Preleukemic Hematopoietic Hyperplasia Induced by Moloney Murine Leukemia Virus Is an Indirect Consequence of Viral Infection

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We previously showed that neonatal mice inoculated with Moloney murine leukemia virus (M-MuLV) exhibit a preleukemic state characterized by splenomegaly and increased numbers of hematopoietic progenitors. An M-MuLV variant with greatly reduced leukemogenic potential, Mo+PyF101 M-MuLV, does not generally induce this preleukemic state. In order to investigate the mechanism involved in M-MuLV induction of preleukemic hyperplasia, we tested the CFU-mixed myeloid and erythroid (CFU_{mix}) from M-MuLV- and Mo+PyF101 M-MuLV-inoculated mice for the presence of virus by antibody staining and for the release of infectious virus. The majority of CFU_{mix} colonies from both M-MuLV- and Mo+PyF101 M-MuLV-inoculated mice contained infectious virus even though M-MuLV-inoculated mice showed elevated levels of CFU_{mix} while the Mo+PyF101 M-MuLV-inoculated mice did not. This indicates that direct infection of hematopoietic progenitors was not sufficient to induce hyperplasia. Rather, hematopoietic hyperplasia may result indirectly from infection of some other cell type.

Moloney murine leukemia virus (M-MuLV) induces T-lymphoblastic lymphoma when inoculated into neonatal mice. We previously described a preleukemic state in M-MuLV-inoculated NIH Swiss and NFS inbred mice, as characterized by generalized hematopoietic hyperplasia (3). From 6 to 10 weeks after inoculation, animals developed mild splenomegaly. Agar colony assays showed increased numbers of myeloid and erythroid progenitors (CFU_{mix} and CFU_E, respectively) in preleukemic spleens. On the basis of these observations, we proposed a model for M-MuLV-induced leukemogenesis involving two infection events. The first infection, in bone marrow or spleen cells, would lead to generalized hematopoietic hyperplasia (erythroid, myeloid, and presumably lymphoid). Hyperplastic lymphoid progenitors migrating to the thymus could undergo a second infection. During the latter infection, provirus would integrate adjacent to one or more cellular proto-oncogenes, leading to their activation (2, 12, 14).

Our studies on M-MuLV-induced leukemia have been aided by an M-MuLV variant, Mo+PyF101 M-MuLV, which we described previously (5, 8). Mo+PyF101 M-MuLV contains enhancers from the F101 variant of polyomavirus inserted into the U3 region of the long terminal repeat between the M-MuLV enhancers and the proximal promoter elements. In contrast to wild-type M-MuLV, Mo+PyF101 M-MuLV showed greatly reduced leukemogenic potential even though Mo+PyF101 M-MuLV virions are identical to the wild type (5). Moreover, the variant virus could efficiently establish infection in animals, as evidenced by high-level infection of splenocytes and thymocytes approximately equal to wild-type M-MuLV at preleukemic stages (6 weeks) (4). Thus, Mo+PyF101 M-MuLV has been a useful tool for identifying critical events and cell types for M-MuLV leukemogenesis. Indeed, in our previous experiments Mo+PyF101 M-MuLV did not induce the splenic hematopoietic hyperplasia typical of wild-type M-

MuLV, supporting the importance of this process in leukemogenesis. As measured by agar colony assays, 6- to 9-week-old M-MuLV-inoculated NIH Swiss mice showed 5- to 10-fold elevated levels of CFU_{mix} compared with those of controls, whereas Mo+PyF101 M-MuLV-inoculated mice showed no increases in CFU_{mix} (3).

In these experiments, we wished to investigate the mechanism by which M-MuLV induces preleukemic hyperplasia in the spleen. Possible mechanisms can be divided into two classes. One possibility is that hyperplasia results from direct infection of hematopoietic stem cells, e.g., perhaps due to mitogenic effects of an M-MuLV protein (e.g., *gag* or *env* glycoprotein [13]). Alternatively, infection of nonhematopoietic cells might indirectly lead to increased hematopoiesis, e.g., perhaps due to increased production of growth factors such as interleukin 3 (6, 7). One way to distinguish between these possibilities was to test whether hematopoietic stem cells in preleukemic animals are virus infected; with the first possibility, all progenitors should be infected; in the latter case, this would not necessarily be the case.

To determine whether hematopoietic progenitors in preleukemic spleens are virus infected, individual CFU_{mix} progenitor colonies from spleens of M-MuLV- and Mo+PyF101 M-MuLV-inoculated NIH Swiss mice were tested for viral infection by antibody staining or infectious center assays. In the first method, splenocytes from preleukemic spleens (6 to 7 weeks) were plated for CFU_{mix} in methylcellulose suspension in the presence of WEHI 3B cell conditioned medium as a source of interleukin 3 (9). After 7 days, the numbers of CFU_{mix} colonies were counted, as described previously (3), to determine whether the mice were undergoing generalized hyperplasia. As expected, M-MuLV-inoculated mice exhibited generalized hematopoietic hyperplasia with two- to sevenfold increases in CFU_{mix} while Mo+PyF101 M-MuLV-inoculated mice demonstrated essentially normal levels of these progenitors.

For the plates used in immunoperoxidase staining, methylcellulose was removed by gradual dilution with buffer. CFU_{mix} colonies remaining on the bottom of the culture

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FIG. 1. Staining of CFU_{mix} colonies for M-MuLV p30^{gag} antigen. Methycellulose colonies were fixed and stained as described in the text by the immunoperoxidase method, using anti-M-MuLV p30^{gag} as the primary antibody. Colonies positive for expression of p30^{gag} antigen turned orange-brown in color in the presence of 3,3'-diaminobenzidine tetrahydrochloride. Shown are representative CFU_{mix} colonies derived from an uninoculated mouse and stained with anti-p30^{gag} (a), an M-MuLV-inoculated mouse and treated with normal rabbit serum (b), an M-MuLV-inoculated mouse which stained positively with anti-p30^{gag} (c), and an Mo+PyF101 M-MuLV-inoculated mouse which also stained positively with anti-p30^{gag} (d). Bar = 100 μ m.

dishes were fixed with a 4:1 mixture of methanol-3% H₂O₂ and air dried. The fixed CFU_{mix} colonies were then stained for M-MuLV p30^{gag} protein by the immunoperoxidase method by using a polyclonal rabbit anti-p30^{gag} serum (10) as the primary antibody. NIH Swiss mice do not readily express endogenous MuLV-related sequences, so the anti-p30 staining should be specific for exogenously infecting M-MuLV. Those colonies from M-MuLV- and Mo+PyF101 M-MuLV-infected animals expressing p30 showed orange-brown staining in the presence of 3,3'-diaminobenzidine tetrahydrochloride (Fig. 1c and d). No staining was observed when control normal rabbit serum was used (Fig. 1b). In addition, when CFU_{mix} colonies from uninfected animals were stained with anti-p30 (Fig. 1a), no staining was observed, confirming the fact that p30 expression was from the exogenously infecting M-MuLV. When these results were quantified, 87 and 91% of CFU_{mix} colonies from two M-MuLV-inoculated mice were positive for p30^{gag} antigen while 68 and 92%, respectively, of CFU_{mix} colonies from Mo+PyF101 M-MuLV-inoculated mice were p30^{gag} positive (Table 1). Thus, the great majority of hematopoietic progenitors in preleukemic M-MuLV mice were virus infected, which would be consistent with a direct infection mechanism for hyperplasia. However, Mo+PyF101 M-MuLV mice showed comparable high levels of viral infection with the progenitors, even though there was no hematopoietic hyperplasia. Moreover, the intensity of staining for viral protein in the CFU_{mix} colonies from Mo+PyF101 M-MuLV-inoculated animals was comparable to that for CFU_{mix} colonies from wild-type M-MuLV-inoculated preleukemic animals,

indicating similar levels of virus expression. Thus, infection of CFU_{mix} per se was insufficient to result in hematopoietic hyperplasia.

As an independent test for infection of CFU_{mix}, colonies were also assayed for production of infectious M-MuLV. In this case, splenocytes from preleukemic M-MuLV-inoculated NIH Swiss mice were plated in CFU_{mix} assays in agar suspension in the presence of WEHI 3B conditioned medium at a density resulting in 30 to 60 colonies per 35-mm (diameter) dish. After 7 days, individual well-separated

TABLE 1. Assay for p30^{gag}-expressing CFU_{mix} by immunoperoxidase staining^a

Virus	Animal	No. of CFU _{mix} colonies staining with anti-p30 ^{gag}
Uninoculated	1	0/74
M-MuLV	1	69/76
	2	69/79
Mo+PyF101 M-MuLV	1	61/66
	2	48/71

^a Rabbit anti-p30^{gag} antiserum was used in immunoperoxidase staining to detect MuLV p30^{gag}, as shown in Fig. 1 and described in the text. Normal rabbit serum was used as a control. Number staining of the total number of CFU_{mix} colonies on a plate is indicated. In this assay, Mo+PyF101 M-MuLV splenocytes were seeded at 5 times the density of M-MuLV splenocytes in order to obtain equivalent numbers of CFU_{mix} colonies for staining. Parallel colony assays were used to quantify the number of CFU_{mix} colonies; for these, splenocytes from the different mice were seeded at equal densities. The results verified that Mo+PyF101 M-MuLV splenocytes contained essentially normal levels of CFU_{mix} while M-MuLV CFU_{mix} were elevated two- to sevenfold.

TABLE 2. Assay for infectious virus in CFU_{mix} by cocultivation with NIH 3T3 cells^a

Origin of CFU _{mix}	CFU _{mix} colonies	
	No. assayed	No. positive ^b
Uninfected animal	5	0
Wild-type M-MuLV animal	17	15
Mo+PyF101 M-MuLV animal	16	13
Wild-type M-MuLV assay plate, agar plug lacking CFU _{mix} cells	3 ^c	0

^a Individual 7-day CFU_{mix} colonies were picked and cocultivated with NIH 3T3 cells as described in the text. Agar plugs containing no CFU_{mix} cells were also picked to investigate the ability of virus to spread through the agar.

^b Cultures were assayed for infectious ecotropic virus by the UV-XC plaque assay (11).

^c Number of agar plugs assayed.

CFU_{mix} colonies (ca. 2,000 cells per colony) were picked with a finely drawn Pasteur pipette, freed from the agar by repeated pipetting in the medium, and seeded onto NIH 3T3 cell monolayers (5×10^4 NIH 3T3 cells per 4.5-mm² well in 12-well microtiter plates seeded 4 h earlier in medium containing 2 µg of Polybrene per ml). As controls, similar-sized agar plugs containing no CFU_{mix} colonies were also picked from the same cultures. These agar plugs would contain any putative surviving cells and virus from the initially seeded splenocytes which might score in the assay as well. After 24 h of incubation, the nonadherent hematopoietic cells were removed by washing and the NIH 3T3 cells were allowed to grow to confluency and then passaged once. Upon reaching confluency a second time, the cultures were assayed for the presence of M-MuLV by UV irradiation and overlay with XC cells (11). XC plaques specifically detect the exogenous M-MuLV, since infectious endogenous XC⁺ virus is not carried in the genome of NIH Swiss mice (1). As shown by a representative experiment in Table 2, greater than 80% of the CFU_{mix} colonies from preleukemic M-MuLV-inoculated mice were productively infected with M-MuLV, as indicated by the release of infectious XC⁺ virus. This was completely consistent with the antibody staining seen in Fig. 1. As expected from Fig. 1, the great majority of CFU_{mix} colonies from Mo+PyF101 M-MuLV-inoculated mice were also virus infected (Table 2). The agar plugs that did not contain CFU_{mix} colonies from the same plates were negative for infectious virus. This indicated that initially seeded non-CFU_{mix} cells and/or M-MuLV virions were not the source of infectious M-MuLV detected in this assay.

These experiments indicate that infection of hematopoietic progenitors in the spleen of M-MuLV-inoculated animals is extensive at preleukemic times. However, the results from the Mo+PyF101 M-MuLV-inoculated animals indicate that infection of CFU_{mix} is not in itself sufficient to lead to hyperplasia. Thus, they suggest that hematopoietic hyperplasia results indirectly from infection of some other cell type.

One potential alternative explanation for these results could be that M-MuLV infection of hematopoietic progenitors does cause hyperplasia, but with a lag. In this case, if Mo+PyF101 M-MuLV established infection in hematopoietic progenitors later than wild-type M-MuLV, it might induce hyperplasia later than tested here. However, in previous experiments, we showed that at significantly later times (9 to 10 weeks versus the 6 to 7 weeks postinoculation of Fig. 1 [3]), Mo+PyF101 M-MuLV-inoculated mice still did not show hematopoietic hyperplasia of either myeloid or erythroid lineages.

Since these results suggest infection of an additional cell type(s) in the generation of preleukemic hyperplasia, it will be important to identify those putative cells. The poorly leukemogenic Mo+PyF101 M-MuLV will be useful for these experiments, since this virus does not generally induce preleukemic hyperplasia. Cells that are permissive for infection by wild-type but not Mo+PyF101 M-MuLV would be candidates for the critical cells that must be infected in order to establish the preleukemic state.

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