9-(1-3-Dihydroxy-2-Propoxymethyl)Guanine Prevents Death but Not Immunity in Murine Cytomegalovirus-Infected Normal and Immunosuppressed BALB/c Mice

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Immunosuppressed (from treatment with cortisone acetate and anti-thymocyte globulin) and control adult female BALB/c mice, latently infected with murine cytomegalovirus (MCMV) or lethally challenged (10^6 PFU) with MCMV intraperitoneally, were treated with 9-(1-3-dihydroxy-2-propoxymethyl)guanine (DHPG) intraperitoneally. A dose of 3 mg/kg reduced mortality by 50% in lethally challenged normal mice; 10 mg/kg was required in immunosuppressed mice. When 15 mg/kg was given, the onset of treatment could be delayed for 64 h after lethal challenge. DHPG did not prevent the establishment of latent MCMV infection or immunosuppression-induced reactivation. The antibody titer to MCMV in DHPG-treated mice which survived lethal challenge was 41 (reciprocal of geometric mean) 4 to 5 weeks after inoculation; such mice survived a second lethal challenge. When antiserum treatment was begun 64 h and DHPG was begun 72 h after a lethal challenge, most mice survived; most did not when either treatment alone was begun at those times. In summary, DHPG effectively treated lethal MCMV infection even in immunosuppressed mice and even when treatment onset was delayed for 64 h. Treatment did not alter the establishment or reactivation of latent infections or the induction of effective immunity. The administration of DHPG coupled with antiserum treatment may be even more effective than the administration of either alone.

The most important cause of infectious disease morbidity and mortality complicating organ transplantation is the human cytomegalovirus (HCMV) (10). These infections may be due to primary infections in the nonimmune individual, to activated latent cytomegalovirus (CMV) infections in a previously immune person given immunosuppressive therapy, or to superinfection with exogenous HCMV in individuals whose immunity is ablated by immunosuppression (1). Pneumonia, fever, and chorioretinitis are the direct results of such HCMV infections, infections which can also induce a range of serious bacterial and fungal diseases as a result of their immunosuppressive capacity. Currently available methods to prevent or treat these CMV infections have limited efficacy. A vaccine is not yet available, and adenine arabinoside and acyclovir, agents which are effective against other human herpesviruses, are not sufficiently effective against HCMV (2, 8).

A newer agent, 9-(1-3-dihydroxy-2-propoxymethyl)guanine (DHPG), has been shown to be effective in vitro (6, 7, 9) and has had limited effectiveness in treating HCMV infections (3, 8). In addition, alpha interferon and anti-HCMV immunoglobulin have been shown to have some effectiveness when administered prophylactically (4, 16).

The murine CMV (MCMV) system provides a useful model for exploring the potential utility of these approaches. Shanley et al. (14) have reported an anti-MCMV effect of DHPG in vitro and that DHPG reduced MCMV titers in the lung, although it did not prevent the pneumonitis induced by the dual effects of MCMV challenge and cyclophosphamide administration. Katzenstein et al. (5) demonstrated that

DHPG prevented the death of BALB/c mice inoculated with a lethal dose of MCMV if therapy was begun no later than 24 h after viral challenge. Such treatment, however, did not prevent the establishment of latent infection. Using this model, we have previously reported that antisera could effectively treat MCMV, resulting in both an immediate passive and a long-term active immunity (11).

In the present series of experiments, we sought to determine the effectiveness of DHPG in both normal and immunosuppressed mice challenged with lethal doses of MCMV, to ascertain the impact of treatment on long-term immunity and viral latency, and to explore the possible utility of combined DHPG and antiserum administration.

MATERIALS AND METHODS

Mice. Adult female BALB/c mice obtained from Cumberland View Farms, Clinton, Tenn., were used in all experiments. They were kept in bonneted cages to prevent cross infection, with no more than six per cage; mice were given Purina Laboratory Chow and water ad libitum.

Virus. The Smith strain of MCMV was maintained in this laboratory by serial passage in CD-1 Swiss Webster mice (Charles River Breeding Laboratories, Inc., Wilmington, Mass.). Three-week-old CD-1 mice were inoculated intraperitoneally (i.p.) with 10^3 to 10^4 PFU of MCMV. Twenty-one days after infection, the salivary glands were harvested and homogenized in Dulbecco medium containing 10% fetal calf serum and 10% dimethyl sulfoxide. The virus stock was a 10% (wt/vol) homogenate of infected salivary gland with a titer of 10^8 PFU/ml.

Virus assay. Secondary mouse embryo cell cultures were prepared in Dulbecco medium plus 10% fetal calf serum and

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TABLE 1. Effect of DHPG on MCMV-induced mortality in normal and immunosuppressed mice

Treatment group	Dose[a]	No. of survivors/total (%)
Normal mice		
DHPG	20	13/13 (100)
	19.2	8/8 (100)
	12.8	8/8 (100)
	6.4	7/7 (100)
	5.0	6/7 (86)
	2.5	6/15 (40)
	1.25	2/8 (25)
	0.625	2/8 (25)
	0.3125	0/8 (0)
Saline		2/25 (8)
Immunosuppressed mice		
DHPG	20	6/10 (60)
	15	4/5 (80)
	10	6/10 (60)
	7.5	1/10 (10)
	5	0/9 (0)
Saline		1/10 (10)

[a] Dose in milligrams per kilogram per day given i.p.

plated onto six-well plates. A plaque assay utilizing these secondary mouse embryo cell cultures overlaid with tragacanth solution was used as previously described to determine the quantity of MCMV in tissue samples (12, 13).

Antibody assay. Antibodies to MCMV were determined by an indirect immunofluorescence slide technique using acetone-fixed MCMV-infected primary mouse embryo cells as previously described (11). Titers are given as the reciprocal of the highest dilution of serum which demonstrated specific immunofluorescence.

Latently infected mice. Adult mice, 4 to 6 months after inoculation with 10^4 PFU of MCMV (when the mice were 5 weeks old), had no recoverable virus in their salivary glands and had a positive antibody response to MCMV by immunofluorescence assay. Immunosuppression with cortisone acetate and anti-thymocyte globulin caused reactivation of the MCMV infection manifest by recovery of MCMV from the liver, heart, and salivary gland, but the infection was clinically inapparent (12).

Treatment. DHPG (BW B759U) was kindly supplied by Burroughs Wellcome Co., Research Triangle Park, N.C. The dry powder was solubilized in phosphate-buffered saline. The solution was stored at 4°C. Fresh stock was prepared every other day. In most experiments, DHPG treatment was given in one i.p. injection per day, beginning the day after challenge with virus. The control mice were inoculated with an equivalent volume of saline.

Cortisone acetate (125 mg/kg) given 6 days/week (Merck Sharp & Dohme, West Point, Pa.) and anti-lymphocyte serum (0.2 ml) given twice weekly (kindly provided by Henry Winn, Transplantation Unit, Massachusetts General Hospital) were inoculated i.p. for immunosuppression of mice.

Antiserum preparation. Antiserum used in these studies was prepared and tested by two different methods (11). The first method was to inoculate already latently infected mice i.p. weekly for 4 to 5 weeks with 10^4 to 10^5 PFU of MCMV. Four to 6 weeks later, blood was obtained by ocular bleeding, the serum was separated and pooled, and the antibody titers were determined by indirect immunofluorescence assay. The serum was stored at −70°C until used. The second

method for preparing antiserum was to inoculate latently infected mice i.p. weekly for 2 months with a mixture of MCMV and complete Freund adjuvant. Ascites developed after 5 to 7 weeks, and the ascitic fluid was removed from the abdominal cavity at weekly intervals. The weekly tappings of ascitic fluid were pooled, aliquoted, and stored at 4°C overnight to allow for clot formation. After centrifugation, the titer of the supernatant was determined and the supernatant was stored at −70°C until used. Ammonium sulfate precipitation was used to obtain the immunoglobulin G component of the ascites fluid preparation. The titers of all antiserum preparations used in these experiments were determined by two different antibody assays, the indirect immunofluorescence assay previously described (12) and a standard plaque-reduction (virus neutralization) assay (13).

RESULTS

Determination of effective dose of DHPG in normal BALB/c mice. The results of treating normal adult BALB/c mice challenged with 10^6 PFU of MCMV i.p. (which killed 95% or more of the mice) are shown in Table 1. DHPG was administered i.p. in doses ranging from 0.3125 to 20 mg/kg for 4 days, beginning the day after virus challenge. Using those data, it was calculated that a dose of 3 mg/kg would reduce mortality by 50%.

Effective dose determination in immunosuppressed mice. The DHPG dose required when mice were challenged with MCMV (10^6 PFU i.p.) and then immunosuppressed with cortisone acetate and anti-thymocyte globulin beginning on the same day is also shown in Table 1. DHPG treatment was initiated the day after virus challenge and was given for 4 days. A dose of 10 mg/kg was necessary to obtain a 50% reduction in mortality.

Effect of delaying start of DHPG treatment. If mice were treated with a 15-mg/kg dose of DHPG, the onset of treatment could be delayed 64 h after challenge with a lethal quantity of MCMV and still achieve a 50% survival rate (Table 2). Delay in the onset of treatment beyond that resulted in greater than 50% mortality, even when therapy was continued for 2 weeks.

Increasing the dose of DHPG to 30 mg/kg increased the percentage of survivors when treatment was begun 48 h after viral challenge but did not significantly prolong the delay after challenge when that dose of drug could be given and still achieve a greater than 50% survival rate.

Recovery of MCMV from viscera during DHPG treatment. The quantity of virus recovered from the liver, heart, and salivary gland of DHPG-treated and untreated MCMV-infected mice 5 days after challenge is shown in Table 3.

Significant multiplication of MCMV continued for weeks after the administration of DHPG. Organ virus titers of

TABLE 2. Effect of delaying the onset of DHPG treatment

Onset of treatment[a]	No. of survivors/total (%) at dose[b] of:	
	15 mg/kg	30 mg/kg
24	7/7 (100)	11/11 (100)
48	4/7 (53)	10/11 (91)
64	3/5 (60)	ND[c]
72	4/17 (23)	1/11 (9)
96	0/10 (0)	0/6 (0)
No treatment	0/15 (0)	0/11 (0)

[a] Hours after viral challenge when treatment was begun.
[b] Dose of DHPG given i.p., 4 days/week for 2 weeks.
[c] ND, Not determined.

TABLE 3. Virus recovered 5 days after challenge

Organ	Virus recovered in mice[a]	
	DHPG treated	Control
Heart	3.5	4.0
Liver	5.6	6.5
Salivary gland	2.7	4.6

[a] Data given as $\log_{10}$ PFU per gram and are average of three mice.

TABLE 5. Effect of treatment with antibody and DHPG

Onset of treatment[a]	No. of survivors/total treated with:	
	DHPG	Antibody and DHPG
64	3/5	
72	0/8	4/5[b]
96	0/8	0/8[c]

[a] Hours after viral challenge when DHPG was begun.
[b] Antibody given at 64 h and DHPG given at 72 h.
[c] Antibody given at 72 h and DHPG given at 96 h.

survivors at 2 weeks were not significantly different between mice given 15 mg and those given 30 mg of DHPG per kg (Table 4). Data are not shown for control mice because all died by day 7 after inoculation.

Effect of DHPG on antibody response to MCMV. The titer of antibody to MCMV in DHPG-treated animals which had survived a lethal challenge of MCMV was 41 (reciprocal of the geometric mean; $n = 14$) 4 to 5 weeks after challenge. Moreover, all such mice survived a second challenge with 10^6 PFU of MCMV (a lethal dose in untreated mice) administered 4 or 5 weeks after the initial challenge. Seven to nine days after that second viral challenge, there was no detectable virus in the livers or salivary glands of those mice.

Effect of DHPG on immunosuppression-induced reactivation of latent MCMV infection. Latently infected mice received immunosuppressive treatment as indicated, and on the next day, DHPG treatment was started. Doses ranging from 5 to 45 mg/kg were administered 4 days per week, and immunosuppressive therapy was continued. Control animals received phosphate-buffered saline and immunosuppressive therapy. At weekly intervals for 3 weeks, the animals were sacrificed and the liver, heart, and salivary glands were tested for virus. There was no difference in the quantity of virus recovered or in the anti-MCMV antibody titers between DHPG- and phosphate-buffered-saline-treated mice. No deaths occurred in either group, and the infections were inapparent.

Effect of DHPG on establishment and reactivation of latent infection. Twenty-eight mice were inoculated with 10^4 PFU of MCMV, and the next day, nine were given a 10-mg/kg dose of DHPG; treatment was continued for 4 days. Control animals were treated with phosphate-buffered saline. One month later, three animals from each group were sacrificed, their livers, hearts, and salivary glands were tested for virus, and their sera were tested for antibody titers. There was no difference in the findings in the two groups.

Immunosuppressive therapy of the remaining mice was begun and continued for the duration of the experiment. At weekly intervals for several weeks, two to five mice per group were sacrificed and studied virologically and serologically.

One month after the initial virus challenge, both groups of

mice had a small quantity of MCMV in their salivary glands. Increased concentrations of MCMV were found in the organs of the mice tested during the continued administration of immunosuppressive treatment, but there was no significant difference between the treatment and control groups.

Effect of treatment with DHPG and antibody. Four of five mice survived a lethal challenge when antiserum was given 64 h and DHPG was begun 72 h after MCMV challenge (Table 5). In the other experiments reported herein (Table 2), most mice had died when DHPG alone was started 72 h after lethal MCMV challenge; in previously reported experiments, all mice died when antiserum was given 48 h or later after challenge (14).

DISCUSSION

These data establish the following findings. (i) The effective dose of DHPG for reducing the death rate in otherwise lethal MCMV infections is 3 mg/kg per day i.p. in BALB/c mice. (ii) Beginning DHPG treatment with doses of 15 mg/kg per day can be delayed for 64 h after injection of a quantity of virus which regularly kills 100% of the mice and still be effective. (iii) A DHPG dose of 8 mg/kg per day i.p. prevents death induced by the injection of a lethal quantity of MCMV even when immunosuppressive treatment is given concomitantly. (iv) This DHPG treatment does not prevent an antibody response, and antibody response is associated with the subsequent resistance of the animals to lethal viral challenge. (v) DHPG treatment did not prevent the establishment or reactivation of a latent MCMV infection. (vi) The concurrent administration of antibody and DHPG may be more effective than administration of either alone.

These data expand the observations of those who had shown the in vitro effectiveness of DHPG against MCMV and are consistent with the observations of Katzenstein et al. (5), who had found that DHPG prevented death in MCMV-infected BALB/c mice. In contrast to the work reported herein, Katzenstein et al. administered DHPG subcutaneously or i.p. and found that treatment begun 24 h postchallenge saved less than 50% of the mice. They did not report whether the animals were tested for an antibody response,

TABLE 4. Recovery of virus and antibody response of survivors

Onset of treatment[a]	MCMV recovered from tissue[b]						Antibody titer[c]	
	Liver		Heart		Salivary gland			
	15 mg/kg	30 mg/kg	15 mg/kg	30 mg/kg	15 mg/kg	30 mg/kg	15 mg/kg	30 mg/kg
24	3.8	3.6	<3	2	6.5	7.2	38	47
48	4.4	4.8	<3	2	6.3	7.3	38	53
72	4.8	5.3	<2	2	6.8	7.2	90	32

[a] Hours after lethal viral challenge when treatment was begun.
[b] Data given as $\log_{10}$ PFU per gram from tissues tested 2 weeks after challenge inoculation from mice treated with 15 or 30 mg of DHPG per kg per day.
[c] Data given as geometric mean of reciprocal of the highest dilution of serum showing effect.

nor did they determine the effect of DHPG on MCMV infection of immunosuppressed mice.

Shanley et al. (14) showed that 5 mg of DHPG per kg per day given subcutaneously (or an apparently equivalent dose provided in the drinking water) significantly reduced the quantity of virus recovered from the lung and salivary gland after the intranasal inoculation of a sublethal quantity of MCMV. It did not prevent the interstitial pneumonitis induced by that inoculation and the concomitant administration of cyclophosphamide, although DHPG did reduce its severity. DHPG may have a beneficial effect in human CMV infection, especially in treating CMV retinitis (3), but its effect on CMV pneumonitis is as yet unproven (15).

DHPG treatment did not prevent the establishment of latent MCMV infection in these studies; Katzenstein et al. (5) have made the same observation. Since DHPG reduces, but does not eliminate, MCMV multiplication, and latent infections regularly follow acute MCMV infection, these results are not surprising. Latent infections of herpesvirus type 1 induced by percutaneous inoculation were prevented by DHPG, but only if the drug was administered topically to the same cutaneous site no later than 6 h after virus inoculation. DHPG treatment that early and in that fashion probably is not feasible in most instances of MCMV and human CMV infections.

It is significant that DHPG did not prevent the animals from acquiring protective immunity even though it was an effective treatment of otherwise lethal infections. It reduced the multiplication of MCMV and thus the potential antigenic mass but apparently did not significantly alter the antibody response. In this regard, it was similar to treatment with hyperimmune anti-MCMV antiserum. This is an important finding which could be relevant for human CMV infectious disease and its treatment.

A recent review of antiviral chemotherapy noted that the concurrent administration of antiserum and an antiviral chemotherapeutic agent is beginning to be addressed more systematically (2). In that context, the preliminary observation reported here is promising. Most mice survived when antiserum was given 64 h and DHPG was given 72 h after a lethal MCMV challenge, whereas all mice died when antiserum alone was given 48 h after lethal MCMV challenge and most mice died when DHPG alone was given 72 h after such a challenge. Therefore, we believe this observation merits additional exploration, and those experiments are planned.

In summary, DHPG effectively treated lethal MCMV infections of BALB/c mice even when the mice were immunosuppressed; it did so even when the initiation of treatment was delayed for 64 h after lethal virus challenge. Moreover, this did not prevent the induction of protective immunity. When coupled with hyperimmune antiserum, DHPG may be even more effective than when it is used alone. DHPG treatment did not prevent the establishment or reactivation of latency.

ACKNOWLEDGMENTS

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