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Pathogenicity of Fibroblast- and Lymphocyte-Specific Variants of Minute Virus of Mice

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We tested two strains of the minute virus of mice (MVM) for pathogenic effects and patterns of infection in laboratory mice. The two strains differ in their ability to infect differentiated cultured cells: the prototype virus, MVMp, infects only fibroblasts, while its variant, MVMi, is restricted to lymphocytes. We find that neither strain has any demonstrable effects on the T-cell function of mice infected as adults. In contrast, MVMi, but not MVMp, is able to induce a runting syndrome accompanied by mild immune deficiencies upon the infection of newborn mice. After neonatal infection, MVMi spreads to many organs, and the presence of viral replicative form DNA is evident in nucleic acid hybridization experiments. In contrast, replication of MVMp can be detected only by the seroconversion of infected animals. Newborn mice that grow abnormally as a result of MVMi infection also have low circulating antibody titers to the virus. This phenomenon may be a consequence of the lymphotropism of MVMi.

The genus *Parvovirus* of the family *Parvoviridae* groups a large number of nondefective single-stranded DNA viruses that infect vertebrates (18). A characteristic feature of these viruses is that they grow in rapidly dividing tissues, being dependent for their own replication on functions transiently expressed during the S phase of the cell cycle. In addition to this requirement, their growth seems to be restricted to a limited number of differentiated cell types.

Several parvoviruses have been implicated in the etiology of socially and economically important animal diseases. The syndromes associated with these infections include fetal and neonatal death, congenital malformations, enteritis, hepatitis, myocarditis, cerebellar ataxia, hemorrhagic encephalopathy, dwarfism, and panleukopenia (17). Recently, a human parvovirus (strain B-19) was shown to be the etiological agent of fifth disease, an erythematous rash that affects mostly children, and of the aplastic crises of sickle-cell anemia patients (1). The pathogenic effects of the virus can be attributed to its tropism for precursor cells of the erythroid line.

The object of this work, the minute virus of mice (MVM), has been studied in great detail at the molecular level as a paradigm for the genus *Parvovirus*. However, its pathogenic potential in mice remains unclear. It has been reported that intracranial injection of MVM into newborn mice can cause growth retardation and cerebellar lesions (10), but there is no solid evidence that infection via more natural routes can cause disease. Serological evidence from laboratory mouse colonies suggests that MVM is highly prevalent but persists in a clinically inapparent state (12).

Two independent isolates of MVM have been described. The first strain, called MVMp (for prototype), was isolated from a murine adenovirus stock and was subsequently adapted for growth in mouse fibroblasts (4). More recently, another strain was isolated as a contaminant of an EL-4 T-cell lymphoma culture and was shown to have an inhibitory effect on the generation of cytotoxic T cells in mixed leukocyte cultures and on a number of T-cell functions measured in vitro (2, 6, 11). For this reason, it was called MVMi (for immunosuppressive). It now appears that inhibition of T-cell functions by MVMi can be ascribed to the tropism of the virus for lymphocytes. MVMp, on the other hand, grows only in fibroblasts and has no effect on immune reactions in vitro. There is some precedent for parvoviruses affecting the immune response of infected hosts; for example, in the course of a feline panleukopenia virus infection, lymphocyte proliferation in response to lectins is decreased but not the humoral response to soluble antigens (3, 16). Infection by the Aleutian disease virus of mink leads to immune complex disease accompanied by a depressed response to various antigens (14).

The purpose of the experiments described in this paper was to find out whether the different tissue specificities of MVMp and MVMi in vitro would be reflected by different patterns of infection in vivo. In particular, we were interested in the effects of MVMi on T-cell function in the whole animal and in the tissue distribution of the two virus strains during the productive phase of the infection.

MATERIALS AND METHODS

Mice. Mice of the inbred strains C57BL/6 and BALB/c were bred in the animal colony of the Swiss Institute for Experimental Cancer Research. The original breeding pairs came from the Jackson Laboratory, Bar Harbor, Maine. The BALB/c mice used in the in situ nucleic acid hybridization experiments were obtained from the animal colony at the University of Colorado Health Sciences Center, Denver, Colo. All of the adult mice used for evaluations of immune responsiveness, as well as the mothers of the litters used in neonatal infection experiments, were seronegative to MVM by the radioimmunoassay (RIA) described below.

Viruses and cells. The MVMp strain was provided by P. Tattersall and was routinely grown on the A9 murine fibroblast cell line. MVMi was isolated in our laboratory as a contaminant of EL4(G−) (C57BL/6, *H-2*^{b}) ascites cells obtained from G. Bonnard, National Institutes of Health,

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Bethesda, Md., and was subsequently purified by limiting dilution on EL4(G−) × A9 hybrid cells (11). MVMi was propagated in EL-4 cells in Dulbecco modified Eagle medium containing 10% fetal calf serum. Stocks of MVMp and MVMi virions were purified on CsCl density gradients and titrated by using the plaque assay described below.

Plaque assay. The following protocol for the plaque assay was modified from Tattersall and Bratton (20). 324K cell monolayers (permissive for both MVMp and MVMi) were grown to 25 to 35% confluency in six-well disposable plates. They were inoculated with 0.2 ml of a virus dilution in medium containing 2% fetal calf serum. The virus was allowed to bind to cells for 60 min at 37°C, with gentle rocking every 15 min. The cells were overlaid with a mixture containing 0.8 to 0.9% agarose (Seakem 49-052-2; FMC Corp., Marine Colloids Div., Rockland, Maine) in Dulbecco modified Eagle medium plus 5% fetal calf serum, nonessential amino acids (GIBCO Laboratories, Grand Island, N.Y.), vitamins, penicillin, and streptomycin. When the plaques were approximately 1 mm in diameter, the plates were overlaid with 0.3% neutral red in Tris-buffered saline and 0.8 to 0.9% agar, and the plaques were stained for approximately 45 min and counted under a dissecting microscope.

RIA. We developed the following RIA to measure free anti-MVM antibody titers in sera. CsCl-purified empty MVM capsids were used as antigen; 1.5 μg of capsid protein in 50 μl of coating buffer (50 mM Na_2CO_3, pH 9.6) was added to each well (96-well plastic plates, Linbro; Flow Laboratories, Inc., McLean, Va.) and allowed to attach for 1 h. After being washed, the wells were coated for 1 h with phosphate-buffered saline containing 30 mg of egg albumin per ml. The wells were then incubated with primary antibody (50 μl of serum dilution in phosphate-buffered saline) for 90 min. Known positive and negative control sera were included in each run. The second antibody was a rabbit anti-mouse Fab preparation labeled with ^{125}I (kindly provided by J.-P. Mach, Ludwig Institute for Cancer Research, Lausanne, Switzerland). Approximately 25,000 cpm of labeled antibody was added per well, and the mixture was incubated for 90 min. Between each step, the plates were vigorously washed with phosphate-buffered saline; all incubations were done at 37°C. After the final wash, individual wells were cut out, and the bound ^{125}I was measured in a gamma counter.

Growth rate and mortality. Two litters of seven and nine BALB/c mice were infected at birth with MVMp or MVMi, respectively, by letting them inhale a droplet containing approximately 10^4 PFU of virus. A third litter of seven mice was used as an unexposed control. Individual mice in all three litters were weighed weekly for 12 weeks, and the litters were monitored for infant mortality. Antibody titers against MVM were measured on day 30 by using the RIA.

Measurement of DTH responses. We analyzed the delayed-type hypersensitivity (DTH) response of MVM-infected adult mice to both heat-inactivated herpes simplex virus (HSV) and sheep erythrocytes (9). BALB/c mice were used to measure the response to HSV, while C57BL/6 mice were used to measure the response to sheep erythrocytes. Six groups of five 10-week-old mice were treated as follows: one day 0, groups A, B, and C were given 10^7 PFU of heat-inactivated HSV emulsified in complete Freund adjuvant subcutaneously at the base of the tail. Groups D, E, and F were immunized intravenously with 200 μl of a 0.01% suspension of sheep erythrocytes. In addition, groups A and D received 2×10^8 PFU of MVMi intraperitoneally, while groups B and E received an equivalent dose of MVMp. Groups C and F were uninfected controls. On day 7, groups A, B, and C were injected subcutaneously in the hind footpad with an eliciting dose of 10^6 PFU of heat-inactivated HSV, and groups D, E, and F received 50 μl of a 4% suspension of sheep erythrocytes by the same route. On day 8 (24 h after challenge, at the peak of DTH response), footpad swelling relative to the untreated contralateral control was measured with vernier calipers.

Rejection of allogeneic tumor cells. We studied the rejection by C57BL/6 mice (*H-2*^{b} haplotype) of two allogeneic cell lines, the P-815 mastocytoma and the S-194 plasmacytoma (both *H-2*^{d}). Both lines were tested for permissiveness to MVM infections by monitoring the production and amplification of viral replicative-form (RF) DNA. The Hirt supernatant of cells infected with 10 PFU of MVM per cell was examined for the presence of RF DNA by Southern blotting and hybridization (19). To further examine their permissiveness, the S-194 cells were labeled with [^3H]thymidine from 24 to 48 or 96 h postinfection, and progeny viral DNA was looked for by fluorography of Hirt supernatant DNA separated on agarose gels. By these criteria, the P-815 line is permissive for both MVMi and MVMp infections, while the S-194 line is not permissive for either virus.

Elimination of the tumor cells was monitored as follows. Six groups of five C57BL/6 mice were injected intraperitoneally with 2×10^6 P-815 (groups G, H, I) or S-194 (groups J, K, L) cells that had been labeled with [^{131}I]iododeoxyuridine to a specific activity of 5×10^4 cpm per 10^6 cells. On day 1, groups G and J were injected intraperitoneally with 2×10^8 PFU of MVMi, while groups H and K received an equivalent dose of MVMp. Groups I and L were used as controls. Survival of tumor cells was monitored by taking whole body counts of the mice, whose drinking water had been supplemented with 0.1% NaI to minimize uptake of the released ^{131}I.

Evolution of M-MSV-induced tumors. Sarcomas were induced in three groups of five 10-week-old C57BL/6 mice by injecting them in the hind footpad with 10 μl of a tumor homogenate containing Moloney murine sarcoma virus (M-MSV) and Moloney murine leukemia virus (M-MLV) complex (13). Group M received 2×10^8 PFU of MVMi intraperitoneally, group N was injected with MVMp, and group O served as the control. Footpads were measured every 2 days with a vernier caliper.

In situ hybridization to mouse slices. To study the organ distribution of MVM after infecting newborn BALB/c mice, we used the whole-body slice hybridization technique described by Dubensky et al. (5). Two litters of newborn mice were exposed intranasally to MVMi or MVMp. At days 1, 3, 6, 9, and 12, one mouse from each group was asphyxiated with CO_2 and quick-frozen in liquid nitrogen. After being embedded, serial sections were made with a refrigerated microtome, and nucleic acids were transferred to nitrocellulose filters under denaturing conditions. Individual sections were probed with one of two ^{32}P-labeled DNAs. (i) Nick-translated cloned MVMi DNA (0 to 99 map units) detects both virion and RF DNA. (ii) A strand-specific probe made from a subgenomic clone (40 to 70 map units) of MVM DNA by the method of Hu and Messing (7) hybridizes only to the complement of the encapsidated strand and thus identifies areas of viral replication. Nonfat dry milk was used as a blocking agent in all of the hybridizations (8).

RESULTS

Effects of MVM on mouse development. Since several parvoviruses have been reported to cause growth retarda-

tion, we infected newborn mice intranasally with MVMi or MVMp and observed their growth and humoral response to the infecting virus. In the experiment shown in Fig. 1, three of nine newborn mice exposed to MVMi died (on days 1, 7, and 14) compared with none in the MVMp-exposed and control litters. Surviving mice from litters infected with MVMi could be separated into two groups based on growth rate. The difference in average weight between the two groups started being statistically significant at 21 days after infection. The growth-retarded mice never reached a normal adult weight or stature, while their littermates grew at a rate indistinguishable from that of controls. In separate experiments, two other litters were infected at birth with MVMi. Of the 15 mice that were infected, 4 died, 6 were growth retarded, and 5 grew normally.

At 30 days after exposure, antibody titers against MVM were measured in all of the mice by testing a 1:4 serum dilution in the RIA. Mice infected with MVMp developed a good humoral response against the virus (2,800 ± 550 cpm bound), as did MVMi-infected mice that grew normally (2,760 ± 530 cpm). The growth-retarded mice, however, had low antibody titers (250 ± 30 cpm). Since the RIA detects circulating antibody only, we cannot be sure whether these low titers reflect the trapping of antibody in virus-infected tissue as a result of active replication of MVMi or failure of the animals to respond to the antigen. Uninfected and negative control serum titers were approximately 10 to 30 cpm above a no-serum control. Taken together, these data indicate that mice infected at birth with MVMi, but not with MVMp, can develop a syndrome leading to severe growth retardation or death and that the occurrence of this syndrome correlates with a low level of circulating antibody to the virus. We cannot exclude the possibility that higher doses of MVMp could produce effects similar to those seen with MVMi. In any case, with equivalent doses of virus, MVMi is more virulent than MVMp.

Tissue distribution of the virus. We explored the spread of

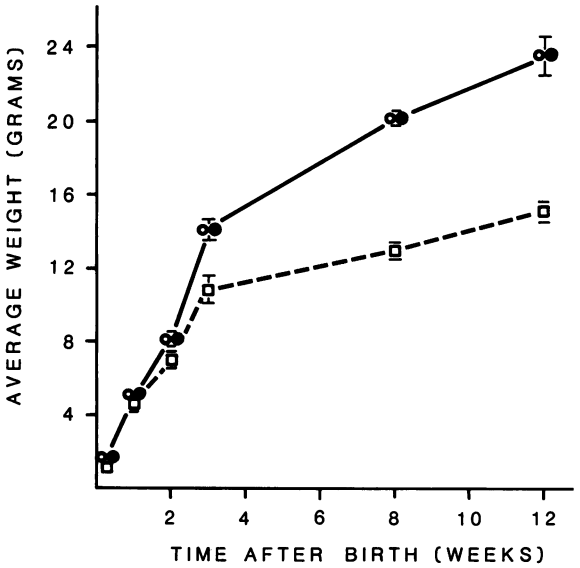

FIG. 1. Weight gain of mice infected intranasally with MVM at birth. Symbols: (○), uninfected controls; (●), mice infected with MVMp; (□), mice infected with MVMi that developed a runting syndrome. Mice infected with MVMi that did not develop a runting syndrome (two of nine in this experiment) grew exactly like the uninfected controls.

TABLE 1. Spread of MVMi to various organs in newborn mice infected intranasally[a]

Days after birth	Probe detects	
	All viral nucleic acids	RF DNA + mRNA
1	NAOC, stomach	No signal
3	NAOC, liver, blood	No signal
6	NAOC, liver, blood, lung, spleen	No signal
9	NAOC, liver, blood, lung, intestine	NAOC
12	NAOC, thymus, eye, liver, intestine, bone	NAOC, bone[b]

[a] Mice were infected at <18 h after birth, and the distribution of viral nucleic acids was determined by the mouse slice hybridization technique.
[b] See Fig. 2.

MVMi or MVMp after intranasal inoculation by using the sliced mouse in situ hybridization technique. This approach also revealed profound differences between the two virus strains. MVMi infections, as measured by the presence of viral DNA, reached many organs, starting from the site of inoculation (nasal-oral cavity [NAOC]) on day 0 and spreading to most well-vascularized organs by day 6, probably as a result of viremia (Table 1). MVMp, by contrast, was detected in the NAOC and stomach only, and only during the first 3 days after inoculation (data not shown). We assume that this DNA represented input virus. It disappeared rapidly and could not be detected at any of the later times tested. However, some exposure of lymphoid organs to the viral antigens must be presumed since the animals seroconverted, but at the level of sensitivity of the tissue slice hybridization assay, we have no evidence that the virus ever spread beyond the initial site of inoculation.

We tried to detect the sites of replication of MVMi by using a strand-specific probe which hybridizes to the complement of the viral strand (only one strand is encapsidated in MVM virions). This strand is present in viral RF DNA and in mRNA, but not in viral particles, and can therefore serve as a marker for sites of replication. The results of these experiments are summarized in the right-hand column of Table 1. The NAOC, which was the site of entry for the virus in these experiments, also seemed to become the primary site of viral replication. The resolution of the hybridization technique used was not sufficient to pinpoint the exact tissue where replication takes place (Fig. 2). On day 12, the probe also detected viral RF in bones (cranium, vertebrae, sternum; Fig. 2), presumably in the marrow. This observation is interesting in light of the effects of viral infection on the growth of the animals, which becomes apparent after approximately 20 days (Fig. 1). By using the same probe on slices from MVMp-infected newborn mice, we were unable to detect any signal at all.

Effect of MVM on cell-mediated immune responses. We used three different techniques to measure the effects of MVMi or MVMp infections on T-cell functions in vivo: (i) DTH response to two different antigens (sheep erythrocytes and heat-inactivated HSV), (ii) rejection of two lines of allogeneic tumor cells, and (iii) rejection of tumors induced by M-MSV or M-MLV.

The effects of MVM infection on DTH responses in adult mice are shown in Table 2. A concurrent intraperitoneal infection with MVMi or MVMp had no effect on the ability of the mice to mount a DTH response to either sheep erythrocytes or heat-inactivated HSV, even though the animals developed antibody to the virus as a result of the infection (data not shown). We also measured the DTH response, but to sheep erythrocytes only, on three groups of

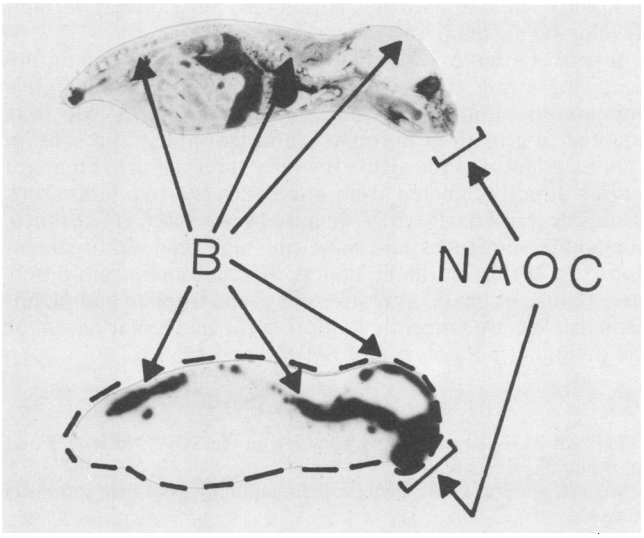

FIG. 2. Hybridization to a mouse slice 12 days after infection with MVMi. The photograph at the top is of a stained section through the midsagittal plane used for hybridization. After being transferred to nitrocellulose, the tissue slice was hybridized with the strand-specific ^{32}P-labeled probe that detects areas of viral replication (see Materials and Methods). B, Bone; NAOC, nasal-oral cavity.

growth-retarded BALB/c mice that had been infected with MVMi at birth and on uninfected and MVMp-infected controls (Table 3). In two of three experiments, footpad swelling was significantly less in growth-retarded mice than it was in unexposed control mice of the same age or in mice that had been exposed intranasally to MVMp at birth. Why the third experiment failed to show this effect is not clear, and more experiments are needed to clarify this point.

Figure 3 shows the effects of MVM infections on the rejection of allogeneic ascites tumors. P-815 mastocytoma cells were cleared more rapidly by C57BL/6 mice with a concurrent MVMi or MVMp infection than by uninfected animals. The differences between the two groups (infected versus uninfected) are statistically significant ($P < 0.005$). This rather surprising result prompted us to test the permissiveness of the P-815 line for viral growth and to repeat the experiment with a tumor line that would not be permissive. By the criterion of RF or viral progeny DNA production (see Materials and Methods), the P-815 line supported the growth of both virus strains, while the S-194 line did not. Clearance of the S-194 cells was correspondingly unaffected by a concurrent infection with MVMi or MVMp (Fig. 3). The most likely explanation for these results is that MVM infection affects the growth of the P-815 cells in the peritoneal cavity, rather than the immune reaction that eliminates them.

TABLE 2. DTH response in mice infected with MVM as adults

Group	Infection with:	Antigen	Increase in footpad thickness (mm)[a]
A	MVMi		1.32 ± 0.20
B	MVMp	Inactivated HSV	1.30 ± 0.19
C	None		1.32 ± 0.13
D	MVMi		1.20 ± 0.14
E	MVMp	Sheep erythrocytes	1.21 ± 0.18
F	None		1.20 ± 0.18

[a] Difference between treated footpad and contralateral control 1 day after challenge. See Materials and Methods for a detailed description of experimental protocols.

TABLE 3. DTH response in mice infected with MVM at birth[a]

Expt no.	Infection at birth with:	Increase in footpad thickness (mm)
1	None	0.91 ± 0.12
	MVMi (runted)	0.32 ± 0.18
	MVMp	0.84 ± 0.14
2	None	1.11 ± 0.14
	MVMi (runted)	0.37 ± 0.20
	MVMp	0.97 ± 0.15
3	None	0.98 ± 0.16
	MVMi (runted)	0.91 ± 0.20
	MVMp	0.94 ± 0.14

[a] The DTH response to sheep erythrocytes was measured as described in Materials and Methods. The groups of MVMi-infected runted mice used in experiments 1, 2, and 3 were 56 days old (three animals), 70 days old (three animals), and 70 days old (two animals), respectively, at the time of the first injection of sheep erythrocytes. Control groups were matched for age and contained five animals each.

The progression and resorption of M-MSV- and M-MLV-induced footpad tumors in uninfected and MVM-infected C67B/6 mice is shown in Fig. 4. Although progression of the tumors seemed to be a little more rapid in infected mice, tumor rejection was certainly not impaired by the infection.

DISCUSSION

The results presented in this paper clearly show a difference between the effects of MVMi and MVMp when the viruses are used to infect BALB/c mice intranasally shortly after birth. Most of the MVMi-infected mice failed to grow normally, and some of them may have died of the infection, while none of these effects were seen with MVMp. These observations are consistent with those obtained from in situ tissue hybridization experiments which show that MVMi can

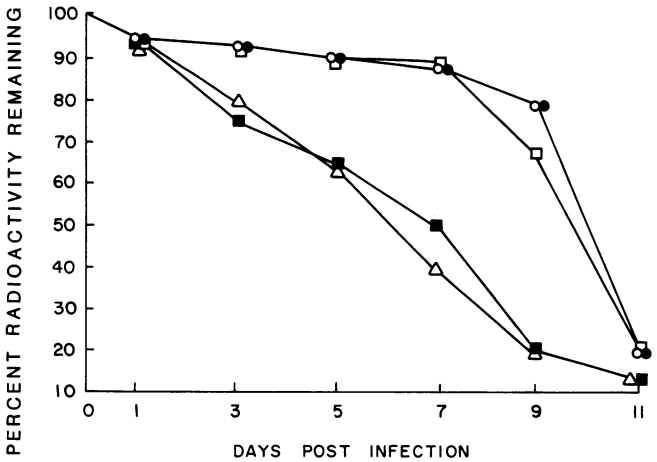

FIG. 3. Clearing of ^{131}I-labeled allogeneic tumor cells from the peritoneal cavity of MVM-infected mice. (See Materials and Methods for a detailed description of experimental protocols.) Symbols: (○), P-815 cells, no virus (group I); (■), P-815 cells, infection with MVMi (group G); (△), P-815 cells, infection with MVMp (group H); (●), S-194 cells, infection with MVMi (group J); (□), S-194 cells, infection with MVMp (group K). Group L (S-194 cells, no virus) gave results identical to those of group I (P-815 cells, no virus).

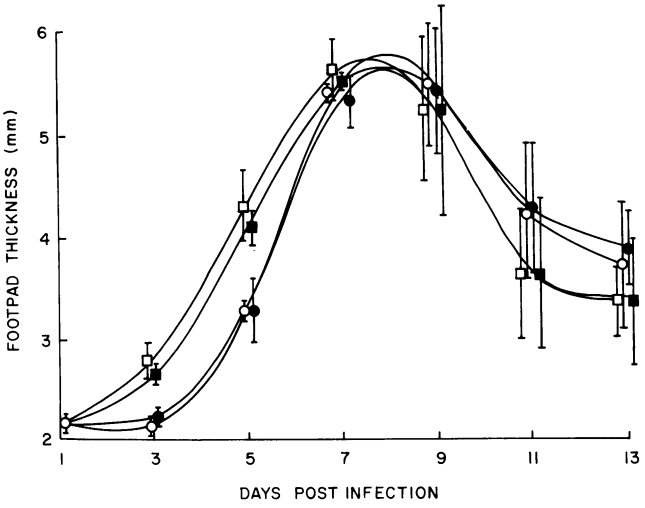

FIG. 4. Formation and regression of M-MSV-induced tumors in the footpads of mice infected with MVM. Symbols: (○), controls infected with M-MLV and M-MSV alone (group O); (●), MVMi-infected mice (group M); (□), MVMp-infected mice (group N); (■), mice injected with MVM empty capsids.

spread to many organs in the body, while MVMp seems to be confined to the original inoculation site. Replication of MVMi can occur at remote sites, in contrast to MVMp for which no RF could be detected by the mouse slice hybridization technique. However, replication of MVMp may have occurred below the threshold at which we can detect it, causing seroconversion of the infected animals. The differences between the pathogenic effects of MVMi and MVMp may be linked to the fact that mice with debilitating MVMi infections failed to develop significant circulating antibody titers against the virus. Whether this is an effect of increased antigenic load or of specific immunosuppressive properties of the virus remains to be determined.

Our data indicate that both laboratory strains of MVM are nonpathogenic in adult BALB/c and C57BL/6 mice. Three independent tests of T-cell function failed to detect differences between infected and uninfected animals. All the infected animals developed normal antibody titers against the virus, and none showed signs of disease. Therefore, it seems that MVMi can have immunosuppressive effects only on newborn mice which are not fully immunocompetent, while adult animals can mount an effective immune response against the virus. Our data on the ability of the growth-retarded mice to mount a T-cell-mediated immune response as adults are still inconclusive, but they suggest that neonatal infections by MVMi may have long-term effects on immunocompetence. It would be interesting to test whether these animals have developed an immunological tolerance to the virus, since the age at the time of infection is a major factor in the pathogenesis of MVM.

Our observations have some bearing on the epidemiology of the virus. Only MVMi seems to be capable of initiating a productive infection in newborn mice and spreading in large amounts to areas of the body from which it could be released into the environment. The presence of MVMi in the intestine at days 9 and 12 after infection coupled with its ability to spread from the NAOC, is consistent with a fecal-oral route of transmission. How the virus is maintained in mouse colonies is still not clear, but it seems reasonable to infer that newborn or very young animals provide a reservoir for productive infections, while adults only seroconvert without developing systemic infections.

It is likely that MVMi is closer than MVMp to a naturally occurring strain that is able to propagate itself in mouse populations. Immediately after its isolation, MVMp was adapted to growth in fibroblasts and may thus represent an attenuated form of the virus. It will be interesting to compare strains directly isolated from mice with the two laboratory strains described here. MVMi and MVMp share 96% of their nucleotide sequences and have the same genetic organization (15). It is remarkable that such small interstrain differences cause dramatic alterations in tissue tropism and pathogenicity. We are currently exploring the molecular bases for the divergent phenotypes of MVMp and MVMi.

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