Ganciclovir Prophylaxis for Cochlear Pathophysiology during Experimental Guinea Pig Cytomegalovirus Labyrinthitis

NIGEL K. WOOLF,[1,2]* JAMES W. OCHI,[1,2] ERIC J. SILVA,[1,2] PATRICIA A. SHARP,[1,2] JEFFREY P. HARRIS,[1,2]
AND DOUGLAS D. RICHMAN[2,3]

Division of Otolaryngology, Department of Surgery,[1] and Departments of Pathology and Medicine,[3] University of
California San Diego Medical Center, San Diego, California 92103, and Research Service, Veterans Administration
Medical Center,[2] La Jolla, California 92161*

The effectiveness of the antiviral agent ganciclovir (9-[1,3-dihydroxy-2-propoxymethyl]guanine) against guinea pig cytomegalovirus was tested in vitro in guinea pig embryonic fibroblasts and in vivo in an experimental guinea pig cytomegalovirus labyrinthitis model. In vitro, ganciclovir completely prevented guinea pig cytomegalovirus infection of guinea pig embryonic fibroblasts at concentrations above 32.6 µg/ml. In vivo, antibody-negative animals had an average 17-dB elevation in their auditory nerve compound action potential thresholds ($P < 0.01$, t test) and showed signs bilaterally of guinea pig cytomegalovirus labyrinthitis 8 days after intrathecal inoculation of virus. Ganciclovir administration starting 1 day before inoculation prevented the development of both cochlear histopathologic change and hearing loss. Guinea pig cytomegalovirus meningitis was observed in both the drug-treated and untreated groups. High-pressure liquid chromatography confirmed the presence of ganciclovir in the serum, perilymph, and cerebrospinal fluid of the drug recipients. Prophylactic ganciclovir thus can protect the cochlea from the histopathologic changes and hearing loss normally associated with experimental guinea pig cytomegalovirus labyrinthitis.

Cytomegalovirus (CMV) is the leading infectious cause of congenital sensorineural hearing loss in humans. As many as 2.5% of all newborns have been estimated to be congenitally infected with CMV (10, 11, 13, 25, 26). About 5% of congenitally infected infants develop symptomatic congenital CMV syndrome. One half of these infants die, and the survivors typically exhibit significant and permanent intellectual, visual, or neurologic sequellae of their infection (26). Ninety-five percent of infants congenitally infected with CMV are asymptomatic at birth. However, as many as 20% of infants who appear normal at birth are later found to have sensorineural deafness, neurologic abnormalities, or mental retardation (11, 18). Recent epidemiological studies have concluded that in the United States alone both congenital CMV infections and primary CMV infections later in life may account for as many as 40,000 new cases of sensorineural hearing loss per year (6). CMV may also be a significant factor in the sensorineural hearing loss and central auditory dysfunction found in up to 68% of patients with acquired immunodeficiency syndrome (J. Gardi, C. D. Sooy, D. Morledge, P. Chambers, R. Gorter, and I. Medina, Assoc. Res. Otolaryngol. 10:93, 1987), over 90% of whom are infected with CMV (20). The pathogenesis of CMV-induced sensorineural hearing loss is poorly understood, and there is no known effective treatment.

The species specificity of CMV has prevented the development of animal models using human CMV. However, it was recently demonstrated that the virology and histopathology of guinea pig CMV (GPCMV) closely resemble those of human CMV disease (2, 27). Using GPCMV, we established a model of virus labyrinthitis in guinea pigs after inoculation of seronegative animals with GPCMV via intracochlear or intrathecal routes (12, 29, 30). The degree of cochlear pathophysiology observed in these studies was dependent upon both the infectious dose and the site of virus inocula-

tion. Intrathecal inoculations have the advantage that they do not interrupt the integrity of the cochlea and that the resulting labyrinthitis is less extensive. Unpublished studies have shown that intrathecal inoculation into seronegative guinea pigs of a $10^{2.2}$ 50% tissue culture infective dose (TCID$_{50}$) of GPCMV produced no cochlear pathology or hearing loss in six animals, while all seven animals inoculated with $10^{3.2}$ TCID$_{50}$ developed labyrinthitis with a significant hearing loss. Cochlear inflammation and fibrosis in the $10^{3.2}$ TCID$_{50}$ group animals was primarily confined to the basal turn scala tympani. Hearing losses in this group were restricted to the auditory nerve compound action potential (AP) thresholds, which were significantly elevated an average of 17 dB. Their cochlear microphonic potential (CM) thresholds were not affected.

Having established the dose-response kinetics of GPCMV labyrinthitis, it is possible to use this model to evaluate the efficacy of the antiviral agent ganciclovir, also called DHPG. Ganciclovir (9-[1,3-dihydroxy-2-propoxymethyl]guanine) is an acyclic nucleoside which has been shown to inhibit CMV replication in humans (14, 15, 17, 24) and other mammals, including guinea pigs (8, 9, 23). This study was designed to determine whether prophylactic ganciclovir could prevent the labyrinthitis and hearing loss normally associated with intrathecal inoculation of $10^{3.2}$ TCID$_{50}$ of GPCMV into seronegative animals. The treatment protocol used was chosen to optimize the potential effectiveness of the drug.

MATERIALS AND METHODS

Animals. Randomly bred, female Hartley guinea pigs were purchased from Charles Rivers Breeding Laboratories, Inc., Wilmington, Mass. The animals were tested on arrival for the presence of antibody to GPCMV. Only animals negative for antibody to GPCMV and free from obvious middle ear inflammation were used in the study. Throughout the study, animals infected with live virus were housed separately from uninfected animals. For all experimental procedures, the

guinea pigs were anesthetized with a combined regimen of sodium pentobarbital (Nembutal; 30 mg/kg [body weight], intraperitoneally) and fentanyl-droperidol (Innovar-Vet; 0.3 ml/kg, intramuscularly).

Virus inoculum. (i) Live virus. The prototype strain of GPCMV (ATCC 22122) was obtained from B. P. Griffith and G. D. Hsiung of Yale University. Viral suspensions for inoculation were prepared as described previously (12). Virus stock solutions with infectivity titers of 6.5 $\log_{10}$ $TCID_{50}$/ml were stored in 150-μl aliquots at -70°C until needed.

(ii) Inactivated virus. GPCMV stock solution was inactivated by being mixed with 4' aminomethyl-4,5',8-trimethyl psoralen (10 μg/ml) (Calbiochem-Behring, La Jolla, Calif.) followed by a 10-min exposure to long-wavelength UV light (19). The inactivated virus stock was then divided into aliquots and frozen at -70°C until needed.

Enzyme immunoassay for antibodies to GPCMV. The assay of antibody in microliter quantities of serum and perilymph was performed by using an enzyme immunoassay with a filtration system originally described by Cleveland et al. (3) and described in detail for GPCMV elsewhere (12).

CM and auditory nerve compound AP recordings. CM and auditory nerve compound AP are electrophysiological measures of the functional integrity of the inner ear sensory cells and auditory (eighth cranial) nerve, respectively (5). The surgical and electrophysiological recording procedures were detailed previously (30). Briefly, the external ear was left intact, and a small opening was made in the inferior-posterior and tympanic chambers of the bulla to provide access to the cochlear round window. A silver ball recording electrode was placed adjacent to the round window membrane, and a reference electrode was placed in contact with the dorsal neck muscles. CM and AP were amplified 1,000 times and filtered at 0.10 to 30 kHz. CM and AP were measured with a Hewlett-Packard 3561A Signal Analyzer.

Calibrated auditory stimuli were delivered to the bony external meatus through a closed acoustic system (30). Stimuli were tones presented continuously for CM or gated (6 kHz, 30-ms duration, 3/s, 1-ms rise/fall time) for AP recordings. All recordings were conducted inside a double-walled International Acoustics Co. sound-attenuated chamber.

Sampling and inoculation techniques. For sampling of cerebrospinal fluid (CSF), an incision was made in the neck and the muscles were blunt dissected down to the dura at the level of the foramen magnum. The dura was cleaned of all traces of blood. A clear blood-free CSF sample was obtained by inserting a glass micropipette through the dura into the cisterna magna. When necessary, live or inactivated virus inoculations were done after CSF sampling by inserting another glass micropipette, attached to a 100-μl Hamilton syringe with a repeating dispenser, through the dura and injecting 50 μl of the appropriate suspension into the cisterna magna. After the inoculation micropipette was removed, the small hole in the dura was teased closed and the area was sealed with Gelfoam. For sampling of perilymph, a small hole, 250 μm in diameter, was hand drilled into the basal turn scala tympani, and approximately 25 μl of perilymph was collected with a micropipette.

Drug administration and assay. Ganciclovir sterile sodium salt was a generous gift from L. E. Kirk of Burroughs Wellcome Co., Research Triangle Park, N.C. Injections of 100 mg/kg were given twice daily, at 8:00 a.m. and 4:00 p.m. Levels of ganciclovir were kindly assayed by Charles H. Sherwood and James D. Connor, University of California

San Diego Medical Center Department of Pediatrics, Division of Infectious Diseases. A 100-μl sample of plasma at 4°C and 900 μl of 0.44 M perchloric acid were added to a 2.5-ml plastic tube. The contents were vortexed and then centrifuged at 2,500 $\times$ g for 20 min. The supernatants were added to twice their volume of 20% (wt/wt) trioctylamine in Freon 113 and then vortexed for 1 min. The neutralized sample-trioctylamine-Freon emulsion was then separated by low-speed centrifugation at room temperature. The top layer containing the neutralized, deproteinated sample ready for analysis was removed and transferred to autosampler vials. Twenty microliters of plasma extract from each sample was used for analysis. The perilymph and CSF samples were diluted with 20 μl of saline and 30 μl of water. The samples were analyzed by reverse-phase high-pressure liquid chromatography by using an Adsorbosphere RP18 column (250 by 4.6 mm; Alltech Associates, Inc.). Each sample was eluted isocratically with 10 mM ammonium acetate-acetic acid buffer (pH 3.45). With the flow rate held constant at 1.2 ml/min, the ganciclovir retention time was 11.2 $\pm$ 0.3 (standard deviation) min. The eluate was monitored at 254 nm on a UV detector (Kratos Spectroflow 757). The column was purged by switching to 100% acetonitrile over 1 min, held at 100% for 6 min, and then returned to 100% buffer over 1 min. The column was reequilibrated for 15 min. The UV absorbance data were collected and analyzed by a Shimadzu Corp. integrator (model C-R1A). The ganciclovir concentration in each sample was determined by comparing sample peak area to a standard ganciclovir curve. There is a linear relationship between peak area and concentration of ganciclovir between 1 and 100 μg/ml. The standard curve was constructed for each assay by performing linear regression on concentration-versus-peak-area data for aqueous standards of known areas. The slope and intercept values were entered into the integrator. Ganciclovir levels for subsequent analysis were then calculated and printed by the integrator. Ganciclovir standards were determined regularly throughout the experimental samples to verify accuracy.

Antiviral assay in vitro. Subconfluent monolayers of guinea pig embryonic (GPE) fibroblasts were incubated with GPCMV at a multiplicity of infection of 0.01 $TCID_{50}$ per cell for 3 h at 36°C in 5% CO_2. The monolayers were then rinsed with Dulbecco modified Eagle medium. Serial twofold dilutions of ganciclovir from 2 to 1,024 μM in Dulbecco modified Eagle medium containing 5% fetal bovine serum were then added to the wells. Five days later, the cells were fixed in graded alcohols and stained immunohistochemically for anti-GPCMV antigen by using biotinylated anti-GPCMV, streptavidin-horseradish peroxidase conjugate (Bethesda Research Laboratories, Inc.), and 3-amino-9-ethylcarbazole as a chromagen (21).

Histology. At the end of the experiment, the deeply anesthetized animals were perfused with intracardiac warm saline, followed by 5% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.3). The cochleas and brains were then dissected and stored overnight in cold paraformaldehyde. The cochleas were decalcified in phosphate-buffered 5% EDTA with 2% paraformaldehyde. The cochleas and brains were dehydrated in graded ethyl alcohols and embedded in Paraplast Plus (Lancer) by using an Autotechnicon Mono (Technicon Corp.). Sections (8 μm) were mounted on slides and stained with hematoxylin and eosin.

Experimental design. Guinea pigs were randomly assigned to one of three experimental groups: (i) a control group (n = 5) which received an intrathecal inoculation directly into the cisterna magna through the foramen magnum with $10^{3.2}$
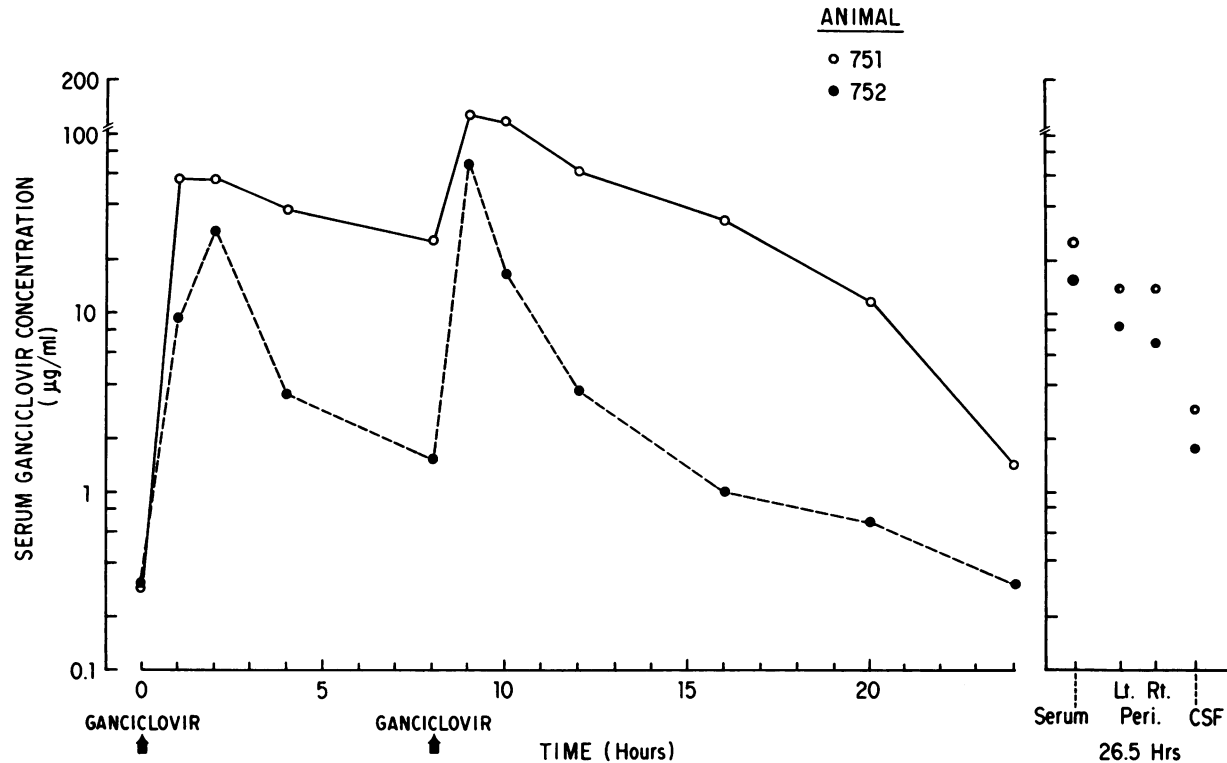
FIG. 1. Pharmacokinetics of ganciclovir in two guinea pigs that received ganciclovir twice daily for 2 days. Time 0 is day 3. Final ganciclovir injection at 24 h (day 4). Peri, Perilymph; Lt., left; Rt., right.

TCID$_{50}$ of GPCMV that had been inactivated in 50 μl (inactivated virus control); (ii) a live virus group ($n = 7$) which was inoculated intrathecally with $10^{3.2}$ TCID$_{50}$ of GPCMV in 50 μl (infected-untreated group); and (iii) a prophylactic drug group ($n = 6$) which, in addition to intrathecal inoculation with live GPCMV, also received 100 mg of ganciclovir per kg twice a day intraperitoneally starting 1 day before virus injection and continuing throughout the 8 days of infection (infected-ganciclovir group). On day 0, before intrathecal inoculation, the base-line CM and AP thresholds were determined for each animal, and serum and CSF samples were taken. On day 8, the terminal day, CM and AP thresholds were remeasured; serum, right and left perilymph, and CSF samples were taken; and then each guinea pig was fixed by cardiac perfusion with warm saline and 5% paraformaldehyde. Brains and cochleas were processed in paraffin for histopathologic evaluation.

RESULTS

Activity of ganciclovir against GPCMV in cell culture. The activity of ganciclovir against GPCMV replication in GPE fibroblasts was assessed in vitro. In comparison to drug-free, virus-infected controls, ganciclovir inhibited replication of GPCMV in GPE fibroblasts at drug concentrations above 16.3 μg/ml and completely inhibited GPCMV replication at concentrations above 32.6 μg/ml. Ganciclovir did not inhibit GPE fibroblast proliferation even at 261.1 μg/ml, the highest concentration used.

Pharmacokinetics of ganciclovir. The pharmacokinetics of ganciclovir were examined in two guinea pigs (Fig. 1). The guinea pigs were injected intraperitoneally with 100 mg of ganciclovir per kg twice daily (8 a.m. and 4 p.m.) for 2 days. Starting on the third day, serum samples were taken sequen-

tially for 24 h. At the end of the third day, the animals received a final ganciclovir inoculation, and 2.5 h later they were anesthetized and sampled for serum, right and left perilymph, and CSF. Serum peak and trough ganciclovir concentrations, as determined by high-pressure liquid chromatography, ranged from 127 to 0.28 μg/ml, respectively. At 2.5 h after the final injection, significant concentrations of ganciclovir were observed in serum, perilymph, and CSF. A clear hierarchy existed for ganciclovir levels in these fluids: serum had the highest concentration, approximately twice that of perilymph; CSF had the lowest drug levels, approximately one-quarter that of perilymph.

Antibody responses to GPCMV. We previously demonstrated that immunoglobulin G to GPCMV can first be detected by enzyme immunoassay at 2 weeks after GPCMV infection (N. K. Woolf, J. P. Harris, E. M. Keithley, A. E. Magit, and D. D. Richman, Assoc. Res. Otolaryngol. **9**:182, 1986). Consistent with this, none of the guinea pigs from any of the three experimental groups exhibited measurable GPCMV antibody in serum, perilymph, or CSF on day 8.

Histopathologic evaluation. (i) Cochleas. Light microscopic evaluation of the hematoxylin and eosin-stained cochleas revealed essentially normal morphology for the animals in the inactivated virus control and infected-ganciclovir groups (Fig. 2B and 3B). A small number of lymphocytes and plasma cells were occasionally seen in the scala tympani of the extreme basal turn. There was no evidence of labyrinthitis. The perivascular spaces within the modiolus were clear and free of lymphocytic infiltration.

The infected-untreated group (Fig. 2A and 3A) uniformly showed evidence of acute labyrinthitis in the basal turn at 8 days after GPCMV inoculation. The scalae tympani in these guinea pigs showed numerous inflammatory cells, extensive
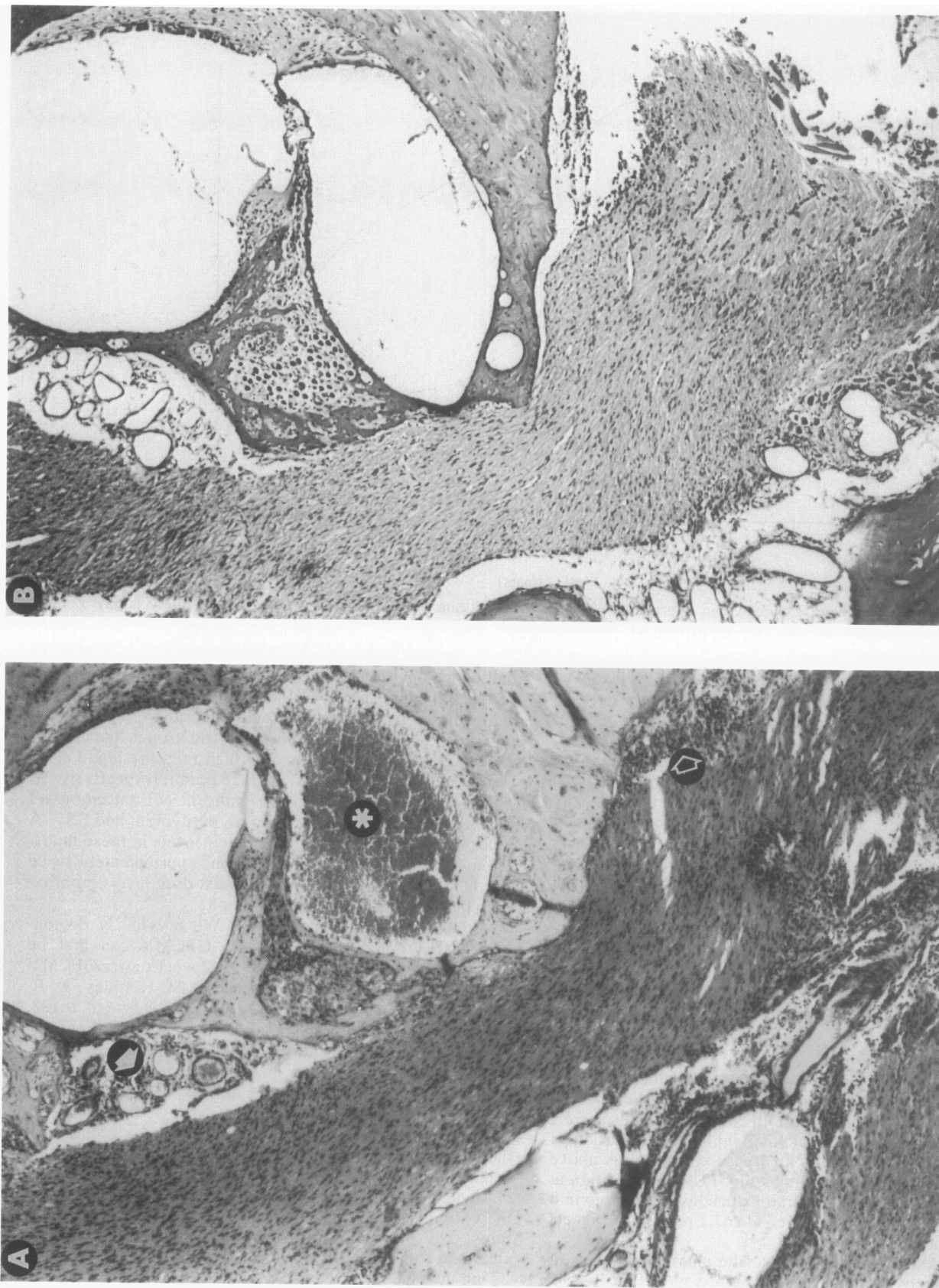
FIG. 2. Comparison of representative cochlear histopathologies in a guinea pig which was inoculated with GPCMV alone (A) and an animal which received GPCMV and prophylactic ganciclovir (B). Shown for each animal is the cochlear upper basal turn and the auditory nerve within the modiolus. The animal not treated with ganciclovir has acute labyrinthitis with inflammatory infiltrates in scala tympani (star) and lymphocyte infiltration around the perivascular spaces (filled arrow) and throughout the auditory nerve root within the internal meatus (open arrow). The cochlea of the ganciclovir-treated animal has normal morphology. Original magnification, ×12.5.
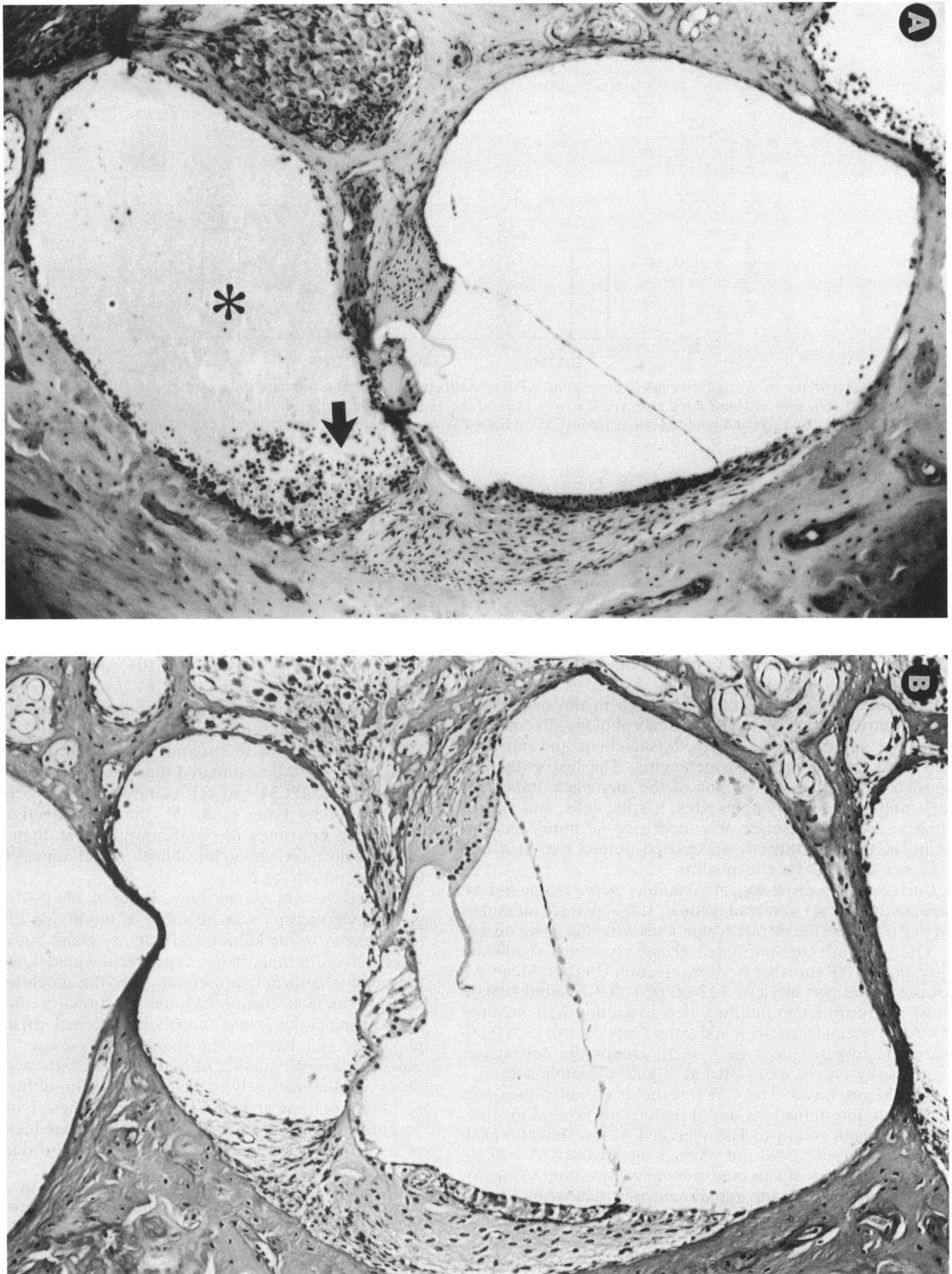
FIG. 3. Higher magnification of the cochlear upper basal turn in two guinea pigs other than those shown in Fig. 2. The animal not treated with ganciclovir (A) has good preservation of the organ of Corti but acute labyrinthitis in the scala tympani (star), including inflammatory infiltrates consisting of lymphocytes, plasma cells, and macrophages and fibromyxoid reaction (arrow). The guinea pig treated with ganciclovir (B) has normal cochlear morphology. Original magnification, ×25.
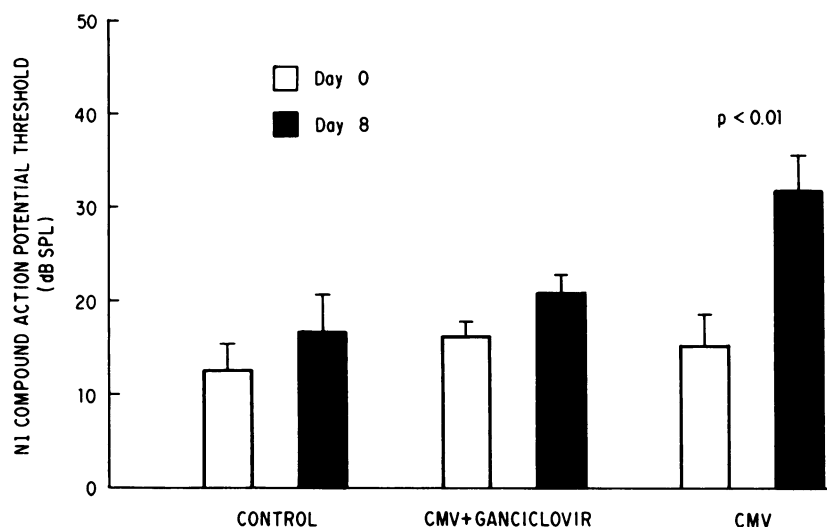
FIG. 4. Comparison of the auditory nerve compound AP thresholds (mean plus one standard deviation) for the three experimental groups on days 0 and 8. The infected-untreated group (CMV; $n = 14$ ears) showed a significant threshold elevation on day 8, while AP threshold shifts for the control ($n = 10$ ears) and infected-ganciclovir (CMV+Ganciclovir; $n = 12$ ears) groups were not significant. SPL, Sound pressure level re. 0.0002 dynes/cm^2.

perilabyrinthine fibrosis, and hemorrhage. However, the organ of Corti, the region where the sensory hair cells are located, showed good preservation. Within the modiolus, the regions around the perivascular blood vessels showed extensive lymphocytic infiltration. These perivascular spaces, which were normally seen as clear regions around the blood vessels in inactivated virus control and infected-ganciclovir group animals, were completely filled with lymphocytes, macrophages, and plasma cells for animals in the infected-untreated group (Fig. 2A).

(ii) **Brains.** The brains of guinea pigs in the inactivated virus control group showed normal morphology. In contrast, all of the animals in the infected-ganciclovir and infected-untreated groups exhibited meningitis. The histopathology consisted primarily of swelling of the meninges; inflammatory infiltration with lymphocytes, plasma cells, and macrophages; and the presence of a subarachnoid fibroid matrix. None of the intrathecally inoculated guinea pigs had any evidence of GPCMV encephalitis.

Cochlear pathophysiology. (i) Auditory nerve compound AP thresholds. The AP thresholds for all subjects were measured on day 0, before the intrathecal inoculation, and again on day 8. Only the infected-untreated group showed a significant elevation in AP thresholds after infection (Fig. 4). Mean AP for this group was increased 17 dB ($P < 0.01$, paired-sample t test), indicating that auditory nerve function was impaired (5). AP thresholds for the inactivated virus control ($P > 0.50$) and infected-ganciclovir ($P > 0.20$) groups did not change significantly between days 0 and 8 (paired-sample t tests).

(ii) **CM responses.** The CM thresholds for all guinea pigs were also determined on day 0, before intrathecal inoculation, and again on day 8. The average CM threshold for each of the three groups did not change significantly ($P > 0.10$, two-way analyses of variance) between days 0 and 8 (Fig. 5), indicating no significant impairment of cochlear outer hair cell function (5).

DISCUSSION

Ganciclovir is being used to treat immunosuppressed patients with serious CMV infections. The most extensive

experience has been with patients with acquired immunodeficiency syndrome who have CMV retinitis. Ganciclovir promotes healing of retinal lesions with resolution of viremia (1, 7, 22). The only serious adverse effects associated with ganciclovir treatment have been neutropenia and possible sterility, which are dose related and reversible (4).

The present study evaluated the antiviral activities of ganciclovir against GPCMV in vitro and in vivo. In vitro, ganciclovir was not toxic to GPE cell lines at even the highest concentrations tested (261.1 μg/ml), levels significantly above human therapeutic levels. Ganciclovir was observed to inhibit replication of GPCMV in GPE fibroblasts at concentrations of 16.3 μg/ml, consistent with the data of Fong et al. (8), who estimated the 50% effective dose of the drug against GPCMV in cell cultures to be 18.1 μg/ml. As pointed out by Fong et al. (8), the 50% effective dose in guinea pig cell lines is significantly higher than the 50% effective dose for human fibroblasts, which ranges from 0.04 to 3.3 μg/ml (9, 16, 28).

In studies with guinea pigs, Fong et al. (8) found that ganciclovir therapy was effective in modifying histopathologic lesions in the kidney and salivary gland but not in the spleen, liver, or lung. In our experimental model, ganciclovir provided significant protection for the cochlea against GPCMV-induced anatomical and functional pathology. Infected-ganciclovir group animals had normal cochlear morphologies and hearing thresholds 8 days after infection; however, a small number of lymphocytes were occasionally observed floating freely in the scala tympani of the cochlear basal turn of these animals. The source of these lymphocytes was presumably the CSF via the cochlear aqueduct, since all of the infected-ganciclovir group animals had documented GPCMV meningitis.

With the GPCMV dose and infection period used, the pathophysiology in the cochlea was confined to AP changes, while CM thresholds were not affected, consistent with our previous observations (Woolf et al., Assoc. Res. Otolaryngol. 1986). Both CM and AP are cochlear potentials. However, CM originates with the outer hair cells of the organ of Corti, which are relatively isolated from the central
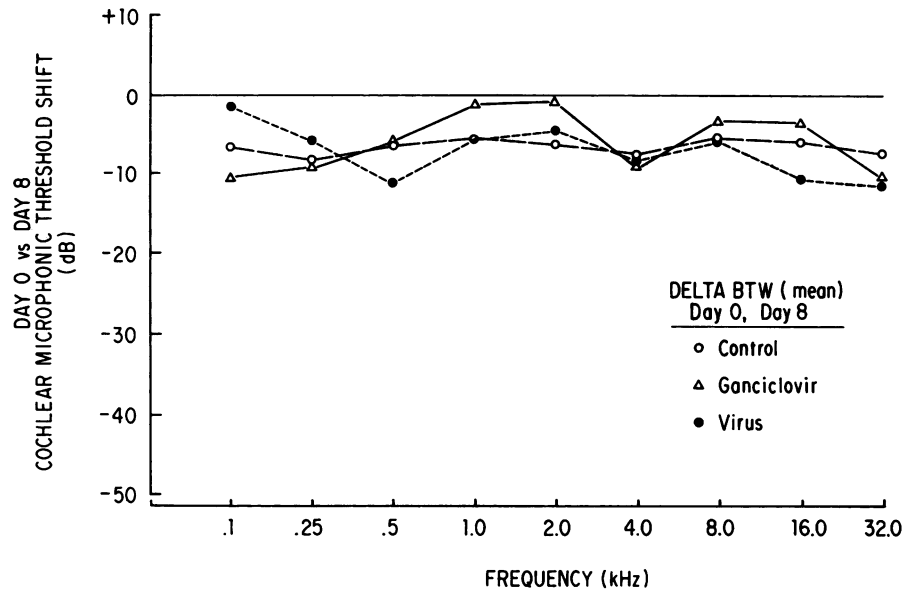
FIG. 5. Comparison of mean CM threshold shifts for the three experimental groups between days 0 and 8. Mean CM thresholds on day 8 were not significantly elevated in either the infected-untreated (Virus; $n = 14$ ears), control ($n = 10$ ears), or infected-ganciclovir (Ganciclovir; $n = 12$ ears) group. DELTA BTW (mean) Day 0, Day 8, Mean threshold shift between days 0 and 8.

nervous system, while AP reflects the evoked activity of the auditory nerve. The virus can enter the inner ear directly from the brain via two routes: along the auditory nerve projection to the brain stem and through the cochlear aqueduct which runs between the CSF and the perilymph. It is quite probable that in the presence of meningitis the virus enters the cochlea along one of these routes to produce labyrinthitis.

The level of GPCMV infection in the cochleas and brains of the infected-ganciclovir guinea pigs correlated well with the pharmacokinetic data. The level of ganciclovir measured in serum was approximately twice that in perilymph and approximately eight times that in CSF. If we hypothesize that a critical level of drug must be maintained to prevent GPCMV labyrinthitis and meningitis, then it is possible with the fourfold difference in drug level between perilymph and CSF that the drug level in the ear could be adequate to prevent labyrinthitis while the drug level in the CSF is inadequate to protect the brain from meningitis. Alternatively, inner ear infection follows sequentially infection of the meninges at the site where the virus was injected. The viral load may therefore have been relatively higher in the brain, with less virus available in the inner ear to initiate infection.

Although we have demonstrated that ganciclovir can be an effective agent for the prevention of acute GPCMV labyrinthitis, we do not yet know whether ganciclovir permanently prevents GPCMV labyrinthitis or only suppresses the infection for the duration of drug therapy. This is particularly relevant since in studies of patients without the benefit of effective immunity, such as patients with acquired immunodeficiency syndrome, it has been found that ganciclovir suppresses CMV retinitis for the duration of therapy but that the retinitis usually returns if the drug is withdrawn (14, 15). The guinea pig experimental model of CMV labyrinthitis and meningitis provides an excellent system in which to assay the efficacy and kinetics of ganciclovir and other chemotheraputic agents against virally induced functional and histologic pathologies in the inner ear and central nervous system, regions where much human disease occurs.

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