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Generalized cytomegalovirus (CMV) infection and CMV-induced pneumonitis in the rat: Combined effect of 9-(1,3-dihydroxy-2-propoxymethyl)guanine and specific antibody treatment

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Abstract

The combined effect of 9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG, ganciclovir) and hyper immune serum (HIS) was studied in two different rat models. In the first model, a lethal generalized rat cytomegalovirus (RCMV) infection was established in immunosuppressed Brown Norway (BN) rats. Treatment with DHPG or hyper immune serum (HIS) effectively reduced both mortality rate and virus titers in the liver and lungs. By combined treatment the effective dose of both DHPG and HIS was reduced to 25%. The fractionary effective dose was 0.5, indicating a moderate synergistic effect on survival. Combined treatment also established a significant reduction of virus titers in lungs and liver ($P < 0.01$), but not in spleen. In the second model, interstitial pneumonia (IP) was established in RCMV-infected immunosuppressed BN rats after allogeneic bone marrow transplantation. IP was characterized by infiltration of mononuclear cells and diffuse thickening of the alveolar septal wall. DHPG reduced virus titers in the lungs but had no effect on IP. In contrast, HIS significantly reduced both virus titers and histopathologic changes in the lungs. Combined DHPG and HIS treatment minimized virus titers in internal organs and CMV-induced IP. Likewise, combined DHPG and control immune serum treatment significantly reduced immunopathologic changes in the lungs.

Keywords: Cytomegalovirus; Antibodies; Ganciclovir; Pneumonia; Rat

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1. Introduction

Cytomegalovirus (CMV) has been recognized as one of the most common pathogens causing a wide spectrum of symptoms in immunocompromised patients, depending on the state of the host immunity. In neonates CMV infections are characterized by hepatosplenomegaly, neurologic disorders and thrombocytopenia (Ho, 1991). In patients with the acquired immunodeficiency syndrome (AIDS), CMV-induced retinitis, pneumonia and gastrointestinal ulcers frequently occur (Wiley et al., 1988). In allogeneic bone marrow transplant (BMTx) recipients CMV-induced interstitial pneumonia (IP) is an important feature, representing a major cause of death with a mortality rate of up to about 80% (Ho, 1991; Winston et al., 1990).

Although the clinical use of the agent is limited by adverse reactions (Balfour, 1990; Collaborative DHPG Treatment Group, 1986), the antiviral drug, 9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG, ganciclovir) has been shown to be therapeutically effective against CMV disease in man (Collaborative DHPG Treatment Group, 1986; Goodrich et al., 1991; Shepp et al., 1985). Alternatively, intravenous treatment with hyper immune serum (HIS) containing polyclonal antibodies directed against CMV seems plausible, especially for treatment of CMV-induced IP after allogeneic BMTx (Snydman et al., 1987). In addition, several reports about the combined action of HIS and DHPG gave promising results for treatment of CMV-induced pneumonitis (Emanuel et al., 1988; Ljungman et al., 1992; Reed et al., 1988; Rubin, 1991). To study the therapeutical approach of CMV infections, we used rat cytomegalovirus (RCMV), isolated and characterized in our laboratory (Bruggeman et al., 1982; Meijer et al., 1984). Similarly to CMV infections in man, the course of RCMV infections in the immunocompetent host is asymptomatic, but in immunocompromised animals severe generalized infection occurs. Symptoms are characterized by splenitis, hepatitis and thrombocytopenia (Stals et al., 1990; Stals et al., 1991). In addition, CMV infection in allogeneic bone marrow transplantation (BMTx) recipient rats results in severe IP (Stals et al., 1993).

The therapeutical effect of single and combined treatment with DHPG and HIS on lethal generalized RCMV infection was investigated. Finally, the effect of these agents on the development of CMV-induced IP was investigated in an allogeneic bone marrow transplant model.

2. Materials and methods

2.1. Viruses

RCMV was isolated and characterized in our laboratory (Bruggeman et al., 1982; Meijer et al., 1984; Meijer et al., 1986). For in vitro studies and for the production of HIS, supernatant of RCMV-infected rat embryonal fibroblasts (REF) was used. For in vivo experiments RCMV was obtained from suspensions of salivary glands after several in vivo passages (Bruggeman et al., 1985). Uninfected salivary gland suspensions were used for control experiments.

2.2. Antiviral agents

DHPG (Syntex, Palo Alto, California, USA) was dissolved in sterile phosphate-buffered salt solution (PBS) and administered intraperitoneally (i.p.) at different dosages as indicated.

HIS was obtained from immunized male specific pathogen-free (SPF) Brown Norway (BN) rats after intradermal inoculation with RCMV as described before (Stals et al., 1990). Control immune serum (CIS) was prepared by administration of a non-infected REF suspension.

2.3. Virus plaque inhibition, antibody neutralization and cytotoxicity assays in vitro

Plaque inhibition assays were performed as described before (Stals et al., 1991). In short, 200 plaque forming units (PFU) of RCMV were incubated in basal medium Eagle's (BME) + 2% newborn calf serum (NCS) on REF monolayers in a 24-wells plate (Costar Europe, Badhoevendorp, The Netherlands) for 1 h at 37°C. Then, the medium was replaced by BME + 2% NCS containing DHPG in concentrations ranging from 200 to 0.01 μg/ml medium. After mixtures were removed the monolayers were covered with a BME-agarose mixture. After 8 days the monolayers were fixed in formalin 3.7% in PBS and stained with methylene blue. Experiments were carried out in triplicate. The minimal inhibitory concentration of the compound was expressed as the concentration required to inhibit plaque formation by 50% (EC_{50}). The EC_{50} was estimated from the number of plaques on semi logarithmic graphs as a function of the concentration of the antiviral compounds.

To test the concentration of neutralizing antibodies in HIS and CIS, the plaque-reduction assay was used (Plummer et al., 1964). In short, 2-fold dilutions of heat-inactivated serum were incubated with 300 PFU RCMV in BME + 2% NCS for 1 h at 37°C. These mixtures were absorbed on a REF monolayer in a 24-wells plate (Costar Europe) for 1 h at 37°C and replaced by a BME-agarose mixture. After 8 days of incubation the number of RCMV-induced plaques was monitored and the neutralization titer was expressed as the serum dilution at which the number of plaques was reduced by 50% (NT_{50}).

The inhibitory effects of DHPG and HIS on REF cell growth were determined as described before (Stals et al., 1991). The cytotoxic concentration of the compound is expressed as the IC_{50} or concentration required to reduce cell growth by 50%. The selectivity index corresponds to the ratio of IC_{50} for cell growth to the EC_{50} for RCMV replication.

2.4. Animals and CMV infection

Eight week old inbred SPF male BN rats with a total body weight between 140 to 180 g were used in all experiments. Adult Lewis and BN rats were used as donors for allogeneic and syngeneic bone marrow cells, respectively. Neither the donor animals nor the recipients had antibodies to RCMV in their peripheral blood at the start of each experiment, as determined by enzyme immunosorbent assay. Each rat received 10^5 plaque forming units (PFU) RCMV i.p. as described before (Bruggeman et al., 1983).

To prevent bacterial infections all animals received enrofloxacin 2 mg/kg/day (Baytril^R, Bayer, Mijdrecht, The Netherlands) and acidified drinking water (Stals et al., 1990; Vancutsem et al., 1990) during the whole experiment, starting 1 week before the onset of the experiment. Furthermore, all animals were kept in sterile cages and received sterile food.

The experiments were approved by the University Ethical Committee for animal experiments.

2.5. Preparation of donor-bone marrow cells

Bone marrow cells from donors were prepared as described earlier (Bos et al., 1989; Stals et al., 1993). One day after 9.6 Gy total body irradiation (TBI) and 6 h post-infection (p.i.) each rat received $5 \cdot 10^7$ viable cells intravenously.

2.6. Design of the experiment

In vivo combined treatment of DHPG and HIS was evaluated in two different infection models. In the first model generalized infection was induced by i.p. inoculation with RCMV. For immunosuppression animals received 5.0 Gray (Gy) TBI 1 day before infection. DHPG treatment (administered intraperitoneally) was started 6 h p.i. and continued twice daily for 5 days. One ml of HIS, diluted 2-fold in basal medium Eagle's with 2% newborn calf serum (BME + 2% NCS), was administered intravenously at 6 h p.i. To determine the effect of combined treatment, DHPG was administered twice daily for 5 days starting at 6 h p.i. and HIS was administered in a single dose at 6 h p.i.

The following controls were used: (1) Irradiated RCMV-infected rats, (2) irradiated non-infected animals and (3) RCMV-infected non-irradiated rats. In addition, irradiated RCMV-infected animals, which received undiluted negative control serum were included into the study.

The effect of (combined) treatment on survival was recorded over a period of 21 days p.i. The effect of treatment on virus titers in organs was determined at 8 days p.i., as described earlier (Stals et al., 1991). For this purpose spleen, liver and lungs were removed aseptically, homogenized in a tissue grinder and suspended in BME + 2% NCS and plaque assays were performed. The amount of virus was expressed as the number of PFU per gram organ tissue.

The in vivo activity of DHPG is expressed as the minimal effective dose or the daily dose at which at least 50% of the animals survived the lethal infection (ED_{50}). In addition, the mean day of death (MDD) was calculated. For histological studies material was processed as described below.

In the second model interstitial pneumonia (IP) was induced in allogeneic BMTx recipient rats. One day before RCMV inoculation and BMTx, animals received 9.6 Gy TBI. At the day of infection RCMV was administered i.p., followed by i.v. administration of freshly harvested viable bone marrow cells (Bos et al., 1989). DHPG, HIS and combined treatment were administered as described above. Uninfected allogeneic BMTx

recipients and infected syngeneic BMTx recipients were included in the study as well as CIS-treated animals and combined DHPG- and CIS-treated animals.

Rats were examined daily for signs of infections as described elsewhere (Stals, 1991) and survival was recorded. For plaque studies and histopathology, spleen and lungs were harvested 8 days p.i.

2.7. Histological examination

Samples of the organs mentioned above were fixed in paraformaldehyde-lysine-periodate (PLP) and embedded in paraffin. Serial 4 μm thick sections were prepared for hematoxylin-eosin (HE) staining and immunoperoxidase staining using monoclonal antibodies (McAb) to RCMV antigens as described earlier (Stals et al., 1990; Stals et al., 1991). Specificity of the McAb (numbers 8 and 35) was described before (Bruning et al., 1987). Monoclonal antibodies ED-1 and W3/13 were used for identification of rat monocytes/macrophages and rat T-cells, respectively (Brown et al. 1981; Dijkstra et al., 1985).

For morphometric analyses a reticulin staining was performed and the size of the alveolar wall was measured as described before (Stals et al., 1991). The relative amount of inflammatory infiltrate in alveolar septa was assessed semi-quantitatively in each section and was expressed in score units: absence of cells was scored as 0, slight inflammatory infiltrate as 1, moderate dense infiltrate as 2, and severe dense inflammatory infiltrate as 3.

2.8. Statistical analysis

For statistics on the in vitro results, the Mann-Whitney test and Fisher's exact test were applied.

Combined interaction was calculated from the fractionairy effective dose (*FED*):

$$FED = \left(ED_A^{comb}/ED_A^{alone}\right) + \left(ED_B^{comb}/ED_B^{alone}\right)$$

ED_A^{comb} and ED_A^{alone} stand for the minimal effective dose of agent A when used in combination or alone (Sühnel, 1990). The in vivo activity of a drug is expressed as the minimal effective dose or the daily dose at which at least 50% of the animals survived the lethal infection (ED_{50}).

FED values between 0.75 and 1.25 were considered to express an additional effect. *FED* values below 0.75 were considered to indicate a weak synergistic effect, *FED* values of 0.5–0.25 indicated a moderate synergistic effect and *FED* values of 0.25 or below were associated with a strong synergistic effect. For comparison of survival curves, the generalized Wilcoxon test by Gehan was used (Gehan, 1956; Gross et al., 1975). For distribution of discontinue variables by one-way ANOVA the Kruskall-Wallis test was used (Wallis et al., 1956). For two-tailed differences the Mann-Whitney U-Wilcoxon rank sum W test was used. *P*-Values < 0.05 were considered statistically significant.

3. Results

3.1. Activity of DHPG and HIS in vitro

DHPG inhibited RCMV plaque formation by 50% at a concentration (EC_{50}) of 25 μg/ml. The cell growth inhibitory concentrations (IC_{50}) of DHPG in uninfected REF was 1900 μg/ml. The selectivity index (ratio of IC_{50} for cell growth to EC_{50} for CMV plaque formation) of DHPG for RCMV was 76.

For HIS the neutralization titer (NT_{50}, the titer at which serum neutralizes 50% of the free virus) of the serum pool was 1:640 ($1.6 \cdot 10^{-3}$). However, at a HIS concentration as high as 20% (v/v) in BME the growth of REF cells was not inhibited ($IC_{50} > 0.2$), resulting in a selectivity index of > 125.

3.2. Effect of treatment on generalized CMV infection

Survival. RCMV-infected irradiated rats died at 7.6 ± 0.7 days p.i. As indicated in Table 1, DHPG therapy at a dose of 40 mg/kg/day, started 6 h p.i. and continued for 5

Table 1
Effect of treatment on survival of rats after generalized RCMV infection

Teatment	Dosage (mg/kg/day)	Neutralization titer	Generalized infection		
			survival	(%)	MDD [a]
DHPG [b]	5		0/10	(0)	7.8 ± 1.3
	10		0/10	(0)	8.7 ± 2.2
	20		10/14 [e]	(75)	14.0 ± 2.6 [e]
	40		7/8 [e]	(88)	17.0
HIS [c]		5	0/10	(0)	8.1 ± 1.0
		10	2/10	(20)	9.3 ± 2.5
		20	7/10 [e]	(70)	15.3 ± 2.1 [e]
		40	10/10 [e]	(100)	–
DHPG + HIS	2,5	2,5	1/8	(13)	8.3 ± 1.3
	5	5	7/9 [e]	(78)	15.5 ± 0.7 [e]
	10	10	8/9 [e]	(89)	10
	20	20	ND		
	40	40	ND		
PBS [b]			0/12	(0)	7.6 ± 0.7
CIS [d]			0/10	(0)	7.9 ± 1.2
uninfected, TBI control				5/5 [e]	(100)

Note: Groups of 6 BN rats each received 5.0 Gy TBI 24 h before either intraperitoneal inoculation with 10^5 PFU RCMV. Survival was recorded for 21 days p.i.

[a] Mean day of death $\pm$ S.D.

[b] DHPG 20 mg/kg/day twice daily for 5 days, started at 6 h p.i. Controls received PBS.

[c] HIS 1 ml with a neutralization titer of 640 was diluted in BME + 2% NCS and administered in one single dose at 6 h p.i. The corresponding titer after dilution is presented.

[d] CIS with a neutralization titer < 10 and a negative immunofluorescence titer was administered undiluted in one single dose at 6 h p.i.

[e] $P < 0.01$.

Table 2
Effect of combined treatment on organ virus titers during generalized infection

Therapy	Virus titers [a]		
	Spleen	Liver	Lungs
untreated	6.2 ± 0.5	5.5 ± 0.3	5.1 ± 0.3
CIS [b]	5.6 ± 1.3	5.3 ± 1.4	4.5 ± 2.9
HIS [c]	5.3 ± 1.1	< 1.3 [e]	< 1.3 [e]
DHPG 20 mg/kg [d]	4.0 ± 1.5	2.9 ± 1.2 [e]	2.9 ± 1.8 [e]
DHPG + HIS	4.2 ± 1.8	< 1.3 [e]	< 1.3 [e]

Note: Groups of 6 BN rats each received 5.0 Gy TBI 24 h before intraperitoneal inoculation with 10^5 PFU RCMV. Organs were harvested at 8 days post-infection.
This table is representative for 2 repetitive experiments.
[a] Log PFU/g tissue.
[b] Control immune serum (CIS) 1 ml with a neutralization titer < 10 was administered in one single dose at 6 h p.i.
[c] HIS 1 ml with a neutralization titer of 640 was administered in one single dose at 6 h p.i.
[d] DHPG 20 mg/kg.day twice daily for 5 days, started at 6 h p.i.
[e] $P < 0.01$.

days with a dose interval of 12 h prevented mortality from RCMV infection in 88% of the cases (survival: 7/8; MDD = 17.0). DHPG treatment for 3 days was not effective (survival: 0/10). If the onset of treatment was delayed for 3 days p.i., no survival occurred (survival: 0/10, MDD = 7.7 ± 1.3 days), (data not shown).

For DHPG the ED_{50} was 20 mg/kg/day (twice daily for 5 days). In addition, the MDD (14.0 ± 2.6) was extended significantly by DHPG treatment ($P < 0.01$), as compared to untreated animals.

The ED_{50} of HIS was 40 times the NT_{50}. At this dose, the MDD was 15.3 ± 2.1 ($P < 0.01$). However, CIS treatment did not affect survival. Analogously to DHPG treatment, HIS had no effect on survival when administration was delayed for 3 days p.i. and no effect on the MDD could be recorded (survival: 0/10, MDD = 8.0 ± 1.2 days).

The FED_{50} for the combination of DHPG and HIS was 0.50, indicating a moderate synergistic effect.

Control rats, which received either TBI or RCMV, all survived (5/5) as did the animals which received CMV-negative salivary gland homogenate (5/5).

Virus titers. Table 2 shows the effects of DHPG and HIS treatment regimens on the virus titers in several organs at 7 days p.i. After DHPG treatment at dosages of 40 and 20 mg/kg administered for 5 days, RCMV titers in the liver and lungs were reduced by 3 log values vs. untreated controls ($P < 0.01$), but RCMV titers in spleen were not significantly affected. DHPG dosages of 10 mg/kg/day or less administered for 5 days, did not markedly reduce virus titers in any organ (data not shown).

Likewise, administration of HIS (1 ml serum with a NT_{50} of 160) caused a significant reduction of virus titers in the liver and lungs as compared to untreated controls ($P < 0.01$), but had no effect on virus titers in the spleen. In addition, combined treatment with DHPG and HIS significantly reduced virus titers in lungs and liver to below detection level ($P < 0.01$), but again RCMV titers in the spleen were not significantly affected.

Table 3
Effect of treatment on infectious CMV in allogeneic BMTx recipient rats

Group	Treatment	Infection (10^5 PFU)	BMTx	(n)	Virus titers [a]	
					lung	spleen
1	none	RCMV	allogeneic	(22)	3.8 ± 0.3	4.3 ± 1.1
2	DHPG [b]	RCMV	allogeneic	(12)	1.3 ± 0.5 g [e]	3.2 ± 0.3
3	HIS [c]	RCMV	allogeneic	(11)	1.7 ± 0.6 [e]	1.6 ± 0.6 [e]
4	CIS [d]	RCMV	allogeneic	(6)	3.4 ± 0.3	4.0 ± 1.0
5	DHPG + HIS	RCMV	allogeneic	(6)	< 1.25 [e]	< 1.25 [e]
6	DHPG + CIS	RCMV	allogeneic	(6)	1.4 ± 0.9 [e]	2.4 ± 0.6 [e]
7	none	RCMV	syngeneic	(8)	4.1 ± 0.2	3.9 ± 0.2
8	none	none	allogeneic	(8)	n.d.	n.d.

Note: Groups of 6 BN rats each received 9.6 Gy TBI and 2.10^7 viable allogeneic and syngeneic bone marrow cells 2 h before inoculation with 10^5 PFU RCMV i.p. Organs were harvested at 8 days post-infection. This Table is representative for 2 repetitive experiments.

[a] Log PFU/g tissue.

[b] DHPG 20 mg/kg/day twice daily for 5 days, started at 6 h p.i.

[c] HIS 1 ml with a neutralization titer of 640 was administered in one single dose at 6 h p.i.

[d] CIS 1 ml with a neutralization titer < 10 was administered in one single dose at 6 h p.i.

[e] $P < 0.01$.

3.3. Effect of DHPG and HIS treatment on RCMV-induced IP

Virus titers in lungs and spleen. The effect of DHPG and HIS treatment on virus titers in lungs and spleen of RCMV-infected allogeneic BMTx recipient rats was measured using treatment schedules shown to be adequate for treatment of generalized infection, i.e., DHPG 20 mg/kg twice daily administered for 5 days and one dose HIS with a NT of 160. As shown in Table 3, under these conditions treatment with DHPG and HIS resulted in a significant decrease in RCMV titers in lungs, which were reduced from 3.8 ± 0.3 log PFU/g tissue in untreated rats to 1.3 ± 0.5 and 1.7 ± 0.6 log PFU/g in the DHPG- and HIS-treated groups, respectively ($P < 0.01$). However, after CIS treatment no effect on virus titers was recorded (3.5 ± 0.1 log · PFU/g). In the spleen virus titers decreased only slightly from 4.3 ± 1.1 to 3.2 ± 0.3 log PFU/g after DHPG treatment. In contrast, HIS treatment resulted in a significant decrease of virus titers in the spleen to 1.6 ± 0.6 log PFU/g ($P < 0.01$). Again, no significant reduction of virus titers was noted after CIS treatment (4.0 ± 1.0 log PFU/g).

After combined treatment with DHPG and HIS, a complete reduction was established of virus titers in the lungs. In addition, RCMV titers in the spleen were decreased to below detection level ($P < 0.01$). Combined DHPG and CIS treatment decreased RCMV titers to 1.4 ± 0.9 and 2.4 ± 0.6 log PFU/g in lung and spleen, respectively ($P < 0.01$).

Pathology. After allogeneic BMTx and subsequent RCMV infection more than 90% of the recipients died, at 8–10 days p.i., showing signs of severe dyspnea. Moreover, all rats displayed macroscopic signs of infection in internal organs consistent with symptoms after generalized RCMV infection (Stals et al., 1993).

Table 4
Effect of treatment on CMV-induced alveolar cell infiltration in allogeneic BMTx recipient rats

Group	Treatment	Infection (10^5 PFU)	BMTx	(n)	Alveolar septal thickness (μm) [a]	MN infiltration score [b]	ED-1 score [b]	W3/13 score [b]	Pattern diffuse focal/ no.
1	none	RCMV	allogeneic	(22)	6.0 ± 1.6	2 (3)	2 (2)	2 (3)	d
2	DHPG [c]	RCMV	allogeneic	(12)	4.7 ± 1.7	1 (2) [g,h,k]	1 (2) [g,h]	1 (3) [g,h]	f
3	HIS [d]	RCMV	allogeneic	(11)	2.9 ± 0.5 [f]	1 (4) [g,h,i,l]	1 (4) [g,h,l]	1 (3) [g,h,l]	f
4	CIS [e]	RCMV	allogeneic	(6)	5.8 ± 1.1	3 (1) [l]	2 (3) [l]	2 (1) [l]	f
5	DHPG + HIS	RCMV	allogeneic	(6)	2.6 ± 0.2 [f]	0 (1) [g,h]	1 (2) [g,h]	1 (1) [g,h]	f
6	DHPG + CIS	RCMV	allogeneic	(6)	2.6 ± 0.4 [f]	0 (1) [g,h]	1 (1) [g,h]	2 (2)	f
7	none	RCMV	syngeneic	(8)	2.5 ± 0.4 [f]	1 (0) [g,h]	0.5 (1) [g,h]	1 (1) [g,h]	f
8	none	none	allogeneic	(8)	1.8 ± 0.2 [f]	1 (2) [g,h]	1 (1) [g,h]	1 (1) [g,h]	f

Note: Groups of 6 BN rats each received 9.6 Gy TBI and $2 * 10^7$ viable allogeneic and syngeneic bone marrow cells 2 h before inoculation with 10^5 PFU RCMV i.p. Organs were harvested at 8 days post-infection.
This Table is representative for 2 repetitive experiments.
[a] The alveolar septa from each rat were measured 10 times by 2 independent observers. Each number represents the mean and standard deviation for these measurements in 6 rats.
[b] The presence of infiltrating mononuclear cells was scored on a scale from 0 (absent) to 3 (maximum); mean (variance).
[c] DHPG 20 mg/kg/day twice daily for 5 days, started at 6 h p.i.
[d] HIS 1 ml with a neutralization titer of 640 was administered in one single dose at 6 h p.i.
[e] CIS 1 ml with a neutralization titer < 10 was administered in one single dose at 6 h p.i.
[f] Differences by t-test vs. group 1, $P < 0.05$.
Kruskal-Wallis two way ANOVA: [g] vs. group 1, $P < 0.05$ [i] group 4 vs. 2 and 3.
Significant differences by Mann-Whitney-U-Wilcoxon Rank W Sum Test: [h] group 1 vs. 2,3,4,5,6,7,8. [l] group 3 vs. 4.

At 8 days p.i. RCMV-infected allogeneic BMTx recipient rats showed dilatation of alveolar vessels and infiltration of the perivascular area with MN cells, mainly consisting of W3/13 and ED-1 positive cells. Furthermore, alveolar edema, hemorrhages and diffuse infiltration of alveolar stroma with mononuclear cells (MN), in particular ED-1 and W3/13 reactive cells was noticed, accompanied by severe thickening of the alveolar septal wall (6.0 ± 1.6 μm), (Table 4). Treatment with DHPG 20 mg/kg/day for 5 days significantly reduced the number of infiltrating MN cells, including ED-1 and W3/13 reactive cells. In addition, the pattern of the infiltrating cells was focal. However, hemorrhagic congestion and vascular dilatation was hardly affected and the alveolar septal wall was still thickened (4.7 ± 1.7 μm). HIS administration in one dose significantly reduced the number of infiltrating MN, ED-1 and W3/13 infiltrating cells as compared to untreated rats and reduced the thickness of alveolar septa to 2.9 ± 0.5 μm as compared to untreated, or DHPG-treated or CIS-treated rats ($P < 0.01$). CIS treatment had no effect on the number of alveolar infiltrating MN, ED-1 and W3/13-reactive cells and hemorrhages, nor on the thickness of the alveolar septal wall, nor on perivascular infiltration.

Combined treatment with DHPG and HIS reduced the alveolar septal wall within normal limits (2.6 ± 0.2 μm). In addition, after combined treatment no marked infiltration in the alveolar septal wall, edema and hemorrhages were observed ($P < 0.01$) and

perivascular infiltrates and vascular dilatation were absent. Likewise, combined treatment with DHPG and CIS had similar effects.

Minimal alveolar infiltration and minimal alveolar septal thickening were observed in untreated controls after allogeneic BMTx without RCMV infection (1.8 ± 0.2 μm) and after syngeneic BMTx with subsequent RCMV infection (2.5 ± 0.4 μm).

4. Discussion

In the present study, we evaluated the effect of treatment with either DHPG or HIS or the combination of these two agents in two different infection models, one inducing a generalized RCMV infection (Stals et al., 1990; Stals et al., 1991) and the other specifically inducing RCMV-induced IP (Stals et al., 1993).

The specific action of DHPG for rat and human CMV in vitro, as expressed by the selectivity index, is comparable (80 and 100, respectively), (Balfour, 1990; Plotkin et al., 1985; Stals et al., 1991). HIS also shows a strong selective neutralizing effect on RCMV infection in vitro, since the SI of HIS for RCMV was about 125 (Stals et al., 1990).

In the generalized infection model the ED_{50} of DHPG was 20 mg/kg/day twice daily for 5 days. At that dose 75% of the animals survived infection. To be effective treatment had to be started within 48 h p.i. The therapeutic range of DHPG in generalized RCMV infection was at least 15, as calculated from the ED_{50} and the toxic dose (in earlier studies toxicity was not observed at dosages up to 300 mg/kg/day (Stals et al., 1991)). At a dose of 20 mg/kg/day DHPG treatment significantly reduced virus titers in liver and lungs, but not in spleen. In man, therapeutic activity of DHPG is recognized at 10 mg/kg/day after generalized CMV infections (Collaborative DHPG Treatment Group, 1986; Goodrich et al., 1991). At that dose DHPG treatment reduces mortality from CMV infection (Drew, 1992; Hirsch, 1992; Laskin et al., 1987; Ocular Complications of AIDS Research Group, 1992). So, DHPG seems to be effective in rats at the same dosage as described for man.

HIS treatment significantly reduced both mortality and virus titers in lungs and the liver, but not in the spleen, of CMV-infected rats. Although the mechanism is not clear, HIS treatment was effective only early in the course of infection, emphasizing the importance of timing for the success of therapy. Furthermore, as we pointed out earlier, the success of antibody therapy also largely depends on the degree of immunosuppression of the host (Stals et al., 1990). These aspects may possibly contribute to therapy failure of HIS treatment for generalized CMV infections in man, in whom the onset of treatment is generally late (Snydman, 1990). Another aspect of the limited success of HIS treatment, as applied in man, is due to the HIS preparations used. HIS is generally produced from CMV-seropositive donors with high immunofluorescence and neutralization titers, but the qualification of the preparations is not well established and depends on the donor population used (Snydman, 1990). Furthermore, the effect of HIS treatment may largely depend on the kind of immunosuppression in the host system. The beneficial effect of HIS treatment is most promising in bone marrow transplant and kidney transplant recipients where the viremic phase seems to be significantly shortened and the rate of interstitial pneumonitis is diminished (Snydman, 1990).

Using combined treatment of DHPG and HIS in generalized RCMV infection, the dose of DHPG could be reduced 4-fold to obtain 78% survival. Also the dosage of HIS could be reduced 4 times, resulting in a FED_{50} of 0.5, indicating a moderate synergistic effect of these two agents.

IP is one of the most life-threatening symptoms of CMV infection in man and occurs predominantly in allogeneic BMTx recipients. To study the effect of treatment on CMV-induced IP we have chosen a model in which the animals received a BMT across a large class I major histocompatibility complex barrier (Lewis to BN rats), TBI and RCMV infection, all described to be risk factors for development of IP (Ho, 1991). Severe IP accompanied by high virus titers in internal organs, including the lungs was recorded at eight to ten days p.i. Although DHPG treatment induced a significant decrease in RCMV titers in the lungs, the effect of DHPG on the immunopathological changes by RCMV-induced IP was relatively low. DHPG had only a minor but not significant effect on both mononuclear cell infiltration and thickness of the alveolar septa and no effect on vascular dilatation and alveolar edema and hemorrhages. These findings are consistent with the reports about the limited effect of DHPG treatment on HCMV-induced IP in allogeneic BMTx recipient patients (Crumpacker et al., 1988; Erice et al., 1987; Schmidt et al., 1991). In our view, the failure of response to antiviral treatment emphasizes the role of immunopathologic responses in the pathogenesis of CMV-induced IP (Grundy et al., 1987; Reed et al., 1988).

In contrast to the poor response on DHPG therapy, the effect of HIS administration on CMV-induced IP seemed to be 2-fold: HIS treatment led to a significant decrease of RCMV titers in the spleen and lungs. This phenomenon could be explained by the fact that the presence of specific antibodies in the blood may inhibit the spread of virus infection in the host, resulting in a decreased virus titer in internal organs. We hypothesize that viral spread may be inhibited by antibody treatment. In addition to its antiviral effect, HIS also prevented RCMV-induced IP, as marked by a significant decrease in infiltrating mononuclear cells, being predominantly ED-1 and W3/13 reactive cells, a reduction in vascular dilatation, a reduction in thickness of the alveolar septal wall, alveolar edema and hemorrhages. CIS had no antiviral effect, nor any effect on CMV-induced histopathologic changes in the lungs, indicating that the antiviral action of HIS is very specific. Likewise, in man many clinical reports describe a beneficial effect of HIS on the immunopathologic response to CMV infection in the lungs and a decrease of mortality in BMTx recipients, but HIS induces only a minor decrease in infectious virus shedding (Winston et al., 1987).

Combined treatment of DHPG and HIS resulted in a dramatic decrease in RCMV titers in lungs and in spleen, again indicating a strong inhibitory effect on multiplication of infectious virus. As compared to HIS alone, no significant differences were noted between the inflammatory response in the lungs and vascular dilatation after combined DHPG and HIS therapy, probably because of the strong beneficial effect of antibodies on the immunopathological response in the lungs. A particular observation was that the combination of DHPG and CIS was as effective as the combination of DHPG and HIS and was significantly more effective then DHPG alone in decreasing histopathologic changes in the lungs. This led to the hypotheses that the reduction of immunopathologic changes by HIS is not specific, but that reduction in virus titers by either DHPG or

antibodies are a prerequisite for the immune modulating effect of non specific serum antibodies. In man, various reports indicate the improved therapeutic response on CMV-induced IP after combined treatment with HIS and DHPG in allogeneic BMTx recipients (Emanuel et al., 1988; Ljungman et al., 1992; Reed et al., 1988; Schmidt et al., 1989). Generally, the regulation of the immune system can be manipulated by intravenous administered immunoglobulin in several ways: Antigen-antibody complexes can bind the Fc-receptor for IgG on phagocytic, B, and T-cells. In addition, the idiotype antibodies can be recognized by antiidiotype antibodies, which can neutralize autoantibodies and bind and down regulate B-cell receptors for autoantibodies. Of greater importance, T cells may recognize antiidiotype antibodies or idiotype-antiidiotype complexes, to bind and subsequently suppress certain T cells by lymphokine induction (Dwyer, 1992). This possibility may be supported by the finding that in the rat model lymphokines also affect the course of CMV infection under immunocompromised conditions (Haagmans et al., 1994; Haagmans et al., 1994), but more research on the pathogenesis of IP and the action of immunoglobulins under these conditions is necessary to study the mechanism by which IP is prevented by CIS.

In conclusion, combined treatment of DHPG and HIS early in generalized infection had a moderate synergistic effect on survival and effectively reduced infectious RCMV titers in lungs and the liver, but not in the spleen. We hypothesize that viral spread may be inhibited by antibody treatment. In addition, combined treatment has a remarkable therapeutic effect on CMV-induced IP after allogenic BMTx, as reflected by both local virus titers as well as the immunopathologic response in the lungs.

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