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Effects of Cell Source, Mouse Strain, and Immunosuppressive Treatment on Production of Virulent and Attenuated Murine Cytomegalovirus

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Murine cytomegalovirus pools from various in vitro and in vivo sources were compared for virulence in suckling mice in an effort to identify the conditions which were necessary for the production of virulent and attenuated viruses. Virus passaged in tracheal ring and salivary gland organ cultures, where virus is produced primarily by epithelial cells, was even more attenuated than virus passaged in mouse embryo fibroblasts. The attenuation observed after passage in all three of these in vitro systems did not appear to be due to defective interfering particles. We also found that virus produced in vivo in salivary glands became attenuated with time after infection. Virus harvested from salivary glands 5 to 6 weeks after infection was highly attenuated compared with both salivary gland-passaged virus harvested 2 to 3 weeks after infection and tissue culture-passaged virus. The attenuation of salivary gland-passaged virus with time was reversed when animals were treated with cyclophosphamide before the virus was harvested. A comparison of virus pools harvested from susceptible and resistant mouse strains indicated that the mouse strain had little effect on the virulence of the virus produced. When the various sources of virus tested in this study were ranked in terms of the virulence of the virus produced, salivary glands in intact mice either 2 to 3 weeks after infection or after cyclophosphamide treatment produced the most virulent virus, followed by mouse embryo fibroblast cultures, tracheal ring and salivary gland organ cultures, and, finally, salivary glands in intact mice 5 to 6 weeks after infection.

Because cytomegalovirus is responsible for congenital birth defects in an estimated 1 of every 1,000 babies born in the United States (6) and because this virus poses a significant problem in patients who are immunosuppressed (4, 13, 23), considerable effort is being made to develop a cytomegalovirus vaccine (3, 20, 25). However, a number of reservations have been expressed concerning the risks involved in administering a live cytomegalovirus vaccine when many basic questions concerning attenuation, latency, reactivation, and oncogenic potential remain unanswered (16, 24). Many of these questions are difficult to investigate because human cytomegalovirus does not infect laboratory animals. However, murine cytomegalovirus, a useful laboratory model (29), has been used recently to examine some of these questions (8, 11, 17). In this study, murine cytomegalovirus pools derived from various in vivo and in vitro sources were compared for virulence in an effort to identify the conditions which were necessary for the production of virulent and attenuated virus.

In 1971, Osborn and Walker (22) described the rapid attenuation of murine cytomegalovirus after passage through mouse embryo fibroblast (MEF) cultures and the rapid restoration of virulence by back passage of MEF-passaged virus in mice, with recovery of virus from salivary glands. The features of the tissue culture environment which effected such rapid attenuation and the features of the salivary glands which rapidly reversed the process were not identified. At that time, MEF cultures provided the only means of producing murine cytomegalovirus in vitro. The demonstration that the epithelial linings of tracheal ring organ cultures (TROC) also produce virus in vitro (14) has provided an entirely different tissue culture environment for virus production. We postulated that TROC

might be an in vitro source of virulent virus and that a comparison of MEF- and TROC-produced virus pools might help identify the tissue culture conditions necessary for attenuation.

Similarly, a comparison of the virulence properties of virus pools from various in vivo sources might help identify the features of those environments which are responsible for virulence. Since the original description of virulence and attenuation, resistant and susceptible strains of mice have been described (1, 19, 28), with no indication of the virulence properties of the virus produced by such mice. Other studies have dealt with reactivation of latent virus by various immunosuppressive therapies (12, 15). Included among these studies is a report in which mice vaccinated with attenuated virus produced virulent virus after immunosuppression (11).

This study was undertaken to answer the following questions. How do the virulence properties of virus passaged in vitro in culture systems other than embryo fibroblasts compare with the virulence properties of mouse salivary gland- and MEF-passaged virus? How do the virulence properties of virus pools produced in different strains of mice compare? Is the virus produced in salivary glands of chronically infected mice as virulent as that produced in acutely infected mice? And what impact does immunosuppressive treatment have on the virulence of the virus?

MATERIALS AND METHODS

Mice. Outbred CD-1 mice were obtained from Charles River Farms, Wilmington, Mass. Pregnant mice for MEF cultures and for production of litters for assessing the virulence properties of virus pools were shipped after 15 days of gestation. CD-1 mice were shipped when they were 30 days old and were used within a few weeks of arrival for producing virus pools and organ cultures. C_3H/Hej mice were obtained when they were 4 to 6 weeks old from Jackson Laboratory, Bar Harbor, Maine, and were used shortly thereafter for producing virus pools.

Tissue and organ cultures. Primary and secondary MEF cultures were prepared from CD-1 mouse embryos (16 to 18 days of gestation) by previously described methods (27). TROC containing six to eight individual tracheal rings, each with a single cartilage, were prepared as described by Nedrud et al. (19). Salivary gland organ cultures (SGOC) were prepared by asceptically removing CD-1 mouse salivary glands, mincing these glands into small pieces (approximately 1 mm^3), and placing them on grids (type 3014; Falcon Plastics, Oxnard, Calif.) in organ culture dishes (type 3037; Falcon Plastics). The center well of each dish was filled with basal Eagle medium containing Earle salts (GIBCO Laboratories, Grand Island, N.Y.), 2% fetal calf serum, 200 U of penicillin per ml, and 200 μg of streptomycin per ml. Cultures were incubated at 37°C in an atmosphere containing 5% CO_2.

Virus. The Smith strain of murine cytomegalovirus was obtained from the American Type Culture Association, Rockville, Md., at tissue passage 12. Subsequently, it was passed once in MEF and twice in mouse salivary glands to produce the first wild-type stock virus. Passage of virus through mouse salivary glands was accomplished by inoculating adult mice intraperitoneally with 10^5 plaque-forming units (PFU) of murine cytomegalovirus and then sacrificing these mice either 2 to 3 or 5 to 6 weeks after infection. In some cases, mice were given intraperitoneal inoculations of 150 mg of cyclophosphamide (Cytoxan; Mead Laboratories, Evansville, Ind.) per kg 9 and 4 days before they were sacrificed at 5 weeks. Salivary glands were removed, pooled, ground with a mortar and pestle to a 10% (wt/vol) extract in maintenance medium (medium 199 in Hanks buffer containing 3% fetal calf serum, 200 U of penicillin per ml, and 200 μg of streptomycin per ml), and clarified by low-speed centrifugation. The salivary gland-passaged virus harvested 2 to 3 weeks postinfection is referred to below as wild-type virus. All virus pools were stored in small portions with 10% dimethyl sulfoxide at −70°C. Figure 1 shows the histories of the passages through MEF, TROC, and SGOC to produce the virus pools used in this study. To obtain MEF-passaged murine cytomegalovirus pools, cells were infected at different multiplicities in 3-ml volumes in 150-cm^2 tissue culture flasks. After 2 h of stationary adsorption at 37°C, an additional 10 ml of maintenance medium was added to each flask. The cultures were incubated for 4 to 5 days until 95% cytopathic effects were observed, and then the culture fluids were clarified by centrifugation and stored. TROC and SGOC were infected as previously described. Briefly, cultures were inoculated with 0.1 to 0.2 ml of undiluted seed virus. After adsorption for 2 h at 37°C, these cultures were rinsed and fed with fresh medium. Culture fluids were collected 10 to 16 days later, clarified, and stored. The infectivity titers of all virus pools were determined by plaque assays on MEF, as previously described (19).

Comparison of virulence properties of virus pools. We used two methods to compare the virulence properties of various virus pools in 5- to 6-day-old suckling mice. Animals from five to seven litters were pooled, randomly distributed among the mothers, and inoculated intraperitoneally with 0.05-ml volumes of serial twofold dilutions of a virus pool; these animals were observed for mortality for 4 weeks. This process was repeated several times, and the dose which produced 50% mortality (50% lethal dose [LD_{50}]) was calculated by probit analysis (5). Alternatively, randomized litters of mice were inoculated with a standard number of PFUs of virus from various sources, and the cumulative percent mortalities obtained with virus pools from different sources were compared on progressive days postinfection. The differences in the mortalities produced by the different virus pools were analyzed by the chi-square test. In some cases, passage 3 MEF- or TROC-produced virus at a final concentration of 4×10^3 PFU/mouse was mixed with serial dilutions of wild-type virus before inoculation into suckling mice, and the mortalities obtained after 4 weeks were compared with the mortalities obtained after mice were inoculated with the same dilutions of

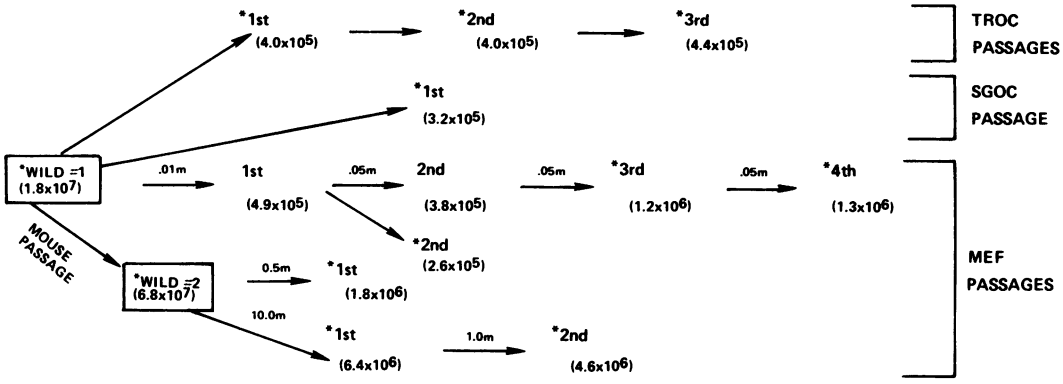

FIG. 1. *Histories of virus pools passaged in vitro and used in this study. Asterisks indicate the virus pools actually used. m, Multiplicity of infection. Parentheses indicate the titers of the virus pools at each passage level (in PFU per milliliter).*

wild-type virus without MEF- or TROC-produced virus. Animals inoculated with normal salivary gland homogenate or maintenance medium containing 10% dimethyl sulfoxide in the absence of virus showed no adverse effects.

RESULTS

Attenuation of virus by in vitro tissue passage. Figure 2A compares the percent mortalities in suckling mice after inoculation with 8 $\times 10^3$ PFU of wild-type virus and after inoculation with the same dose of virus passaged 1 to 4 times in MEF. When these results were analyzed by the chi-square test, the percent mortality due to the wild-type virus was significantly different from the percent mortalities due to all MEF-passaged viruses ($P < 0.00001$), and the percent mortality due to virus passaged once in MEF was significantly different from the percent mortalities due to virus passaged 2 to 4 times in MEF ($P = 0.03$). Hence, the MEF passage 1 virus was intermediate in virulence between wild-type virus and virus pools from subsequent MEF passages, which exhibited the same degree of virulence ($P = 0.34$). Figure 2B shows a similar effect after suckling mice were inoculated with 10^4 PFU of wild-type virus and TROC-passaged virus. However, in this case no virus of intermediate virulence was observed. The difference in percent mortalities between wild-type virus and TROC-passaged virus was highly significant ($P < 0.00001$), but there was no difference in the attenuation of passages 1 through 3 ($P = 0.54$). Mice infected with all attenuated virus showed not only a decrease in mortality but also a delay in the onset of mortality.

Similar results are shown in Table 1, which gives the LD_{50} values for the virus pools produced in SGOC, as well as for the virus pools produced in TROC and MEF and for wild-type virus. Again, virus passaged once in MEF appeared to have intermediate virulence, whereas virus passaged in TROC and SGOC was attenuated after one passage. The LD_{50} values of virus pools from MEF passages 2 through 4 were about 10-fold higher than the LD_{50} for wild-type virus. Virus yields made it impossible to determine the LD_{50} values for SGOC- and TROC-passaged virus beyond one passage; however, the data suggest that these values would be higher than the LD_{50} values for virus pools from comparable passages in MEF.

Role of defective interfering particles in attenuation. Table 1 also shows that similar results were obtained with virus passages 1 and 2 in MEF regardless of whether the virus was passaged at a high or a low multiplicity. There was no indication of interference in pools passaged at a high multiplicity of infection.

Figure 1 shows the passage histories of the virus pools produced in vitro for this study. With the exception of the last series, all MEF passages were made at a low multiplicity of infection, which tends to prevent the accumulation of defective virus. Because the exact number of cells in the TROC and SGOC could not be determined easily, it was not possible to determine the multiplicity of infection for passages in these cultures. There was no evidence for the accumulation of defective interfering particles. A decline in virus titer after passage in TROC did not occur, and there was a slight but gradual increase in virus titer during passage in MEF. Thus, pools of virus which were less able to kill suckling mice than wild-type virus were as productive as or more productive than wild-type virus when they were inoculated into MEF or TROC. Also, the titers obtained at a high multiplicity of infection in MEF were, if anything, greater than those obtained for the same passage at a low multiplicity of infection.

Nevertheless, since accumulation of defective

interfering particles accounted for the attenuation of many viruses during tissue passage, further studies were performed to determine whether defective interfering particles contributed to the attenuation observed in this study. Table 2 shows the results obtained when virus passaged 3 times in MEF or TROC was mixed with varying dilutions of wild-type virus before mice were inoculated and the percent mortalities obtained compared with those obtained when the same dilutions of wild-type virus were inoculated alone. No significant differences in mortality were observed when TROC-passaged virus was mixed with virulent virus; hence, nothing in the TROC-passaged virus inhibited the virulence of wild-type virus. The same was true for two of the three MEF-passaged mixtures. A statistically significant difference was obtained when MEF-passaged virus was mixed with the highest concentration of wild-type virus. However, this difference was probably not related to the presence of defective particles since such particles present in a constant dose of MEF-passaged virus would be expected to have the greatest inhibitory effect on lower doses of virulent virus and the least effect on higher doses.

Attenuation of virus in vivo. In further attempts to improve our understanding of the conditions necessary to produce virulent and attenuated virus, virus pools from various sources of mouse salivary gland virus were compared for virulence. Table 3 shows the LD_{50} values of virus pools produced in vivo in salivary glands of resistant (C_3H) and susceptible (CD-1) mouse strains and also compares the LD_{50} values obtained for virus pools harvested 2 to 3 and 6 weeks postinfection. The data indicate no difference in the LD_{50} values for virus pools produced in C_3H and CD-1 mice; however, the time at which the virus was harvested significantly af-

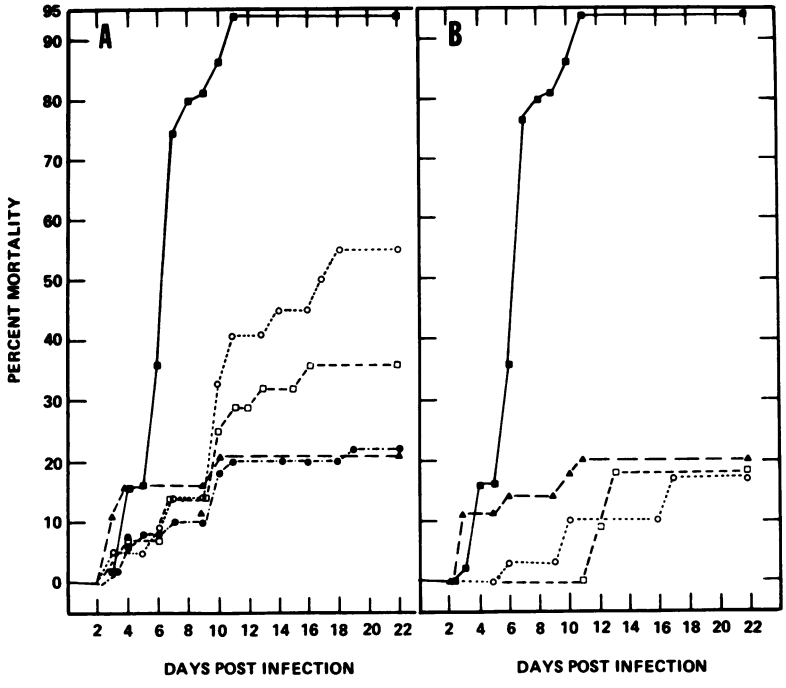

FIG. 2. *Mortality in suckling mice. (A) Mice inoculated with wild-type murine cytomegalovirus and mice inoculated with virus from MEF passages 1 through 4. All doses were 8 × 10^3 PFU. Symbols: ○, virus from MEF passage 1 (12 of 22 mice died); ▲, virus from MEF passage 2 (11 of 51 mice died); □, virus from MEF passage 3 (10 of 28 mice died); ●, virus from MEF passage 4 (4 of 19 mice died); ■, wild-type virus (60 of 64 mice died). (B) Mice inoculated with wild-type virus (8 × 10^3 PFU) and mice inoculated with virus from TROC passages 1 through 3 (1 × 10^4 PFU). Symbols: ○, virus from TROC passage 1 (5 of 30 mice died); ▲, virus from TROC passage 2 (9 of 44 mice died); □, virus from TROC passage 3 (2 of 21 mice died); ■, wild-type virus (60 of 64 mice died). When the results were analyzed by the chi-square test, the mortality due to wild-type virus was significantly different from the mortalities due to all MEF- and TROC-passaged virus ($P < 0.00001$). Also, the mortality due to virus from MEF passage 1 was significantly different from the mortalities due to virus from MEF passages 2, and 4 ($P = 0.03$); however, there was no difference in the mortalities due to virus from MEF passages 2, 3, and 4 ($P = 0.34$) or in those due to virus from TROC passage 1, 2, and 3 ($P = 0.54$).*

TABLE 1. *Comparison of LD_{50} of virus pools derived from various in vitro sources with wild-type virus LD_{50}*

Passage	MOI[a]	LD_{50} (PFU) of virus derived from:[b]		
		MEF	TROC	SGOC
0 (wild type)		1.7×10^3 (1.2×10^3–2.2×10^3)	1.7×10^3 (1.2×10^3–2.2×10^3)	1.7×10^3 (1.2×10^3–2.2×10^3)
1	Low	7.9×10^3 (5.6×10^3–1.1×10^4)	1.6×10^4[c]	$>1.0 \times 10^4$[d]
1	High	4.1×10^3 (2.0×10^3–7.1×10^3)	ND[e]	ND
2	Low	2.1×10^4 (1.4×10^4–2.7×10^4)	$>2.0 \times 10^4$[f]	ND
2	High	2.3×10^4 (1.3×10^4–5.6×10^4)	ND	ND
3	Low	1.5×10^4 (6.5×10^3–2.3×10^4)	$>2.0 \times 10^4$[g]	ND
4	Low	3.5×10^4 (2.7×10^4–4.4×10^4)	ND	ND

[a] MOI, Multiplicity of infection.
[b] LD_{50} values were calculated by using probit analysis. The numbers in parentheses are the 90% confidence limits. Each LD_{50} represents a single virus pool.
[c] There was insufficient virus to get a good value by probit analysis. The LD_{50} was determined roughly from data showing 6 of 10 mice dead at a dose of 2×10^4 PFU and 2 of 21 mice dead at a dose of 1×10^4 PFU.
[d] Highest dose available for inoculation. The percent mortality at this dose was 19% (4 of 21 mice died).
[e] ND, Not done.
[f] Highest dose available for inoculation. The percent mortality at this dose was 27% (3 of 11 mice died).
[g] Highest dose available for inoculation. The percent mortality at this dose was 45% (13 of 29 mice died).

TABLE 2. *Effect of mixing virulent and attenuated viruses*

Dose of virulent virus (PFU)	Expt 1			Expt 2		
	% Mortality with virulent virus alone	% Mortality with MEF-passaged virus[a]	P[b]	% Mortality with virulent virus alone	% Mortality with TROC-passaged virus[a]	P[b]
8×10^3	100 (22/22)[c]	71 (15/21)	0.01	ND[d]	ND	
4×10^3	83 (19/23)	77 (17/22)	0.65	70 (7/10)	80 (8/10)	0.60
2×10^3	76 (16/21)	67 (14/21)	0.49	60 (6/10)	82 (9/11)	0.26
1×10^3	ND	ND		45 (5/11)	36 (4/11)	0.66

[a] Virus from MEF passage 3 or TROC passage 3 at a final dose of 4×10^3 PFU was mixed with varying dilutions of virulent virus before inoculation into suckling mice. The resulting percent mortalities were compared with the percent mortalities of mice which received the same dilutions of virulent virus alone.
[b] Differences were analyzed by the chi-square test.
[c] Numbers in parentheses are number dead/number inoculated.
[d] ND, Not done.

fected virulence. Whereas 8×10^3 PFU of virus harvested 2 to 3 weeks postinfection killed 95% of the inoculated mice (Fig. 2), 6.4×10^4 PFU of virus harvested 6 weeks postinfection did not kill any mice (Table 3). The LD_{50} values for 6-week virus could not be calculated due to virus yields, but they certainly more than exceeded a 60-fold increase over virus harvested 2 to 3 weeks after infection.

Reversal of in vivo attenuation by immunosuppressive treatment. Figure 3 shows that the attenuation which occurred with time in mouse salivary glands could be reversed by treating mice with 150 mg of cyclophosphamide per kg 9 and 4 days before virus was harvested at 5 weeks postinfection. Whereas 2×10^5 PFU of salivary gland-passaged virus from untreated mice killed only 30% of the suckling mice inoculated, the same dose of virus from cyclophosphamide-treated animals killed 100%, a significant difference ($P < 0.00001$). The LD_{50} for virus harvested after cyclophosphamide treatment was 2.1×10^3 PFU/mouse (90% confidence limits, 1.8×10^3 to 2.6×10^3 PFU/mouse), a value comparable to the values obtained for salivary gland-passaged virus pools harvested 2 to 3 weeks postinfection (Table 3). In contrast, the LD_{50} for virus harvested from untreated mice at 5 weeks was more than 2×10^5 PFU/mouse (the highest dose available for inoculation).

A similar reversal in attenuation was observed when mice were treated simultaneously with goat anti-mouse lymphocyte serum (twice weekly for 3 weeks) and 125 mg of hydrocortisone per kg (three times weekly for 2 weeks) immediately before salivary gland-passaged virus was harvested at 5 weeks postinfection.

TABLE 3. *Virulence of virus pools produced in vivo in salivary glands*

Mouse strain	Time between infection and harvest	LD_{50} (PFU)[a]
CD-1	14 days	1.7×10^3 (1.2×10^3–2.2×10^3)
CD-1	17 days	8.2×10^2 (4.7×10^2–1.1×10^3)
C_3H	19 days	1.5×10^3 (6.3×10^2–2.2×10^3)
CD-1	6 weeks	$>6.4 \times 10^4$[b]
C_3H	6 weeks	$>1.2 \times 10^5$[c]

[a] LD_{50} values were calculated by using probit analysis. The numbers in parentheses are the 95% confidence limits. Each LD_{50} represents one virus pool.

[b] Highest dose available for inoculation. At this dose none of 22 mice inoculated died.

[c] Highest dose available for inoculation. At this dose none of 19 mice inoculated died.

DISCUSSION

This study demonstrated that in vitro passage of murine cytomegalovirus in TROC and SGOC resulted in even more rapid attenuation than passage in MEF. The virus pools produced in vivo in the salivary glands of susceptible and resistant strains of mice were equally virulent for outbred suckling mice, suggesting that the mouse strain does not play an important role in determining the virulence of the virus produced. However, the time at which the virus is harvested from the salivary glands appears to be critical, since salivary gland-passaged virus harvested 5 to 6 weeks after infection was highly attenuated compared with salivary gland-passaged virus harvested 2 to 3 weeks after infection. When chronically infected mice were treated with cyclophosphamide before sacrifice, the resulting virus was as virulent as that obtained earlier in infection.

The results which we obtained after passage

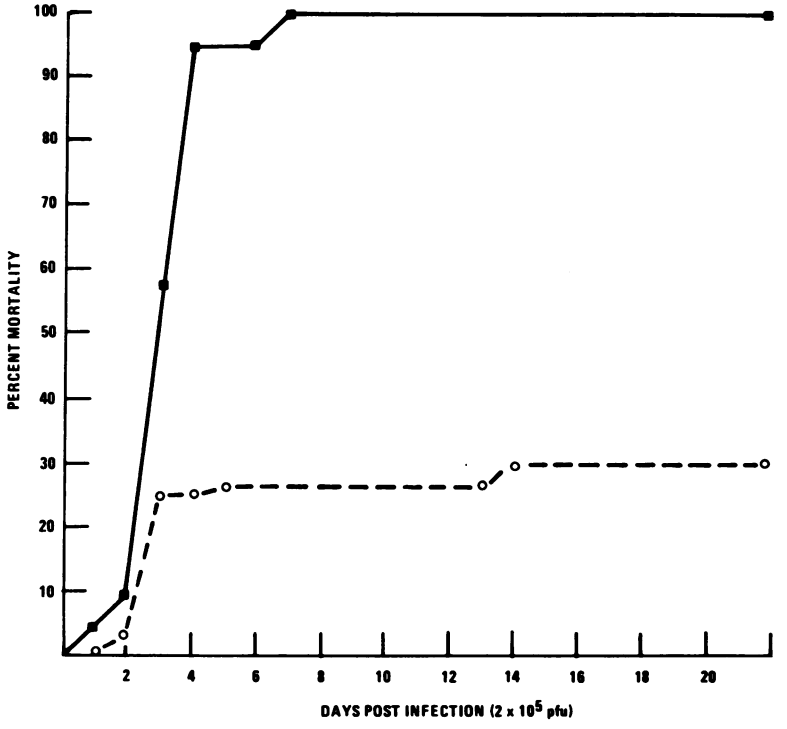

FIG. 3. *Two pools of virus were derived from mouse salivary glands 5 weeks after infection. One pool came from mice treated with 150 mg of cyclophosphamide per kg 4 and 9 days before harvest; the other pool came from untreated mice. Suckling mice were each inoculated with 2×10^5 PFU from one of these pools, and the percent mortalities for the two pools were compared. Symbols: ■, virus derived from cyclophosphamide-treated mice (19 of 19 inoculated mice died); ○, virus from control mice (12 of 36 inoculated mice died).*

of virus in MEF were similar to those reported by Osborn and Walker (22). Not only did MEF passage result in decreased mortality, but the length of time between inoculation and death was extended. Since salivary glands are the sources of virulent virus in intact mice and since much of the virus produced in these glands comes from epithelial cells (18), one might expect that an in vitro infection of epithelial cells would produce more virulent virus than the virus produced in fibroblasts. This was not observed. Fluorescent antibody and histological studies have suggested that epithelial cells are the main sources of virus in TROC and SGOC (14; unpublished data) but virus produced in these cultures appeared to be less virulent for suckling mice than virus produced in fibroblasts in vitro. Also, previous studies (19) indicated that TROC from resistant mouse strains were much less efficient virus producers and showed fewer cytopathic effects than TROC from susceptible mouse strains. Although there seems to be a direct correlation between mouse strain and production of virus in TROC, mouse strain does not appear to influence the production of virulent or attenuated virus.

In all of the cell types tested in vitro attenuation did not appear to be due to defective interfering particles, at least as they are typically described (9). Accumulations of such particles during passage would interfere with in vitro as well as in vivo production of virus and would cause in vitro-passaged virus to interfere with the virulence of wild-type virus when the two were mixed. Neither of these phenomena was observed in this study. We cannot eliminate the possibility that the "multicapsid virions" described by Hudson et al. (10), which appear to be absent from infected salivary gland homogenates but to be prevalent in infected MEF supernatants, are somehow related to attenuation. Although these multicapsid virions may produce plaques and infectious virus in tissue culture with an efficiency similar to the efficiency of single capsid virions, they may be handled differently by intact animals.

In mice, the source of virulent virus appears to be limited to the salivary glands. Other workers have found that virus harvested during acute stages of infection from livers, spleens, and kidneys are more attenuated for suckling mice than MEF-passaged virus (2; J. E. Osborn, personal communication). Furthermore, the results described here indicate that salivary gland production of virulent virus is limited to the first few weeks of infection. By 5 weeks after infection, salivary gland-passaged virus is also more attenuated than MEF-passaged virus. Virulence and attenuation are relative terms which compare the abilities of two or more strains of virus to produce disease. When MEF-passaged virus is compared only with salivary gland-passaged virus harvested 2 to 3 weeks after infection or from mice treated with cyclophosphamide, the MEF-passaged virus is naturally referred to as attenuated. However, it is clear from this discussion that the most attenuated virus is derived from livers, spleens, and kidneys of intact mice and from salivary glands late after infection. Compared with virus produced in these organs and in SGOC and TROC, MEF-passaged virus is virulent.

The modulation of salivary gland-passaged virus from virulent to attenuated during the course of infection may be related to the modulation of the immune response, which is depressed during the acute phase of infection (21, 26) but subsequently recovers and is even enhanced in the chronic stage of infection (30; H. H. Balfour, D. M. Mattsson, and R. J. Howard, Clin. Res. **26**:717A, 1978). The fact that cyclophosphamide treatment and anti-mouse lymphocyte serum–cortisone treatment, both of which depress immune responses (7, 12), caused chronically infected mice to produce virulent virus suggests that host immune responses do somehow affect the virulence of the virus produced. Alternatively, the effects of cyclophosphamide and cortisone could be unrelated to their immunosuppressive effects. Mims and Gould (18) have suggested that there are "sexual factors" present in the salivary glands, which affect the virulence of the virus produced. Cyclophosphamide or cortisone or both could exert an effect by altering these factors.

Whatever the reason for the enhanced virulence of virus after immunosuppressive treatment, it is obviously clinically significant for several reasons. First, in terms of justifying a vaccine, apparently attenuated vaccine virus may revert to virulence in immunocompromised hosts, causing adverse effects in some of the very people that the vaccine is meant to protect. Second, immunosuppressed patients who are seropositive for cytomegalovirus may shed virus having increased virulence and therefore may be more likely to infect seronegative contacts. In terms of managing immunosuppressed patients, it might be beneficial to separate patients who are seronegative from those who are not.

In this paper we raise several questions which have importance relative to the current interest in cytomegalovirus vaccines. The observations that passage of virus in tissue culture produces more virulent virus than passage in the spleens and livers of acutely infected animals and that

chronically infected animals shed more virulent virus when they are immunosuppressed point out several potential pitfalls to successful utilization of a vaccine.

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