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Combined Infection by Moloney Murine Leukemia Virus and a Mink Cell Focus-Forming Virus Recombinant Induces Cytopathic Effects in Fibroblasts or in Long-Term Bone Marrow Cultures from Preleukemic Mice

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We described previously a preleukemic state in mice inoculated with Moloney murine leukemia virus (M-MuLV) characterized by generalized hematopoietic hyperplasia in the spleen. To investigate this further, long-term bone marrow cultures (LTBMC) from preleukemic mice were established. Surprisingly, LTBMC from M-MuLV-inoculated preleukemic mice showed less hematopoiesis than LTBMC from control mice. This resulted from a quantitative defect in establishment of bone marrow stromal cells in the LTBMC. This phenomenon could also be observed in LTBMC from normal mice infected in vitro with a stock of M-MuLV containing a mink cell focus-forming virus (MCF) derivative (M-MCF), but not in LTBMC infected with M-MuLV alone. This implicated MCF derivatives in the reduction in bone marrow stromal cells. The phenomenon could also be detected in infected NIH 3T3 cells. Combined infection of M-MuLV plus M-MCF resulted in fewer cells, in comparison to uninfected cells or cells infected with either virus alone. Further studies indicated that this was predominantly due to an inhibition in cell growth rather than to cell lysis. The cytopathic effect did not appear to result from overreplication of viral DNA, as measured by Southern blots. Thus, combined infection with M-MuLV and an MCF derivative had cytostatic effects on cell growth. This phenomenon might also contribute to the leukemogenic process in vivo.

Moloney murine leukemia virus (M-MuLV) is a typical replication-competent retrovirus, and it causes T-cell lymphoma in mice with relatively long latency (32, 33). This long latency suggests that multiple steps may be involved in disease development. Previous studies identified two characteristic phenomena associated with M-MuLV-induced leukemia: (i) independently induced tumor cells frequently have proviral integrations at common loci, including cellular proto-oncogenes (e.g., c-*myc*, *pim-1*, *pvt-1*, etc.) (5, 14), reflecting the fact that activation of a cellular proto-oncogene(s) is an important event in tumor development; (ii) leukemogenesis is associated with generation of mink cell focus-forming (MCF) viruses (31, 34). MCF viruses are *env* gene recombinants between the infecting M-MuLV and endogenous MuLV-related sequences present in normal mouse cells; MCF derivatives infect cells via surface receptors different from those of M-MuLV (25, 26). A variety of observations have suggested that MCF derivatives may be important in viral leukemogenesis (19), although the exact mechanism is unknown.

Other experiments have investigated early events in the MuLV-induced leukemogenesis process, preleukemic events. Transplantation experiments from preleukemic mice indicated that cells with leukemic potential could first be detected in either preleukemic bone marrow or spleen; only at later times could preleukemic cells be detected in the thymus, where the final tumors frequently appear (4, 18, 24). This suggested an early preleukemic event in bone marrow or spleen. Recently, we described a preleukemic state in M-MuLV-infected mice, consisting of generalized hematopoietic hyperplasia in the spleen and, to a lesser extent, bone marrow (8). Experiments with a nonpathogenic variant of M-MuLV (see below) also supported the view that this hematopoietic hyperplasia plays a role in M-MuLV leukemogenesis. However, the mechanism of preleukemic hyperplasia induction has not been elucidated.

Given the potentially multiple steps in M-MuLV leukemogenesis, more than one viral infection might be necessary. We previously presented a model in which M-MuLV infects the animal twice during leukemogenesis (8): first, to induce preleukemic hematopoietic hyperplasia, and later to reinfect a hyperplastic thymocyte. During the latter infection, long terminal repeat (LTR) activation of proto-oncogenes would occur. An important tool in these experiments was a novel recombinant M-MuLV variant, Mo+PyF101, which contains enhancer sequences from polyomavirus inserted into the M-MuLV LTR but otherwise is identical to wild-type M-MuLV (20). Mo+PyF101 M-MuLV did not induce leukemia in mice, even though it could efficiently establish infection in vivo (7). Importantly, Mo+PyF101 M-MuLV also did not induce preleukemic hyperplasia (8), which supported the relevance of this state to disease development.

Growth of hematopoietic cells in vitro might provide a means to explore mechanisms involved in induction of preleukemic hyperplasia. Procedures for long-term bone marrow cultures (LTBMC) have been developed by Dexter and co-workers (3, 10). These LTBMC support proliferation and differentiation of several hematopoietic lineages, including myeloid and lymphoid, but not erythroid lineages. LTBMC might provide useful in vitro systems for studying viral effects on hematopoiesis and the relationship to leukemogenesis (9, 11, 12, 16). In the experiments described here, we investigated the effects of in vivo and in vitro infection by M-MuLV on LTBMC. The results revealed an unexpected

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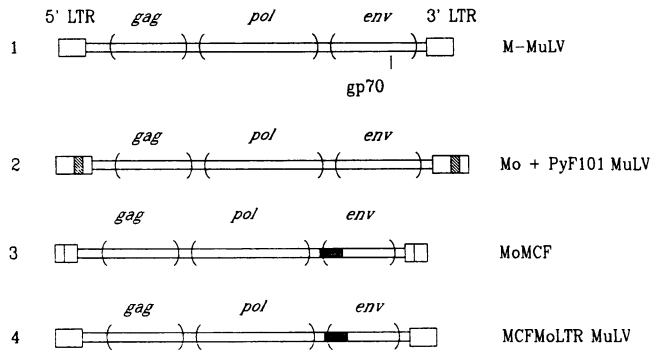

FIG. 1. M-MuLV and derivatives used in our experiments. The genomes of the viruses are shown in DNA forms. 1, Wild-type M-MuLV is a standard replication-competent retrovirus that induces T-cell lymphoma with a latency of 3 to 4 months under the conditions used here. 2, Mo+PyF101 M-MuLV contains enhancer sequences from the F101 mutant of polyomavirus inserted at −150 base pairs in the U3 region of the M-MuLV LTR. 3, M-MCF virus is an MCF recombinant of M-MuLV with substituted endogenous MCF sequences in the *env* region (N-terminal portion of gp70); its LTR also has a 75-base-pair deletion of one tandem repeat in the enhancers. 4, MCFMoLTR virus is M-MCF with a wild-type M-MuLV LTR with two copies of the tandem repeats. Symbols: □, M-MuLV; ■, MCF; ▨, PyF101.

cytopathic effect (CPE), which was attributed to the presence of MCF recombinants. Details of the phenomenon were studied in infected fibroblasts, which also showed the effect.

MATERIALS AND METHODS

Cells and viruses. NIH 3T3 cells and C3H10T1/2 cells were grown in Dulbecco modified Eagle medium supplemented with 10% calf serum.

The M-MuLVs used in these experiments are shown in Fig. 1. These include our original stock of M-MuLV (the A9 stock [15]); molecularly cloned M-MuLV (the 43D stock); Mo+PyF101 M-MuLV (21); and M-MCF, an MCF derivative of M-MuLV (35). The M-MCF stock was obtained from the supernatant of NIH 3T3 cells transfected with a molecularly cloned M-MCF provirus (kindly provided by Marguerite Vogt). MCFMoLTR was also an MCF derivative of M-MuLV, but the LTR contains two tandem repeats (see Results). It was generated from an M-MCF and an M-MuLV viral plasmid by molecular cloning (K. Brightman and H. Fan, unpublished data). Pseudotyped virus stocks were prepared by superinfection of cell lines producing one MuLV with an MuLV of a different interference group. Supernatants from the doubly infected cells were used as the pseudotyped virus stocks. Virus stocks were clarified culture medium (1,200 × *g* for 10 min) from infected cultures. Virus stocks were stored in aliquots at −80°C, and each aliquot was used only once after thawing. Infection of monolayer cells was performed as described previously (15).

Quantification of infected cells. NIH 3T3 cells were seeded at 2×10^5 to 4×10^5 cells per 10-cm dish and infected with undiluted virus stocks (approximately 10^6 PFU) 1 day later. Cells were maintained at 37°C for 3 days. They were trypsinized, suspended in culture media, and quantified in a Coulter Counter. For growth curves, this process was repeated at each transfer, and cumulative records were kept.

For colony formation assays, cells were seeded onto 10-cm dishes 4 days after infection at 100 to 1,000 cells per dish. Cells were allowed to grow for 10 days, at which point colonies were fixed by addition of formaldehyde to the media to a concentration of 3%, and then stained with Giemsa. The number and sizes of colonies were then determined.

Detection of viral DNA. Low-molecular-weight (unintegrated) DNA from virus-infected cells was prepared by Hirt fractionation (20). Undigested Hirt supernatant DNAs were analyzed by electrophoresis in a 0.7% agarose gel followed by Southern blot transfer (30) and hybridization with an M-MuLV probe. Total infected cell DNA was also prepared and analyzed as described previously (15).

LTBMC from virus-inoculated preleukemic mice. NFS inbred mice were inoculated subcutaneously at birth with 0.2 ml of undiluted virus stock (5×10^5 XC PFU of M-MuLV or Mo+PyF101 M-MuLV). At 4 to 7 weeks postinoculation, animals were sacrificed and bone marrow from the femurs was flushed directly into Corning T-25 tissue culture flasks (5×10^6 to 10×10^6 cells/femur per flask) containing Fisher media supplemented with 20% horse serum (GIBCO) and 10^{-6} M hydrocortisone sodium succinate (10). The cultures were maintained at 33°C in a 7% CO_2 atmosphere, and one-half of the media was changed weekly. The cultures were observed microscopically at regular intervals, and the nonadherent cells recovered during the medium changes were collected, counted with a hemacytometer, and also used for hematopoietic colony-forming assays.

For in vitro infection of LTBMC, 1 week after establishment of LTBMC, 200 μl of undiluted virus stock was added directly into each culture flask and the cultures were maintained as above.

Hematopoietic colony assays. Assays for mixed colony-forming cells (CFC_{mix}) in LTBMC were performed as described previously (8). Briefly, nonadherent cells collected from LTBMC were suspended in soft agar in growth medium containing WEHI-3B cell supernatant as a source of interleukin-3. Colonies (>50 cells) were scored microscopically after 7 days of incubation.

RESULTS

LTBMC from M-MuLV-inoculated preleukemic mice. To explore how M-MuLV infection induces hematopoietic hyperplasia, LTBMC from preleukemic mice were studied. LTBMC were established from preleukemic (4- to 7-week-old) M-MuLV-inoculated NFS mice or age-matched uninoculated controls. The cultures were observed microscopically at regular intervals, and nonadherent (hematopoietic) cells recovered during the weekly medium changes were collected, counted, and used in agar colony assays. Multipotential hematopoietic progenitor cells (CFC_{mix}) were detected by agar colony assays in the presence of interleukin-3. Surprisingly, instead of increased bone marrow cell proliferation, LTBMC from M-MuLV-inoculated mice showed less hematopoiesis. The quantification of total nonadherent cells and CFC_{mix} recovered from LTBMC from M-MuLV-inoculated preleukemic or uninfected mice is shown in Fig. 2A and B. We also noticed that LTBMC from preleukemic mice showed significantly fewer stromal cells (Fig. 3B) than those from uninoculated control bone marrow (Fig. 3A), even though the same numbers of total bone marrow cells were originally seeded. The reduction of hematopoiesis from M-MuLV-inoculated preleukemic mice seemed likely due to the depletion of bone marrow stromal cells since the growth of hematopoietic cells in LTBMC is dependent on stromal cells. In fact, the increase in hematopoiesis in LTBMC at later times is associated with establishment of a stromal

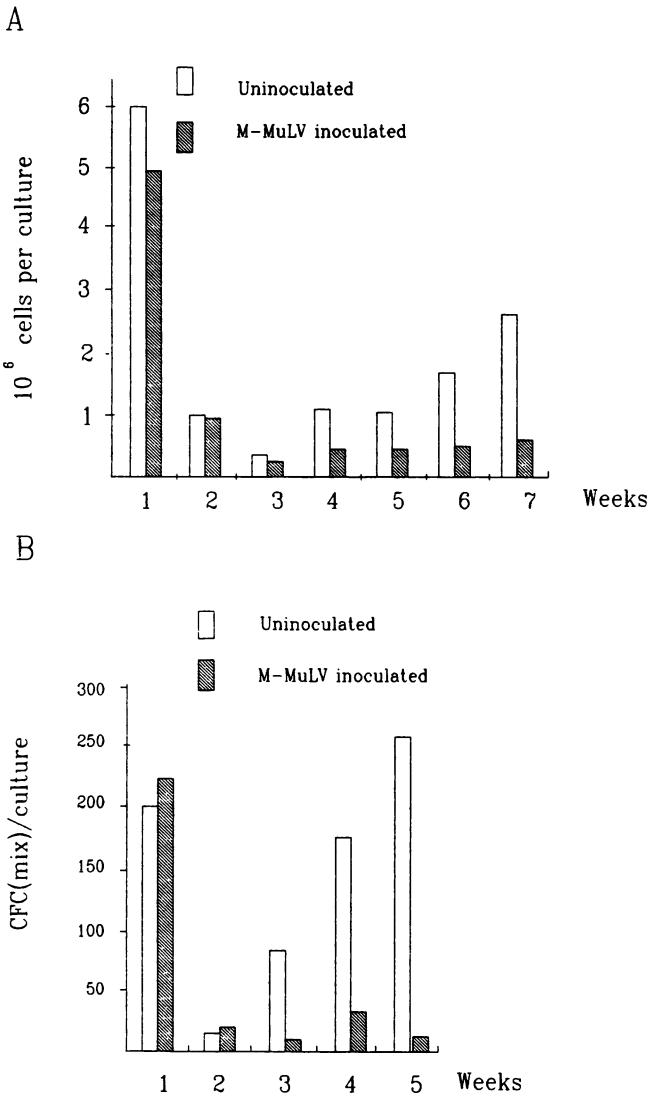

FIG. 2. Hematopoietic cells in LTBMC from preleukemic mice inoculated with M-MuLV. (A) Total nonadherent cells in LTBMC from preleukemic mice. Mice 4 to 7 weeks old were sacrificed, and the bone marrow cells from each femur were collected and cultured in Corning T-25 flasks. Culture conditions are described in the text. One-half volume of media was changed weekly, and nonadherent cells were counted with a hemacytometer. Each data point represents the average of results from two mice and four femurs. (B) Total CFC_{mix} in LTBMC from preleukemic mice. The nonadherent cells collected from the LTBMC of panel A were used in CFC_{mix} agar colony assays, as described in the text. The averages of total CFC_{mix} per culture are shown.

monolayer (3, 10). The stromal cell defect in preleukemic LTBMC appeared to be quantitative: with further incubation, stromal monolayers eventually formed in the LTBMC from preleukemic mice, and hematopoiesis increased at that time (not shown). In addition, once established, the stromal monolayers from preleukemic LTBMC could support hematopoiesis of exogenously added bone marrow cells as efficiently as uninfected LTBMC stroma (not shown). Moreover, bone marrow cells from preleukemic mice showed normal hematopoiesis when cultured on established stromal monolayers from normal mouse LTBMC.

In contrast, LTBMC from an age-matched Mo+PyF101 M-MuLV-inoculated animal did not show the stromal and hematopoietic defect (Fig. 3C), even though the culture was infected (not shown). We showed previously that Mo+PyF101 M-MuLV has greatly reduced leukemogenicity and does not induce splenic hyperplasia in vivo. Thus, there was a correlation between M-MuLV-induced hematopoietic defects in LTBMC in vitro and M-MuLV-induced splenic hyperplasia in vivo.

In vitro M-MuLV infection of LTBMC. We next tested whether the defect in preleukemic mouse LTBMC could be duplicated by in vitro infection with M-MuLV of LTBMC from uninoculated mice. Control mouse LTBMC were infected with an undiluted M-MuLV stock (A9) 1 week after establishment and monitored as above. Like LTBMC from in vivo infected mice, the in vitro infected cultures showed depletion of CFC_{mix} (Fig. 4), total nonadherent cells, and stromal cells as well (not shown). Thus, the same phenomenon could be obtained by in vitro infection of LTBMC with M-MuLV as seen in LTBMC from in vivo infected animals.

Involvement of MCF in in vitro bone marrow depletion. Recently, we found that the M-MuLV stock (A9) used in Fig. 4 contained an MCF derivative, as indicated by a diagnostic MCF-specific restriction fragment in Southern blot analysis. Therefore, it was necessary to test whether the MCF component was involved in any of the observed effects. A new M-MuLV stock (43-D) obtained by transfection of a molecular DNA clone of M-MuLV proviral DNA, and which was free of MCF derivatives, was used. LTBMC were infected by either A9 or 43-D M-MuLV viral stocks (Fig. 5). Surprisingly, no depletion was seen when the molecularly cloned M-MuLV (43-D) was tested. This suggested that the MCF derivative might play a critical role in the LTBMC defect in vitro and possibly an early effect in vivo as well. It is noteworthy that MuLV leukemogenesis is frequently accompanied by the generation of MCF recombinants.

CPE induced by M-MuLV infection of fibroblasts. M-MuLV infection is generally considered noncytopathic in productively infected cells, although some MCF derivatives cause CPE in certain cell lines (37). In light of the depletion of bone marrow stroma in LTBMC after in vitro infection with the M-MuLV A9 stock, it was interesting to test infection of NIH 3T3 fibroblasts. If a similar phenomenon were observed in NIH 3T3 cells, these cells would be more convenient than LTBMC for investigating the mechanism involved. NIH 3T3 cells were infected with the M-MuLV (A9) stock, and 3 days after infection, less cell density was observed in the infected culture dish in comparison to an uninfected culture (Fig. 6B); however, there was no striking change in cell morphology. In contrast, infection with the molecularly cloned M-MuLV 43-D viral stock did not affect the NIH 3T3 cells (not shown; see below). Thus, the same qualitative effects on NIH 3T3 were observed as for the LTBMC, and NIH 3T3 offered a more convenient model system for studying the virus-induced CPE.

To investigate the role of MCFs in CPE induction, we first prepared a pure stock of Moloney MCF recombinant virus. This was obtained by transfecting plasmid DNA containing a molecular clone of M-MCF provirus into NIH 3T3 cells. Supernatant from the transfected cells was then used as the M-MCF virus stock. When this pure M-MCF viral stock was infected into NIH 3T3, no striking CPE resulted (Table 1). Initially, this was somewhat surprising, since the difference between the cytopathic M-MuLV A9 and the noncytopathic M-MuLV 43-D stocks was the presence of M-MCF in the A9

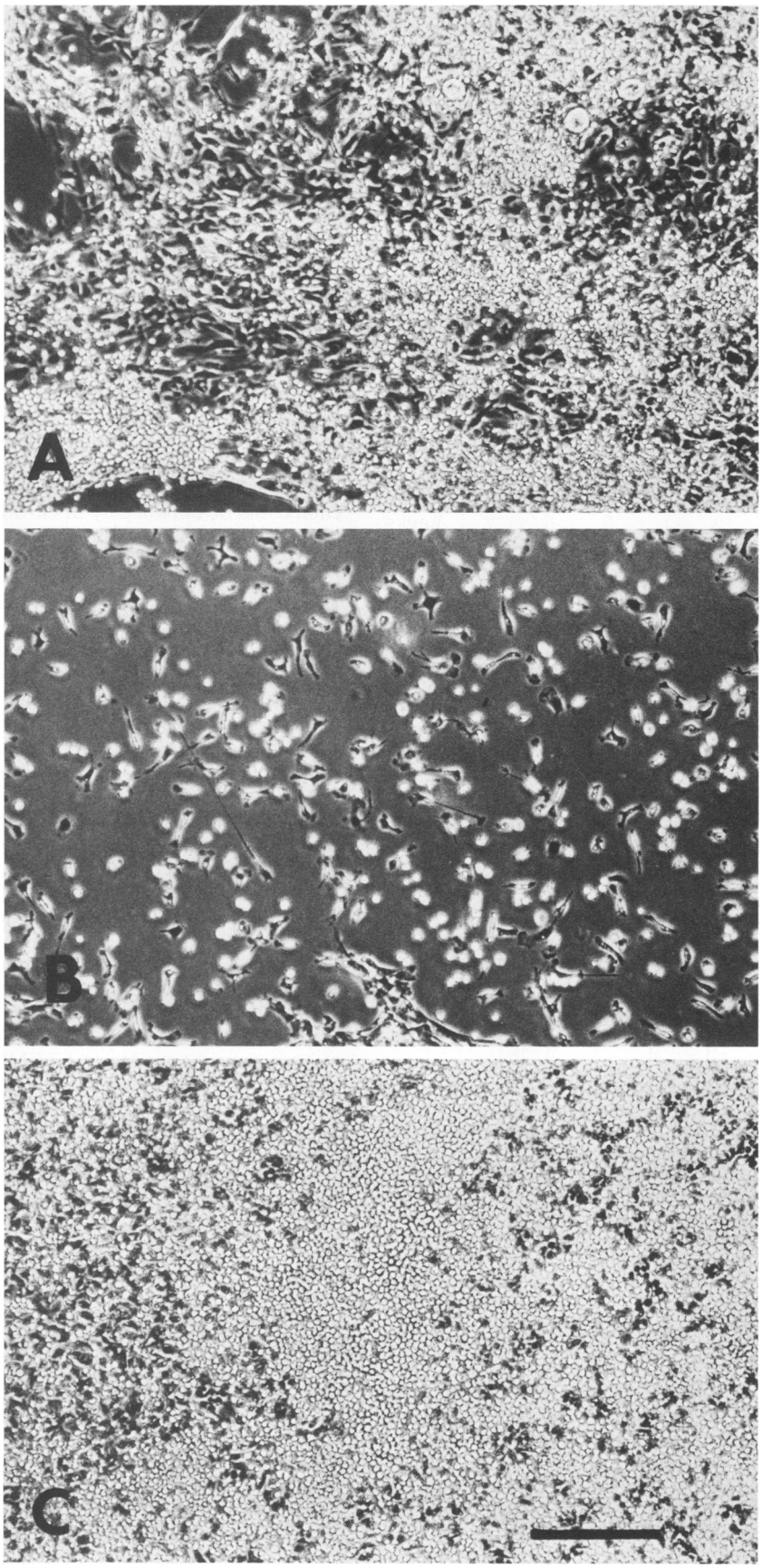

FIG. 3. LTBMC from preleukemic mice. LTBMC were carried for 4 weeks. (A) LTBMC from an uninoculated 7-week-old mouse. (B) LTBMC from an age-matched preleukemic mouse inoculated with M-MuLV (A9). (C) LTBMC from an age-matched Mo+PyF101 M-MuLV-inoculated mouse. Bar, 10 μm.

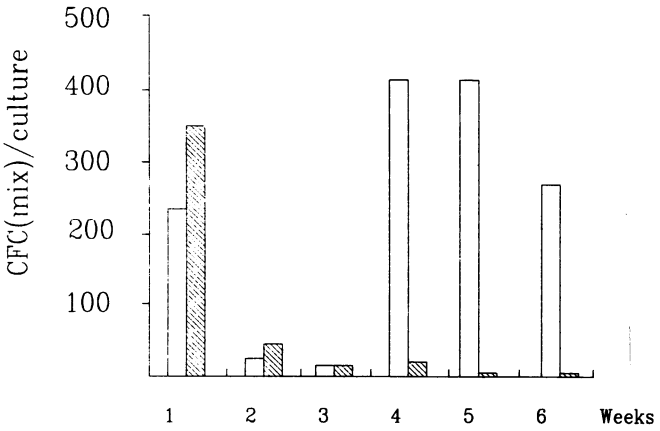

FIG. 4. CFC_{mix} from LTBMC infected in vitro. LTBMC were established from 5- to 7-week-old uninoculated mouse femurs. After 1 week of incubation, one-half of the cultures were infected with M-MuLV (A9). Cultures were carried in the same conditions as in the legend to Fig. 2. The collected nonadherent cells were assayed for CFC_{mix} at weekly intervals. Each time point represents averages of four LTBMC. Symbols: □, uninfected; ▨, M-MuLV infected.

stock. However, another explanation could be that both M-MuLV and MCF together were required for the CPE. Therefore, M-MuLV was infected into the M-MCF-producing cell line to generate cells producing mixed pseudotypes of M-MuLV and M-MCF viral stock. This mixed M-MuLV/M-MCF stock was tested in NIH 3T3 (Table 1; Fig. 6C). The results indicated that coinfection with M-MuLV and M-MCF was responsible for CPE in NIH 3T3 fibroblasts and, by analogy, for the bone marrow depletion in vitro. Recently, a new recombinant virus, MCFMoLTR, was generated which contains the same coding sequences as M-MCF driven by wild-type M-MuLV LTR (shown in Fig. 1). The M-MCF LTR has only one copy of the 75-base-pair tandem repeats in comparison to the two copies in the wild-type M-MuLV LTR. The presence of two tandem repeats would give higher levels of expression for MCF MoLTR. A mixed MCFMoLTR/M-MuLV stock was tested on NIH 3T3 cells (Table 1). This mixture caused even stronger CPE, presumably due to the stronger LTR in MCFMoLTR. Most naturally occurring M-MCFs have a wild-type M-MuLV LTR with two tandem repeats. Another mouse fibroblast cell line, C3H10T1/2, and also rat fibroblasts (R-6) showed the same effect.

Cellular effects induced by M-MuLV/M-MCF. The reduction of cell density after coinfection with M-MuLV and M-MCF virus could represent cell lysis, an arrest of cell growth, or a reduction of cell growth rate. To test whether this resulted from cell lysis, cultures at day 3 after infection were examined in more detail. First, as described above, except for lower cell density, there was no change in cell morphology or an increase in the number of floating dead cells in the doubly infected cultures (not shown). Second, the adherent cells from the culture were trypsinized, stained with trypan blue (which is excluded from live cells), and counted. The result showed the same low percentage of trypan blue-stained cells in the doubly infected culture as in the uninfected culture. These results indicated that the double infection with M-MuLV/M-MCF might not cause detectable direct cell killing. To investigate whether the double infection caused cell growth rate reduction or growth arrest, a colony formation assay was performed. Equal numbers of viable (trypan blue-excluding) cells from uninfected or infected cultures were seeded into 10-cm dishes at low densities and grown for approximately 10 days to allow colony formation. The dishes were then fixed and stained (Fig. 7A). The doubly infected culture (plate d) showed fewer macroscopic colonies than uninfected or singly infected cultures. Microscopic observation indicated that double infection significantly affected the sizes of the colonies, as well as the absolute numbers. Uninfected or singly infected cultures showed a preponderance of large colonies with many cells (e.g., Fig. 7B, a). In contrast, the doubly infected culture showed much smaller colonies with many fewer cells (e.g., Fig. 7B, b). Even when the smaller colonies were counted, there were fewer colonies in the doubly infected cultures. These results suggested that double infection led to growth arrest or cell killing in some cells and reduction in growth rate in others, although it may be difficult to distinguish between these phenomena completely. Occasional larger colonies in the doubly infected culture might reflect uninfected or singly infected cells in the population or cells resistant to the viral effects of double infection.

To characterize the effect of double infection further, growth curves were determined for the infected cultures (Fig. 8). Growth curves of uninfected cells and singly infected cells were indistinguishable. In contrast, doubly infected cells showed a striking drop in the growth rate (the slope of the curve) within 4 days of infection. Interestingly, at later times (e.g., 20 days), growth rate of the doubly infected culture recovered to the normal rate of the uninfected control. The results of Fig. 7 and 8 suggested that the effect of M-MuLV/M-MCF infection is cytostatic and that the effect may be transitory. One possible explanation for the resumption of growth rate is that cells resistant to the effect of double infection grew out of the culture. In any event, other experiments indicated that the cells that grew out were indeed doubly infected.

Quantification of viral replication. Weller et al. (38) previously described cytopathic strains of avian retroviruses that caused transient reductions in infected cell densities. This was reminiscent of the results reported here, although the potential presence of different virus components was not investigated in their studies. Moreover, Weller et al. found a correlation between over-replication of virus through viral superinfection and the induced CPE. Overreplication was reflected by accumulation of unintegrated viral DNA in Hirt supernatants. With regard to those experiments, M-MuLV and M-MCF viruses enter cells via different cell surface receptors. Each virus would render cells resistant to reinfection by viruses using the same receptors by blockage of those receptors, but those cells could still be superinfected by viruses using a different receptor. It seemed possible that a mixed M-MuLV/M-MCF stock might overreplicate (in a limited fashion) due to the presence of two viruses which can superinfect reciprocally. To test this possibility, DNAs were prepared from doubly infected cells showing CPE and analyzed by Southern blot hybridization with an M-MuLV probe. Hirt supernatant DNAs were analyzed without restriction enzyme digestion in Fig. 9A. The results indicated that there was no more than a twofold difference in unintegrated viral DNA (evident as a linear 9.5-kilobase DNA fragment) between singly and doubly infected cultures. Integrated proviruses were analyzed in Fig. 9B, in which total DNAs from infected cells were digested with *Pvu*II. A diagnostic 5′ internal fragment common to both M-MuLV and M-MCF of 1.7 kilobases is indicated (14). Consistent

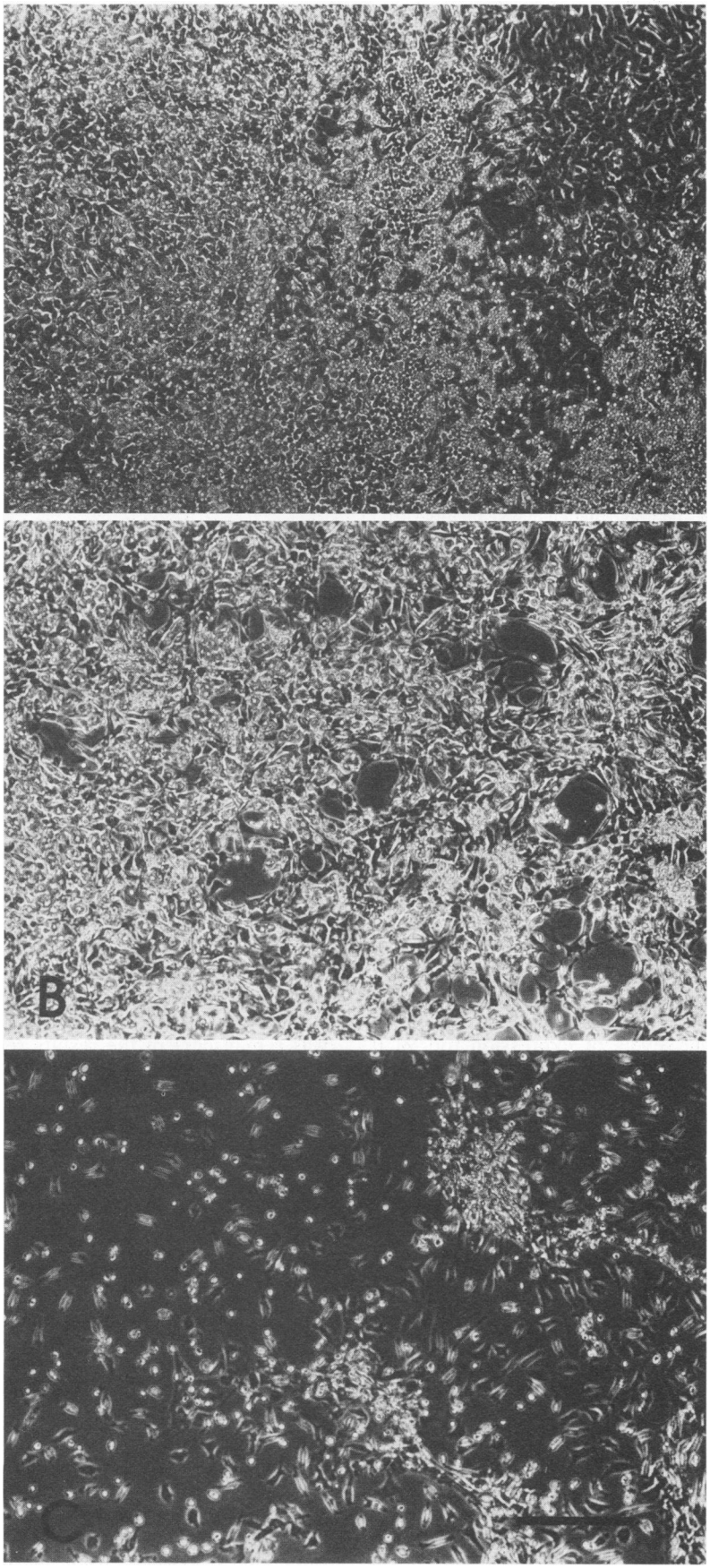

FIG. 5. LTBMC infected by different M-MuLV stocks. LTBMC were carried for 3 weeks. (A) Uninfected LTBMC from an uninoculated 7-week-old mouse. (B) A parallel LTBMC infected after establishment by the molecularly cloned M-MuLV (43-D) stock. (C) LTBMC infected by the M-MuLV (A9) stock. Bar, 10 μm.

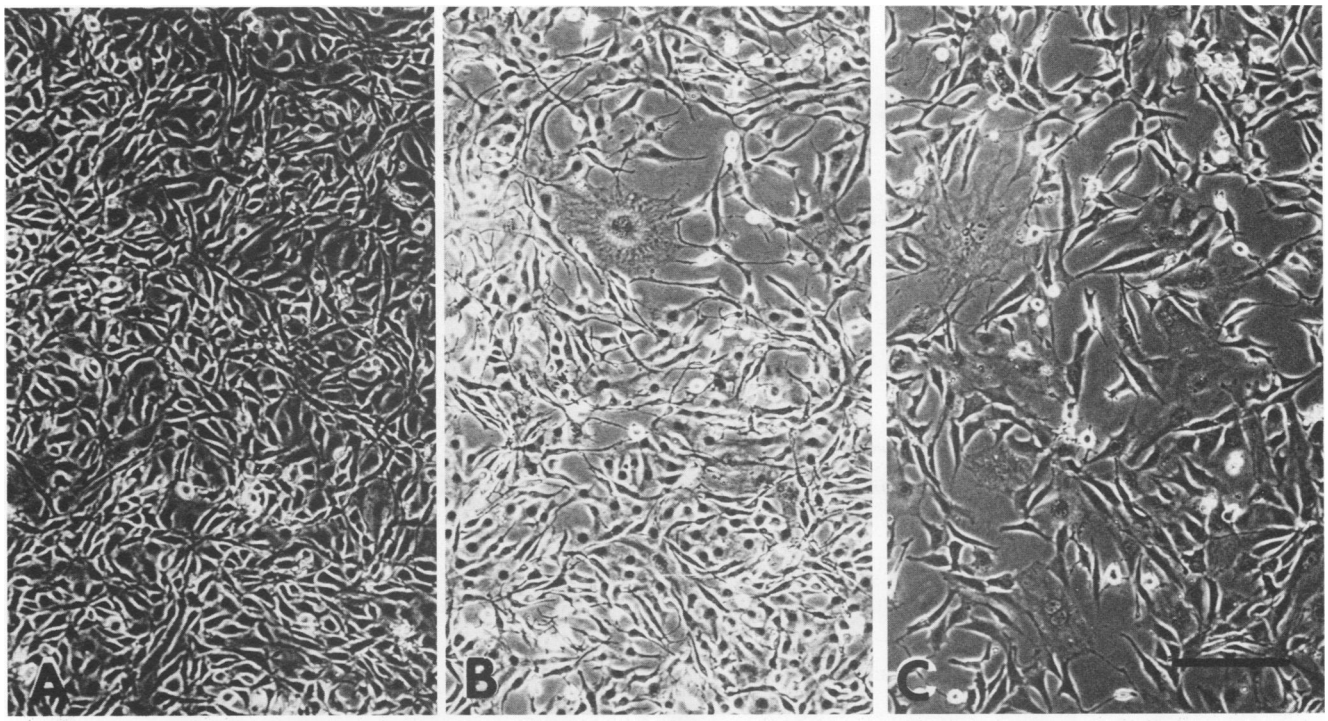

FIG. 6. Infection of NIH 3T3 cells. NIH 3T3 cells, 1.5×10^5, were seeded on 60-mm culture dishes, infected the next day with different undiluted viral stocks, and allowed to grow for an additional 3 days. (A) Uninfected cells; (B) cells infected with the M-MuLV (A9) viral stock; (C) cells infected with a mixed M-MuLV/M-M-MCF stock. Bar, 10 μm.

with Fig. 9A, the amount of total proviral DNA in the doubly infected cultures was approximately twofold more than in the singly infected cultures. In contrast, Weller et al. reported approximately 100-fold overreplication of virus for cytopathic avian retroviruses on day 4. Therefore, this suggested that the CPE induced by M-MuLV/M-MCF did not result from superinfection and overreplication.

DISCUSSION

In the experiments described here, LTBMC from preleukemic animals inoculated with M-MuLV showed defects in hematopoiesis. This was predominantly due to a quantitative defect in stromal cell establishment and appeared to result from coinfection with M-MuLV and an MCF recombinant.

TABLE 1. Quantification of cell density after infection[a]

Viruses	Relative cell density (%)
Expt 1	
No virus	100
M-MuLV (43D)	93
M-MCF	89
M-MuLV (43D)/M-MCF	54
Expt 2	
No virus	100
M-MuLV (43D)	82
MCFMoLTR	95
MCFMoLTR/M-MuLV	11

[a] NIH 3T3 cells were seeded at 1.5×10^5 cells per 60-mm dish on day 1. Cells were infected with various undiluted viral stocks on day 2 and then trypsinized and counted on day 4.

The defect in LTBMC hematopoiesis also appeared to correlate with leukemogenesis, since a poorly pathogenic variant, Mo+PyF101 M-MuLV, did not induce the same effect. In vitro infection of NIH 3T3 fibroblasts with mixed M-MuLV/M-MCF stocks also resulted in CPE.

Initially, the observation of hematopoietic defects in LTBMC from preleukemic M-MuLV-inoculated mice seemed contradictory to our previous observations of in vivo hematopoietic hyperplasia. One possibility is that the preleukemic LTBMC defects could be an in vitro phenomenon, reflecting the presence of MCF recombinants in preleukemic mice, but irrelevant to the disease process. An alternative possibility is that preleukemic animals also have defects in bone marrow hematopoiesis in vivo. Indeed, in our previous study, preleukemic hyperplasia was most pronounced in the spleen (five- to tenfold), while the bone marrow showed little or, at most, twofold changes (8).

The possibility of bone marrow defects in preleukemic animals raises a potential mechanism for the splenic hyperplasia. In other situations, natural or experimentally induced inhibition of bone marrow hematopoiesis results in compensatory extramedullary hematopoiesis in peripheral organs such as the spleen and lymph nodes (1). In humans, dysfunction of bone marrow hematopoiesis has also been associated with preleukemic conditions (22). In the mouse, ablation of bone marrow hematopoiesis with the bone-seeking isotope ^{89}Sr results in marked splenic hyperplasia and increases in CFC_{mix} in the spleen (1, 36). Moreover, administration of ^{89}Sr can induce leukemia in mice (39). Thus, M-MuLV may induce an analogous preleukemic state by generation of MCF recombinants which lead to defects in bone marrow hematopoiesis and compensatory splenic hyperplasia. Consistent with this, recent experiments indicate that preleuke-

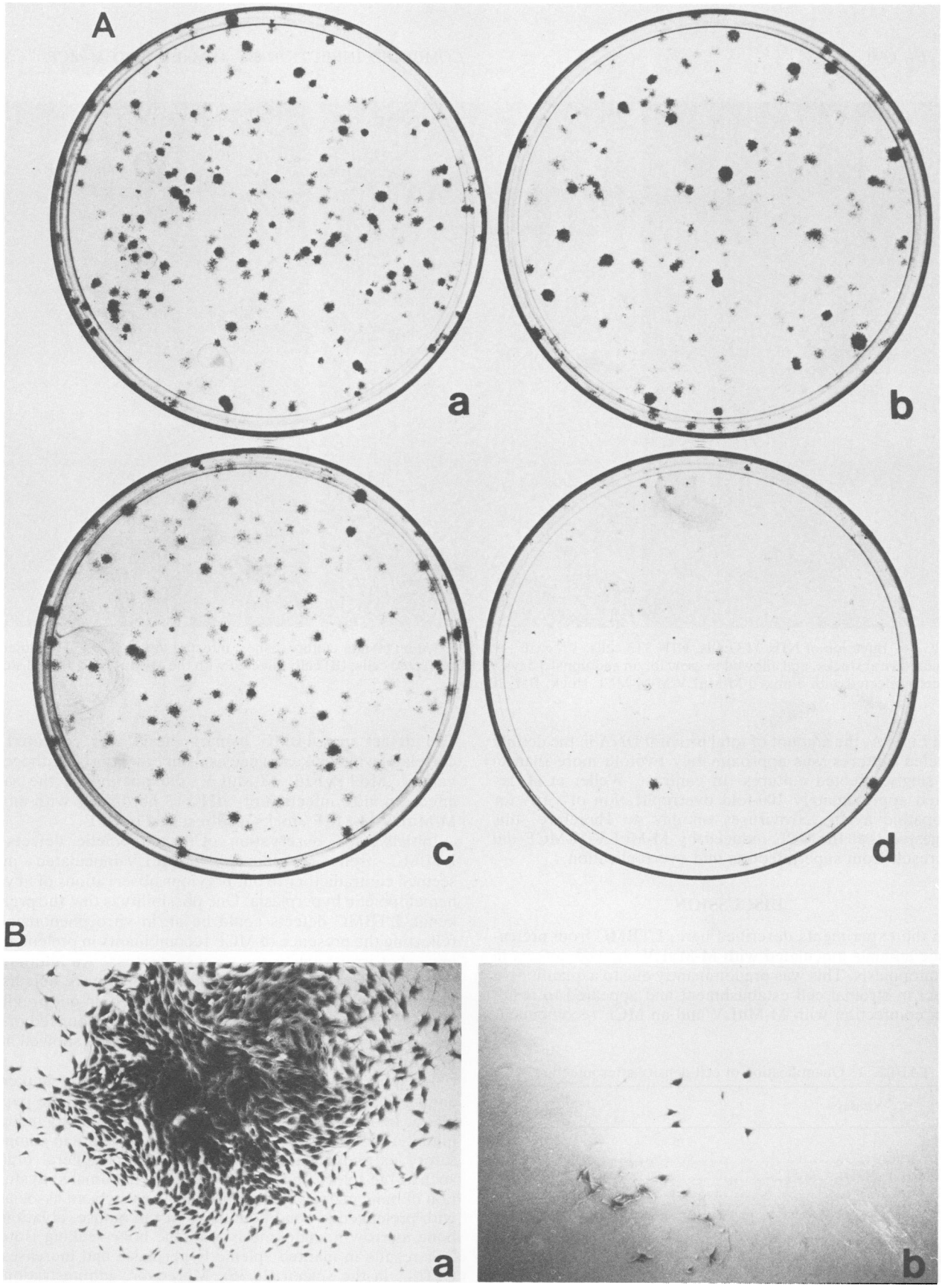

FIG. 7. Colony formation for infected NIH 3T3 cells. NIH 3T3 cells were infected with different virus stocks as in the legend to Fig. 6. Four days after infection, cells were trypsinized and plated at various dilutions on 10-cm dishes; incubation was continued for 10 days. (A) Colony formation by: a, uninfected cells; b, M-MuLV(43D)-infected cells; c, M-MCF-infected cells; d, cells doubly infected with M-MuLV/M-MCF. Stained dishes are shown. (B) Typical colonies from the uninfected (a) (>100 cells) and the doubly infected (b) (<20 cells) cultures are shown at higher magnification.

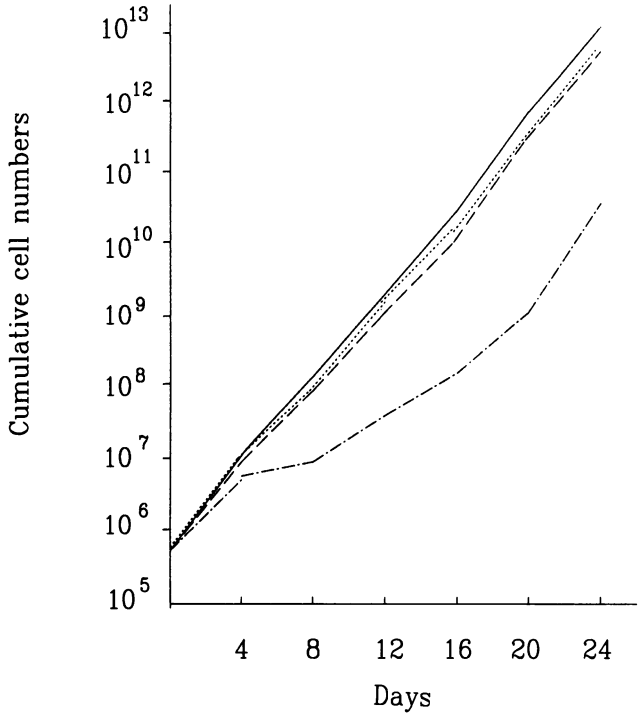

FIG. 8. Growth curves of infected NIH 3T3 cells. The same infection conditions as for Fig. 6 were used. After infection, cultures were transferred and the number of cells was counted every 4 days. The total cumulative number of cells was calculated. Symbols: ———, uninfected cells; – – – –, infection by M-MuLV (43D); . . . . , infection by M-MCF; . – . – ., coinfection with M-MuLV/M-MCF.

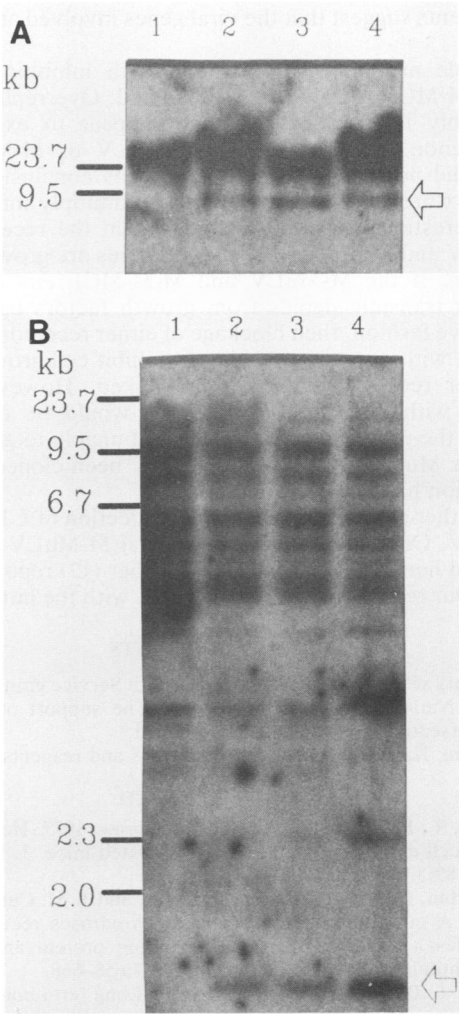

FIG. 9. Proviruses in infected cells. (A) Hirt supernatant DNAs were prepared from infected cells 4 days after infection. The DNAs were analyzed by gel electrophoresis without restriction endonuclease digestion, and Southern blots were analyzed by hybridization with radioactive M-MuLV DNA probe. Lanes: 1, uninfected cells; 2, M-MuLV(43D)-infected cells; 3, M-MCF-infected cells; 4, M-MuLV/M-MCF-infected cells. Positions of molecular weight markers are at the left. The position of unintegrated viral DNA (8.8 kilobases [kb]) is indicated (open arrow at the right). Hybridization to high-molecular-weight DNA (>24 kb) probably represented endogenous MuLV-related virus sequences in contaminating high-molecular-weight cellular DNA (e.g., lane 1), as well as the integrated proviral form. (B) Total infected cell DNA was digested with *Pvu*II and analyzed by Southern blot hybridization, using an M-MuLV *gag* probe. Both M-MuLV and M-MCF would give a diagnostic internal 1.7-kb fragment (open arrow) from −254 to 1,434 base pairs on the viral genome (15). The order of the lanes is the same as for panel A.

mic hyperplasia does not result directly from infection of hematopoietic progenitors, but through an indirect mechanism (B. K. Brightman, B. R. Davis, and H. Fan, submitted for publication).

Our studies on M-MuLV leukemogenesis have been greatly aided by the Mo+PyF101 M-MuLV variant, which is poorly leukemogenic. Mo+PyF101 M-MuLV is identical to wild-type M-MuLV, except that is contains inserted enhancer sequences from polyomavirus in the LTR. LTBMC from Mo+PyF101 M-MuLV-inoculated animals did not show the defect in hematopoiesis (Fig. 3), which correlated with the nonleukemogenicity of the virus. We recently determined that Mo+PyF101 M-MuLV-inoculated mice do not generate MCF recombinants (B. K. Brightman, D. Trepp, and H. Fan, unpublished data). This finding is completely consistent with the results reported here and is supportive of the idea that an in vivo bone marrow defect is involved in M-MuLV leukemogenesis.

The finding that NIH 3T3 fibroblasts also showed the same growth defects as bone marrow stroma when infected with the same viral stocks allowed more convenient analyses of the mechanisms involved. The results indicated that coinfection with M-MuLV and M-MCF was required, and neither virus alone was sufficient. Furthermore, the growth defect probably did not mainly represent killing of infected cells, but rather inhibition or reduction in cell growth. Thus, the effect is better considered cytostatic rather than cytolytic.

The results reported here were for M-MuLV and M-MCF. MCF recombinants have been implicated in leukemogenesis by most other MuLV strains as well (28, 29). It will be interesting to test whether these same phenomena pertain to other MuLVs and their corresponding MCFs. Whether other combinations of MuLVs induce CPE is also interesting. Combined infections with M-MuLV plus amphotropic MuLV (which infects mouse cells via a receptor distinct from those for ecotropic or MCF MuLVs) or M-MCF/Am-MuLV did not cause the growth reduction. Thus, the effect was specific to M-MuLV plus M-MCF. Preliminary

experiments suggest that the viral genes involved are the *env* genes.

Possible mechanisms for the growth inhibition by M-MuLV/M-MCF remain to be elucidated. Overreplication in the doubly infected cells did not appear to explain the phenomenon. That M-MuLV/Am-MuLV or M-MCF/Am-MuLV did not cause CPE supports this conclusion, since these mixtures presumably could also multiply infect cells. One interesting possibility could be that the receptors for M-MuLV and Mo-MCF *env* glycoproteins are growth factor receptors. If the M-MuLV and M-M-MCF *env* receptors normally transmit signals from growth factors used in an alternative fashion, then blockage of either receptor alone by infection with one virus would not inhibit cell growth since the other receptor would be unblocked. However, cells infected with both types of viruses would be unable to transmit the proper growth signals and unable to grow. The ecotropic MuLV receptor has recently been cloned (2), but its function has not been determined.

Two other studies have described infection of LTBMC by M-MuLV. One study (16) reported that M-MuLV infection increased hematopoiesis, while the other (12) reported inhibition. Our results are more consistent with the latter study.

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