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Protection against Retroviral Diseases after Vaccination Is Conferred by Interference to Superinfection with Attenuated Murine Leukemia Viruses

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Cell cultures expressing a retroviral envelope are relatively resistant to superinfection by retroviruses which bear envelopes using the same receptor. We tested whether this phenomenon, known as interference to superinfection, might confer protection against retroviral diseases. Newborn mice first inoculated with the attenuated strain B3 of Friend murine leukemia virus (F-MuLV) were protected against severe early hemolytic anemia and nonacute anemiant erythroleukemia induced by the virulent strain 57 of F-MuLV. Vaccinated animals were also protected as adults against acute polycythemic erythroleukemia induced upon inoculation with the viral complex containing the defective spleen focus-forming virus and F-MuLV 57 as helper virus. Animals were inoculated as newborns, which is known to induce immune tolerance in mice, and the rapid kinetics of protection, incompatible with the delay necessary for the immune response to develop, indicated that protection was not due to an immune mechanism but rather was due to the rapid and long-lasting phenomenon of interference. This result was confirmed by combining parental and envelope chimeric MuLV from different interference groups as vaccinal and challenge viruses. Although efficient protection could be provided by vaccination by interference, we observed that attenuated replication-competent retroviruses from heterologous interference groups might exert deleterious synergistic effects.

A cell culture infected with a retrovirus becomes relatively resistant to superinfection by a related retrovirus (40, 61). This in vitro phenomenon, known as interference to superinfection, has been observed with all retroviral species tested (40, 44, 55, 61). It involves the viral envelope glycoprotein (14, 20, 22, 23, 55), results from a restricted penetration into the cell (53, 54), and has been observed only when both viruses share the same receptor (18, 21, 59, 60). A mechanistic model has been postulated according to which penetration of new virions is restricted as a result of direct interaction of the viral envelope glycoprotein produced by infected cells with its cellular receptor.

Chickens and mice express several endogenous envelope-like glycoproteins which confer resistance to diseases induced by exogenous viruses. It has been suggested that such protection is due to a similar interference phenomenon in vivo (25, 28, 38, 64). We reasoned that protection against retroviral diseases by in vivo interference might also be achieved through vaccinal exogenous infection. Most of the retroviral models would not allow the testing of this hypothesis because involvement of an immune mechanism in protection could not be excluded. We chose the Friend murine leukemia virus (F-MuLV) model because inoculation of mice as newborns with this virus causes distinct pathogenic effects and because these conditions of inoculation have been shown to induce T-lymphocyte immune tolerance against MuLV (10). Also, mice inoculated as newborns do not develop MuLV-specific circulating antibodies (42, 48), although this might also be due to deposition of immune complexes in the kidneys (33). In mice inoculated as newborns, the virulent strain 57 of F-MuLV (34) induces successively a severe early hemolytic anemia (EHA) and an anemiant erythroleukemia, generally readily detected at 2 to 3 and 6 to 8 weeks of age, respectively. Contrastingly and despite good spreading ability, the closely related strain B3 of F-MuLV (30, 51) does not induce severe EHA, and leukemia develops only after a marked latency which generally exceeds 6 months of age (46, 48, 51). Neonatal vaccination of mice with the attenuated F-MuLV B3 conferred efficient protection against both diseases induced upon infection with F-MuLV 57 as well as against acute leukemia induced by viral complexes containing spleen focus-forming virus (SFFV) (17, 26). Furthermore, the use of MuLV strains belonging to different interference groups allowed us to establish that protection was observed only when vaccinal and challenge viruses had envelopes which shared the same receptor. We also described certain limitations in using vaccination by interference with replication-competent retroviruses.

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MATERIALS AND METHODS

Cell cultures, viruses, and viral stocks. All cell lines were cultivated in Dulbecco's modification of Eagle's medium (DMEM) complemented with glutamine (2 mM), penicillin (50 IU ml^{-1}), streptomycin (50 mg ml^{-1}), and 10% heat-inactivated fetal calf serum. The following viral strains were used: the ecotropic F-MuLV 57 (30, 34), F-MuLV B3 (30, 51), and Moloney MuLV 8.2 (M-MuLV) (45), the amphotropic 4070A (Ampho) (21), the polytropic mink-cell focus-inducing virus Fr-MCF-1 (MCF) (58), and the chimera F/MCF Env, which contains the polytropic Fr-MCF-1 envelope in the F-MuLV 57 background (35). Viral stocks were prepared on either NIH 3T3 or *Mus dunni* cells (29). Supernatants were removed from subconfluent infected cells 10 to

18 h after the replacement of the medium, filtered (0.45-μm-pore-size filter), and stored in aliquots at −60°C. SFFV viral stocks were prepared as follows. For SFFV/F-MuLV stock, *M. dunni* cells were infected at a high multiplicity of infection (MOI) with helper-free SFFV stock prepared from the Ψ-2 *trans*-complementing clone 3B2 (63) and superinfected with F-MuLV 57 at a low MOI; stocks were obtained from these cells when F-MuLV infection was confluent as tested by focal immunofluorescence assay (FIA) (50). For SFFV/Ampho stock, *M. dunni* cells were infected at a high MOI with a viral preparation obtained from helper-free NRK/SFFVp cells (2) infected with Ampho. Viral stocks were titrated by FIA as previously described (50) with monoclonal antibodies discriminating among the envelope glycoproteins of F-MuLV, M-MuLV, Ampho, and polytropic viruses (8, 9, 16).

Origin, infection, and clinical evaluation of mice. All experimentation was conducted on ICFW mice, an inbred line derived from Carworth Farms White outbred mice (62). Newborn mice were inoculated intraperitoneally with 0.05 ml of viral stock between 1 to 4 days of age as indicated. Young adult mice (6 to 7 weeks of age) were inoculated intravenously at the retro-orbital sinus with 0.2 ml of viral stock. As described previously (51), hematocrits (the volume of erythrocytes expressed as the percentage of blood volume) were determined on blood samples taken under ether anesthesia by puncture at the retro-orbital sinus with 20-μl heparinized capillary tubes (Drummond Scientific Company, Broomall, Pa.), and EHA was determined from three bleedings performed at 3-day intervals from 16 to 24 days of age. For evaluation of protection, in each litter, only the lowest hematocrits of the bleeding series were taken into account. The anemiant erythroleukemia induced by F-MuLV 57 was monitored by regular spleen palpation under ether anesthesia at approximately 2-week intervals. Mice displaying gross organ enlargement were bled for the determination of hematocrits, and diagnoses were confirmed by sacrifice and autopsy of moribund animals. The diagnosis of anemiant erythroleukemia depended on the association of severe anemia (hematocrits less than 35%) with hepatosplenomegaly and the absence of any enlargement of lymph nodes or thymus. Mice challenged with SFFV stocks were monitored weekly by palpation of the spleen and determination of hematocrits. Acute polycythemic erythroleukemia was characterized by the rapid onset of gross splenomegaly followed by an imposing polycythemy with hematocrits up to 85%.

Infectious center assays. Animals were sacrificed at 21 to 25 days of age, and spleens were collected and dispersed in complete DMEM. Spleen cell suspensions were washed once and adjusted to 10^7 live nucleated cells ml^{-1}. Serial dilutions of splenocytes were prepared in complemented medium, and 10^5, 10^4, 10^3, or 10^2 cells in 1 ml were added to approximately 10^5 NIH 3T3 cells seeded the day before on 60-mm-diameter culture dishes. After approximately 18 h of cocultivation, splenocytes were removed and cultures were permitted to grow to confluency before infectious centers were enumerated by FIA (49, 50) using monoclonal antibodies discriminating between F- and M-MuLV (9, 16) (generous gifts of B. Chesebro).

Statistical analyses. The means of hematocrits were compared by using the two-tailed *t* test. Differences were considered statistically significant when *P* values were less than 0.05.

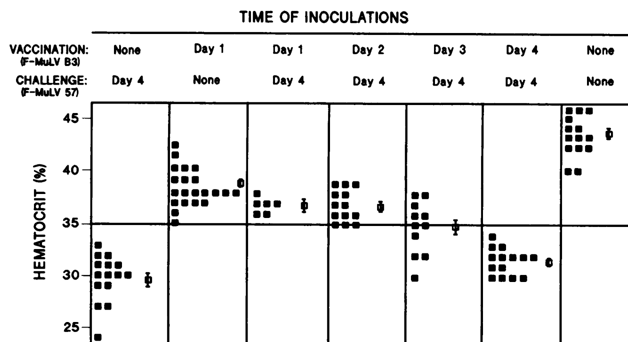

FIG. 1. Rapid protection after vaccination with ecotropic MuLV against severe EHA induced by F-MuLV 57. ICFW mice were challenged with F-MuLV 57 at 4 days of age without vaccination or after vaccination with F-MuLV B3 at 1, 2, 3, or 4 days of age, as indicated. Hematocrits from unchallenged animals vaccinated at 1 day of age and noninoculated animals are also shown. Both viral stocks were adjusted to 10^5 focus-forming units per ml. Hematocrits were determined from bleeding series between 16 and 24 days of age. Only hematocrits of the lowest series are shown for each group. Severe EHA is characterized by hematocrits below 35%. Closed squares correspond to individual mice; open squares indicate the mean hematocrits ± standard error of the mean for each group.

RESULTS

Rapid protection against EHA and nonacute erythroleukemia after preinoculation with F-MuLV B3. Mice inoculated at 4 days of age with the virulent strain 57 of F-MuLV developed a severe EHA at 2 to 3 weeks of age with hematocrits below 35% (Fig. 1) and an anemiant erythroleukemia with gross splenomegaly 3 to 5 weeks later (Fig. 2), whereas F-MuLV B3 induced only mild EHA (Fig. 1). Animals preinoculated as newborns with F-MuLV B3 and challenged 3 days later with F-MuLV 57 did not develop severe EHA (Fig. 1). We evaluated the delay between preinoculation and challenge necessary for the establishment of this protection. As shown in Fig. 1, significant protection against severe EHA, although partial, was observed even when preinoculation and challenge were only 1 day apart

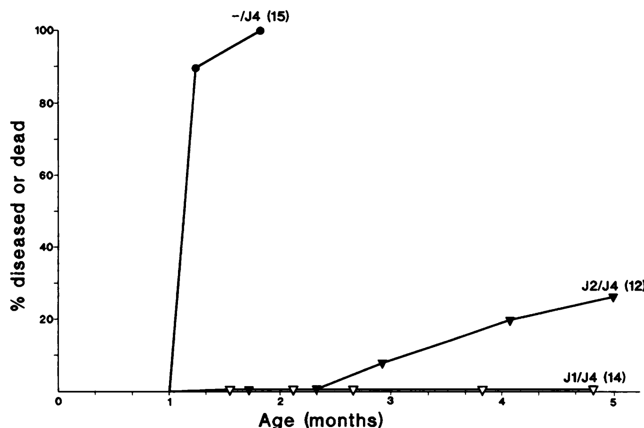

FIG. 2. Protection after vaccination with F-MuLV B3 against anemiant erythroleukemia induced by F-MuLV 57. Occurrence of leukemia was monitored by palpation in nonvaccinated mice (−/J4) and in mice vaccinated with F-MuLV B3 at 1 (J1/J4) or 2 (J2/J4) days of age before challenge at day 4 with F-MuLV 57. The number of animals in each group is indicated in parentheses.

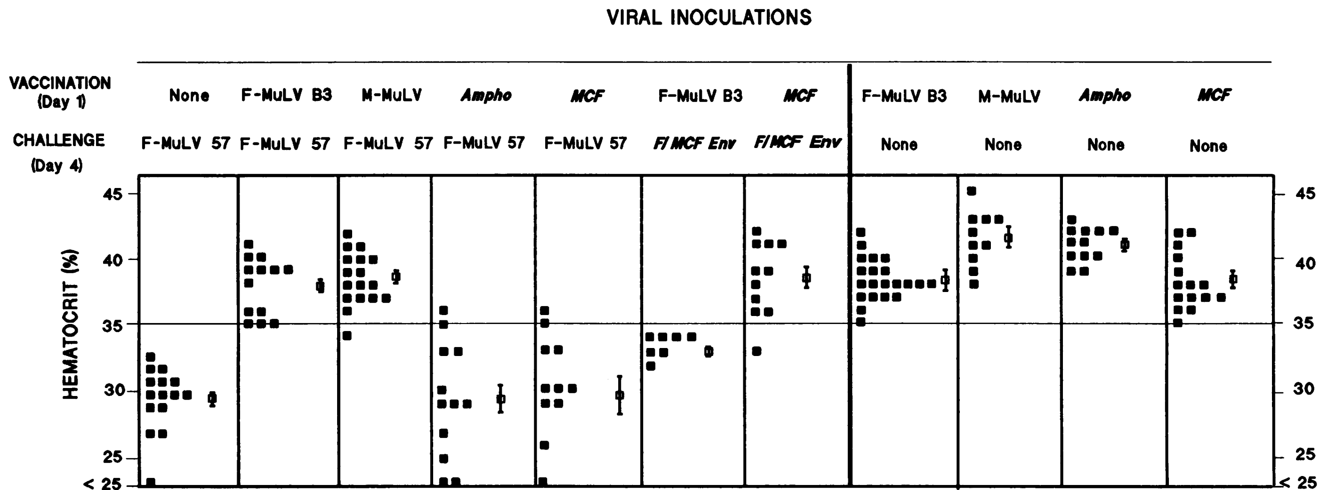

FIG. 3. Protection against severe EHA with vaccinal and challenge MuLV belonging to the same interference group. ICFW mice were vaccinated at 1 day of age with attenuated MuLV from the ecotropic (F-MuLV B3 and M-MuLV), amphotropic (Ampho), or polytropic (MCF) interference groups and were challenged at 4 days of age with either the ecotropic F-MuLV 57 or the polytropic chimeric virus F/MCF Env (35). Vaccinal viral stocks (F-MuLV B3, M-MuLV, Ampho, and MCF) had titers of 10^5 to 7.10^6 focus-forming units per ml; challenge viral stocks (F-MuLV 57 and F/MCF Env) had titers of approximately 10^5 focus-forming units per ml.

(mean hematocrits of 35% versus 30%; $P < 10^{-3}$), and optimal protection with mean hematocrits of approximately 38%, similar to levels observed in unchallenged animals, was observed as rapidly as 2 days after preinoculation. Such rapid establishment of protection after preinoculation of the attenuated F-MuLV B3 was also observed with regard to appearance of erythroleukemia. Thus, preinoculation performed 2 days before challenge considerably increased the latency (Fig. 2), and preinoculation performed 3 days before challenge completely prevented the appearance of erythroleukemia for up to 5 months, similar to what was observed in unchallenged animals. Animals challenged only a few minutes after preinoculation were also partially protected, since they developed erythroleukemia after a significantly increased latency compared with nonvaccinated animals (3.5 months versus 1.5 months) (not shown).

Protection requires that vaccinal and challenge viruses belong to the same interference group. The remarkably rapid establishment of protection indicated that a nonimmune mechanism was involved. We tested whether this nonimmune protection was indeed due to an interference-like mechanism. For this purpose, we used vaccinal and challenge viruses belonging to different interference groups such as MuLV of the ecotropic, amphotropic, and polytropic groups as well as a chimeric virus which substituted the envelope gene of the polytropic MCF for that of the ecotropic F-MuLV 57 (F/MCF Env) (35). As opposed to the results of vaccination with the ecotropic F-MuLV B3, heterologous vaccination was not protective. Thus, newborns vaccinated with either Ampho or MCF were generally not protected against severe EHA after challenge with the ecotropic F-MuLV 57 (Fig. 3). Moreover, animals vaccinated with the ecotropic F-MuLV B3 developed severe EHA upon challenge with the polytropic F/MCF Env, whereas most mice vaccinated with MCF and challenged with F/MCF Env did not develop severe EHA (Fig. 3). Also, animals vaccinated with either of the nonecotropic viruses developed erythroleukemia as rapidly as did nonvaccinated animals upon challenge with F-MuLV 57 (not shown). These results confirmed that protective vaccination developed mostly through in vivo interference.

Efficiency of vaccination by interference may vary between viruses from the same interference group. To further examine characteristics of vaccination by interference, we used as vaccinal virus the ecotropic M-MuLV, which belongs to the same interference group as F-MuLV but has a different target cell spectrum. Thus, animals inoculated as newborns with M-MuLV have only a very slight drop of hematocrits (41.5% versus 43% in noninoculated animals) (Fig. 3) and develop only thymomas after a longer latency than erythroleukemia induced by F-MuLV (45, 47). We observed that mice vaccinated with M-MuLV and challenged with F-MuLV 57 were efficiently protected from severe EHA (Fig. 3). Nonetheless, despite this efficient protection against severe EHA, approximately one-third of the animals vaccinated with M-MuLV developed erythroleukemia within 2 months (not shown).

Protective vaccination by interference may require restriction without suppression of spreading of the challenge virus. Because the protection was only partial after use of a heterologous virus from the same interference group, as described above for M-MuLV, we tested whether such vaccination interfered efficiently with the in vivo overall spreading of the challenge virus. Distinction between vaccinal and challenge ecotropic viruses was possible when M-MuLV and F-MuLV strains were combined by using monoclonal antibodies (9, 16) which allowed the specific quantitation of cells productively infected with either virus (Fig. 4). Although we observed that vaccination with either M-MuLV (Fig. 4A) or F-MuLV (Fig. 4B) interfered significantly with the dissemination of the other, several animals had reduced but still significant levels of cells infected with the challenge virus. Therefore, the actual level of in vivo interference with the spreading of F-MuLV observed after vaccination with M-MuLV was sufficient to fully protect against severe EHA but did not allow efficient protection against erythroleukemia.

Vaccination by interference protects against SFFV-induced

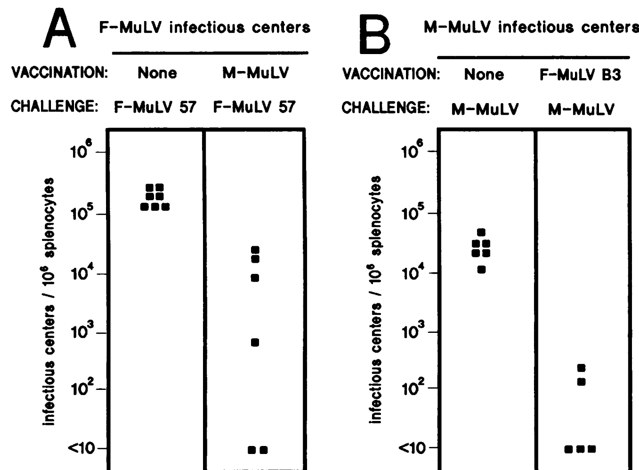

FIG. 4. In vivo interference between spreading of the ecotropic M- and F-MuLV. (A) Nonvaccinated mice or mice vaccinated neonatally with M-MuLV were challenged at 4 days of age with F-MuLV 57. (B) Nonvaccinated mice or mice vaccinated with F-MuLV B3 were challenged at 4 days of age with M-MuLV. The same viral stocks as described in the legend to Fig. 3 were used. Animals were sacrificed at 21 to 25 days of age, and the spreading of the challenge virus was determined by FIA (50) using envelope-specific monoclonal antibodies (generous gift of B. Chesebro).

acute disease but may stimulate the pathogenic properties of challenge viruses from heterologous interference groups. Efficient protection against nonacute diseases induced by MuLV lacking an oncogene could be achieved after vaccination by interference. We also tested the efficiency of vaccination by interference in the protection against a more potent leukemogenic process, such as that triggered through the oncogene-like SFFV defective envelope (26). The SFFV/F-MuLV viral complex induced an acute polycythemic erythroleukemia within 2 to 3 weeks after inoculation of adult mice. Animals vaccinated as newborns with F-MuLV B3 were efficiently protected against disease induced by SFFV/F-MuLV and were still free of disease over 15 weeks after challenge (Table 1). We also tested combinations of vaccinal and challenge helper viruses from different interference groups. The observation that vaccination with Ampho or polytropic MCF did not prevent polycythemic erythroleukemia induced after challenge with the ecotropic SFFV/F-MuLV complex (Table 1) confirmed that protection in this model was also based on an interference mechanism. We further examined this aspect by using the SFFV/Ampho viral complex. The already weak virulence of this SFFV/Ampho complex was no longer observed upon vaccination with Ampho, although the small sample size did not allow evaluation of the statistical significance of this result (Table 1). More interestingly, we observed that all animals vaccinated either with the ecotropic F-MuLV B3 or with the polytropic MCF were rapidly and severely diseased after challenge with SFFV/Ampho. These results indicated that a deleterious synergy occurred in this model when vaccinal and challenge viruses from heterologous interference groups were used.

TABLE 1. Effects of vaccinations with MuLV from different interference groups on induction of polycythemic erythroleukemia by SFFV

Vaccination[a]	Challenge[b]	No. diseased/total inoculated	Time of first appearance of disease (wk after challenge)
None	SFFV/F-MuLV	5/5	3
F-MuLV	SFFV/F-MuLV	0/5	>15
Ampho	SFFV/F-MuLV	3/3	3
MCF	SFFV/F-MuLV	4/4	3
None	SFFV/Ampho	1/5	5
Ampho	SFFV/Ampho	0/5	>12
F-MuLV	SFFV/Ampho	8/8	3
MCF	SFFV/Ampho	4/4	3

[a] Newborn ICFW mice were vaccinated with the ecotropic F-MuLV B3, the amphotropic MuLV 4070A (Ampho), or the polytropic MCF, with titers of 10^5 to 10^6 focus-forming units per ml. Vaccinal strains homologous to the SFFV helper virus are in bold type.

[b] Performed at 45 days of age with SFFV viral complexes containing either ecotropic F-MuLV 57 (SFFV/F-MuLV) or Ampho (SFFV/Ampho) as helper virus.

To test whether these protective and deleterious effects of vaccination by interference to superinfection could be observed even in nontolerized animals, mice were vaccinated with either the attenuated F-MuLV B3 or Ampho at 1 month of age and challenged 2 months later with either SFFV/F-MuLV or SFFV/Ampho. Protective or deleterious effects were similar to those observed in animals vaccinated as newborns (data not shown).

DISCUSSION

Natural protection in mice against several MuLV diseases has been correlated with endogenous expression of glycoproteins homologous to retroviral envelopes (4, 5, 18, 25, 41, 43). In the case of the *Fv-4* locus (56), the resistant allele has been cloned and shown to encode an envelope-like glycoprotein (24, 28). During in vitro experiments, it has been shown that expression of *Fv-4*^{r} in cell lines decreased their susceptibility to infection by ecotropic MuLV (27), suggesting that in vivo protection linked to this gene is due to interference to superinfection. However, since this envelope-like glycoprotein is defective and efficiently incorporated into virions, it has also been suggested that decreased susceptibility might be due to a *trans*-dominant negative effect on production of infectious virions (31). Protection against MuLV-induced pathogenesis has also been reported after exogenous preinoculation, and nonimmune mechanisms have been evoked in certain cases (7, 15, 39). In the present study, nonimmune vaccination against the lytic and nonacute leukemogenic effects of the virulent prototype of F-MuLV was achieved in newborn mice preinoculated with a replication-competent but weakly pathogenic strain of F-MuLV. This neonatal vaccination was also efficiently protective against acute erythroleukemia induced in adult mice by a viral complex composed of the SFFV and a helper virus. Moreover, significant protection against F-MuLV-induced erythroleukemia occurred even when vaccination and challenge were performed within a few minutes of each other, and maximal protection against both the early lytic and late leukemogenic effects was achieved when vaccination and challenge were performed within an interval of only 3 days. That protection following vaccination could be established so rapidly in mice immunologically tolerized by newborn vaccination (10, 42, 48) confirmed that protection in this model was based upon nonimmune mechanisms. Further evidence for an in vivo interference mechanism was obtained by using combinations of vaccinal and challenge viruses from different interference groups including an *env* chimera MuLV. Protective interference is expected to de-

pend upon sufficient spreading of the vaccinal virus in key target cells, which would prevent dissemination of the challenge virus. Accordingly, we observed that vaccination significantly hampered in vivo spreading of the challenge virus and was inefficient when we used an F-MuLV mutant which was altered in its early spreading abilities due to a defect in the production of glycosylated forms of Gag (11).

Because severe EHA and nonacute anemiant erythroleukemia induced by F-MuLV develop only in mice inoculated as newborns and are transient and slow processes, respectively, we further evaluated protection after vaccination by interference against the more virulent acute polycythemic erythroleukemia induced by SFFV. This disease, which is dependent on an oncogene-like activation, develops very rapidly even in mice inoculated as adults (26). Vaccination by interference in newborns also protected against the acute SFFV disease induced after challenge of the animals as adults, with protection extending beyond 5 months. Pathogenesis of the defective SFFV depends on the presence of a replication-competent helper virus, and an SFFV viral complex which comprises the amphotropic virus as a helper, SFFV/Ampho, was weakly pathogenic, most likely because of lower in vivo spreading of Ampho (32). Surprisingly, we found that newborn vaccination with MuLV from heterologous interference groups significantly accelerated the disease induced by the weakly virulent SFFV/Ampho complex. Our results are in agreement with the data reported by Mitchell and Risser on spreading of these SFFV complexes in animals preinoculated as adults with homologous or heterologous viruses (32). The precise mechanism of the synergistic deleterious effect that we observed in the SFFV disease remains unclear. In the models described in both studies, inoculation with F-MuLV leads to production of recombinant polytropic viruses, and an amphotropic-induced polytropic class distinct from the ecotropic-induced polytropic class has also been described (36, 37). It is therefore possible that recurrent superinfections with viruses from distinct interference groups, ecotropic, amphotropic, and polytropic, might lead to formation of new, fast-spreading SFFV complexes. It is also conceivable that although vaccination with the live attenuated virus is minimally pathogenic, it might increase the erythroid cell pool available for infection and transformation by the SFFV-containing viral complex. Finally, immune response defects consequent to tolerization might explain this synergistic effect, similarly to what has been observed in an avian model (12). However, the latter mechanism remains very unlikely, since a synergistic effect was observed even when nontolerized adult mice were vaccinated and challenged.

Although interference is theoretically expected to occur between viruses from the same group, we observed only partial protection against ecotropic F-MuLV diseases when vaccination was performed with the fully replicative ecotropic M-MuLV. Levels of F- and M-MuLV gene expression appear to be significantly different in the erythroid and lymphoid cellular compartments (3, 57). Such differences most likely explain the different cellular specificity of their leukemogenic effects (6, 52). Similarly, less efficient protection against F-MuLV-induced erythroleukemia after M-MuLV vaccination than that observed after F-MuLV B3 vaccination might be due to lower expression of the potentially interfering M-MuLV envelope in the target cells, whose infection is critical for pathogenesis induced by the challenge virus. However, it cannot be excluded that differences between F- and M-MuLV in protection against erythroleukemia reflected intrinsic differences of interaction between their envelopes and the ecotropic receptor (1). Nevertheless, interference sufficient to prevent pathogenesis might not require that expression of the interfering envelope occurred in all potential target cells. Thus, we observed that reduction without abolition of F-MuLV spreading by M-MuLV vaccination was sufficient to protect the animals from severe EHA, and protection against erythroleukemia, although partial, was still significant. Because of lack of reagents allowing distinctive quantitation of the highly related attenuated and virulent strains of F-MuLV (48), precise measurements of spreading of the challenge virus F-MuLV 57 upon vaccination with F-MuLV B3, which conferred maximum protection against erythroleukemia, could not be obtained. Nevertheless, our preliminary data indicated that this more efficient vaccination did not completely abolish spreading of the challenge virus (not shown). This finding suggested that significant protection against retroviral diseases might be conferred by vaccination which would restrict without abolishing accessibility of target cells to infection by the challenge virus.

It has been recently reported that infection by mouse mammary tumor virus was blocked in mice in which target cells essential for dissemination had been deleted by transgenesis of a superantigen (19). Vaccines which would include nonclassical protective mechanisms leading to restricted accessibility of the potential target cells might thus be considered for several retrovirally induced pathogeneses. Among the vaccine strategies developed so far, the use of live attenuated viruses seems to allow such alternative mechanisms to occur in addition to immune responses. In this regard, it is interesting to note that efficient protection against disease induced by simian immunodeficiency virus has been recently achieved by using a live attenuated virus (13). It would be of interest to evaluate whether some nonimmune mechanism(s) played any role in the latter model. It is important to note that vaccination by interference is based on recognition of the cellular receptor, a conserved feature among viruses from the same group, and thus might offer an additional mechanism of protection against hypervariable viral strains. Nevertheless, our results on possible pathogenic synergy between attenuated vaccinal and challenge strains from heterologous groups emphasize that possible facilitation of heterologous viral diseases by replication-competent vaccinal retroviruses should still be of some concern.

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