit anti-goat immunoglobulin G antibodies (Dianova) (1/1000). Nitrocellulose filters were washed twice in PBS–Tween for 10 min at room temperature before revelation with the chemiluminescence ECL kit (Amersham Corp.) following the recommendations of the manufacturer.

Histological examinations

Sections of 5 µm bone marrow were stained with H&E, Giemsa, DiPAS, the Prussian blue iron stain, and the Gomory silver staining method. By light microscopy, the following parameters were evaluated: overall cellularity, maturation in the myelopoiesis, erythropoiesis, thrombopoiesis, the medullary architecture, and the reticulin fiber...
content. The evaluation of the bone included the architecture of the spongiosa and the presence of osteoblasts and osteoclasts on the surface.

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