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Inactivation of *Herpesvirus hominis* Types 1 and 2 by Silver Nitrate In Vitro and In Vivo

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Herpes simplex virus (HSV) types 1 and 2 (two strains each) were inactivated at different rates in vitro by 40 μM $AgNO_3$. The inactivation of HSV type 1 strains was virtually complete in 10 to 15 min, whereas almost half of the infectivity of HSV type 2 strains survived this exposure. One strain of type 1 inoculated into rabbit eyes was almost completely inactivated by 1% $AgNO_3$ solution dropped into the eye 20 min later, so that there was markedly reduced viral replication and less corneal herpetic disease. One strain of HSV type 2 in the rabbit eye was not effectively inactivated by 1% $AgNO_3$. From these results, it seems likely that $AgNO_3$ instillation into the eyes of a newborn who has passed through a birth canal infected with HSV might prevent eye infection with HSV type 1 but not with type 2. The greater resistance of HSV type 2 strains to chemical inactivation in vitro and in vivo may be of medical concern.

The present striking increase in the incidence of venereal diseases in the United States has been accompanied by a marked increase in observed genital infections caused by herpesviruses, mostly *Herpesvirus hominis* (herpes simplex virus [HSV]) type 2. This rise in genital herpetic infections can be expected to produce an increasing exposure of newborns to the virus during passage through the birth canal, with a possible risk of serious illness (6, 7). The eyes of the neonate are one of the highly susceptible potential sites for infection with herpesviruses. However, immediately after delivery, 1% silver nitrate is instilled into the eyes of all newborns. This "Credé prophylaxis" is highly effective in controlling ocular infection due to gonococci acquired from the mother's genital tract (3). The question arises whether this silver nitrate prophylaxis might also have an effect on herpesvirus eye infection of the neonate acquired during passage through the birth canal. As an initial step toward an answer to this question, we compared the inactivation of herpesvirus type 1 and type 2 by silver nitrate in vitro and in a rabbit eye model.

MATERIALS AND METHODS

Virus. Four strains of HSV were used, two each representing type 1 and type 2. PH (type 1) was obtained in 1951 from Harold Pearson (University of Southern California) and was maintained in this laboratory by intracerebral passage in mice. One passage was made in Vero cell culture to serve as the infective inoculum. MS (type 2) was obtained in 1971 from Jang Oh (Proctor Foundation, University of California, San Francisco) in primary rabbit kidney cells. One futher passage was made in this laboratory in Vero cells. Strains Sales (type 1) and Mendoza (type 2) were recent isolates from vesicular lesions in Vero cells. All virus pools were stored at -70 C. They were demonstrated to be mycoplasma-free by appropriate cultures.

Cell culture. Vero (African green monkey kidney) cells were used in all culture procedures and for the preparation of infective pools of virus. The growth medium was Eagle's minimal essential medium (MEM) with the addition of 5% heat-inactivated fetal calf serum, nonessential amino acids, penicillin (100 units/ml), and streptomycin (100 μg/ml). When confluent monolayers were formed, the growth medium was replaced with maintenance medium (MM) consisting of MEM with 2% heat-inactivated fetal calf serum, nonessential amino acids, penicillin, and streptomycin.

For plaque assays, the cells were grown in 16-oz (0.47-liter) prescription bottles. The cell sheets were removed with a mixture of 0.05% pancreatin, 0.05% ethylenediaminetetraacetate, and 0.05% trypsin in phosphate-buffered saline, and were resuspended in growth medium to a concentration of 1.5 million cells/5 ml. This volume was seeded into plastic tissue culture dishes (60 by 15 mm) and incubated at 37 C in 5% CO_2 for 2 to 3 days. The medium was removed from confluent monolayers, and plates were inoculated with 0.2 ml of the material to be assayed. After a 2- to 2.5-h adsorption period at

22 C, 6 ml of a Methocel overlay was placed in each dish. The overlay was 1.5% Methocel (obtained from Dow Chemical Co.) incorporated into MM. After 5 days of incubation at 37 C in 5% CO_2, the overlay was removed from the plates by suction, the cell sheets were rinsed with cold Hanks balanced salt solution (BSS), and then were stained with 1:4,000 neutral red in Hanks BSS. After the plates had been dried overnight in the dark, the plaques were clearly visible and easily counted.

Tube cell cultures for virus isolation attempts were made by seeding 60,000 to 80,000 cells in 1 ml of growth medium into rubber-stoppered tubes. In 3 to 5 days, monolayers were ready for inoculation. Specimens of rabbit corneal and conjunctival epithelium were obtained by rotating cotton-tipped applicator sticks over the entire eye surface and then placing them in 1 ml of MM. After wringing out the swab, the fluid was pipetted onto cell sheets. The tube cultures were examined daily for a period of 10 days for the appearance of typical cytopathic effects.

Silver nitrate. A 1% silver nitrate solution in wax ampoules (pH 5.45) and the vehicle used in its preparation were obtained from Eli Lilly & Co. and used in rabbit experiments. The in vitro tests employed a 40 mM solution of silver nitrate in sterile distilled water.

HSV inactivation in vitro. Virus was diluted either in buffered saline or in MEM with 2% fetal calf serum to contain approximately 10^6 plaque-forming units (PFU)/ml. The silver nitrate solution (or control buffer) was added to the virus suspensions to a final concentration of 40 μM. The mixtures were held at 37 C for up to 20 min. At intervals, samples were removed from the virus-drug and virus-buffer mixtures, immediately diluted 100- to 200-fold in MM, and inoculated onto Vero cell monolayers. Initial assays were made on samples withdrawn from the virus-drug and virus-buffer mixtures, respectively, immediately after addition of the silver nitrate or buffer.

HSV inactivation in the rabbit eye. White New Zealand rabbits weighing about 1.8 kg with normal corneas by biomicroscopy were inoculated with HSV. The untraumatized eye received 0.05 ml of HSV dilutions which contained 1,000 to 200,000 PFU and were dropped from a tuberculin syringe. Both eyes of each rabbit were inoculated and held shut for 30 s; 20 min later one eye of each rabbit received two drops of 1% silver nitrate from commercial wax ampoules, and the other eye received the vehicle. The eyes were examined daily by inspection with fluorescein and biomicroscopy for the appearance of herpetic dendrites. On days 3 and 7 after infection, specimens for virus isolation were obtained from each eye with separate sterile swabs and cultured in Vero cells as described above.

RESULTS

With each strain of HSV, two or three experiments were performed to determine the time course of inactivation of infectivity by 40 μM silver nitrate (0.00068% $AgNO_3$) upon contact in vitro. The results of the different experiments with each strain were in close agreement. One representative experimental result with each strain is shown in Table 1. After 15 min of contact with 40 μM silver nitrate, less than 1% of viable virus could be recovered from the virus suspensions of two HSV type 1 strains. By contrast, only 23 and 69% of viable virus in two HSV type 2 strains was inactivated by this 15-min exposure. Even 20-min exposure to 40 μM $AgNO_3$ inactivated only 62% of viable virus in type 2 strain Mendoza. Thus, the HSV type 2 strains employed here were far more resistant to chemical inactivation by silver nitrate, under the conditions of these exposures, than the type 1 HSV strains.

Type 2 HSV strains grow less efficiently than type 1 HSV strains in Vero cells. It seemed possible therefore that the pools of type 2 strains contained more cell debris and medium constituents than those of type 1 strains for a similar number of viable virus particles. In view of the possibility that the increased cell debris in the type 2 pools could account for their decreased rate of inactivation, we carried out several procedures to determine whether this was a significant factor. First, we estimated the amount of protein in the virus stocks by the Lowry method (5). The average values were 1.1 and 1.6 mg/ml for types 1 and 2, respectively. It

TABLE 1. *Inactivation of HSV type 1 and HSV type 2 strains by 40 μM $AgNO_3$ in vitro*

HSV strain	Time of contact (min)	PFU/0.2 ml		Percent inactivated
		$AgNO_3$	Control	
Type 1				
PH	0	268	270	—
	5	92	209	56
	10	6	206	97
	15	0	196	>99
	20	0	190	>99
Sales	0	181	176	—
	5	117	165	29
	10	23	170	86
	15	2	176	99
	20	0	168	>99
Type 2				
MS	0	186	191	—
	5	127	201	37
	10	93	198	53
	15	56	180	69
	20	27	175	85
Mendoza	0	80	78	—
	5	68	69	—
	10	65	71	8
	15	50	65	23
	20	26	68	62

seemed unlikely that the difference in the rate of inactivation could be due to this small difference in protein content.

Second, we obliterated any possible effect of these minor differences in protein content by testing the inactivation in a medium containing 2% fetal calf serum. Since the protein concentration of this medium is approximately 1.4 mg/ml (Lowry determination), the amount of protein contributed by the virus stocks, which were diluted 1:20 prior to exposure to $AgNO_3$, was negligible.

Third, we repeated the inactivation in buffered saline with no added serum. The amount of protein, therefore, was one-twentieth of that in the original stocks, or approximately 55 to 80 μg/ml. The rate of inactivation of each strain was very similar in the buffered saline and in the medium with 2% fetal calf serum. This indicates that the amount of protein in the solution had little effect on the rate of inactivation within the concentrations tested. For these three reasons, we conclude that the observed differences in the rate of inactivation cannot be attributed to a difference in the amount of cell debris and medium protein contained in the different virus pools.

For a test of in vivo activity of Credé prophylaxis, a model was devised which imitated the sequence of infection and silver nitrate application to the neonate's eye. The results, arranged by inoculum size and HSV type, are shown in Table 2. The results of virus isolation closely paralleled the development of corneal herpetic lesions. A detailed description of these lesions will be presented elsewhere. With the smaller inocula of HSV type 1, the instilled 1% silver nitrate interfered markedly with the development of corneal infection and the subsequent replication of virus. For inocula of 3,000 and 30,000 PFU per eye, these differences are statistically significant. With the largest inoculum of HSV type 1 (200,000 PFU/eye), no difference was demonstrated between silver nitrate and vehicle application.

With HSV type 2, instillation of silver nitrate resulted only in a small and consistent reduction of infection and virus replication with each inoculum size. However, the differences in virus recovery rate from eyes treated with silver nitrate or with vehicle were too small to be statistically significant.

DISCUSSION

Silver nitrate was one of many different chemicals studied by Sery and Furgiuele (10) for anti-HSV activity. They found that, in 1 h of contact in vitro, a herpes strain (type unknown) was inactivated by 10^{-5} M silver nitrate. Such a long contact cannot be achieved in vivo. The most noteworthy finding in our work was the striking difference in the rate of inactivation of HSV type 1 and type 2 strains. Various biological differences between the two types have been recorded (9). The susceptibility of HSV type 1 strains to halogenated pyrimidines and other inhibitors of viral deoxyribonucleic acid synthesis is often greater than that of type 2 (2, 8). Evidently, HSV type 2 strains are also more

TABLE 2. *Inactivation of HSV by $AgNO_3$ in the rabbit eye*

HSV	Inoculum (PFU)[a]	Therapy	No. of positive HSV cultures/no. of eyes cultured on days 3 and 7	Percent positive HSV infections	*P*[b]
Type 1, strain PH	200,000	Silver	36/38	94.7	
		Vehicle	35/38	92.1	NS[c]
	30,000	Silver	24/40	60.0	
		Vehicle	37/40	92.5	<0.002
	3,000	Silver	7/24	29.2	
		Vehicle	18/24	75.0	<0.004
Type 2, strain MS	50,000	Silver	12/20	60.0	
		Vehicle	15/20	75.0	NS
	10,000	Silver	14/30	46.7	
		Vehicle	19/30	63.3	NS
	1,000	Silver	2/34	5.9	
		Vehicle	7/34	20.6	<0.2

[a] Plaque-forming units per dose inoculated.

[b] Chi square test. See N. Bailey, Statistical methods in biology, 2nd ed., p. 67, 1964, The English Universities Press, London.

[c] Difference not significant.

resistant than HSV type 1 to simple chemicals such as silver nitrate.

Nahmias (7) expressed the opinion that the overall risk of neonatal herpes infection, developing in association with maternal infection after 32 weeks of pregnancy, might be 10%; with active viral lesions present at the time of delivery, this risk might be as high as 40%. The protective efficacy of immunoglobulin G antibody transmitted from mother to fetus in utero remains uncertain. The portal of entry for HSV transmitted to the newborn during passage through the birth canal cannot be well defined. Several reported neonates with disseminated herpes had prominent skin lesions or pneumonitis, suggesting skin and respiratory tract as places of early virus replication. Torphy et al. (12) have stressed the wide range of disease which may result from perinatal herpetic infection with HSV type 2, ranging from very mild, self-limited lesions to fatal disease. This spectrum obviously makes it difficult to evaluate the possible efficacy of a prophylactic or therapeutic measure.

Eye involvement in perinatal herpetic infections has not been recorded frequently. In 28 neonatal cases studied in detail, only 5 were found to have definite eye lesions, 4 of them due to type 2 HSV (6). Hagler et al. described five cases of herpetic eye infection in neonates, but four of them also had skin lesions (4). Bobo et al. encountered one neonatal infection limited to the eye (1). Thus, it is not certain how often the eye, rather than skin or respiratory tract, may serve as portal of entry for the virus in perinatal infections.

The prophylactic instillation of 1% silver nitrate into the eyes of the newborn has been strikingly effective against bacterial conjunctivitis, especially gonococcal infection, but has no clear effect on chlamydial inclusion conjunctivitis of the newborn (11). We were surprised to find that herpetic conjunctivitis could be prevented in the rabbit model if 1% silver nitrate followed small inocula of HSV type 1 within 20 min. It was disappointing, however, that this prophylaxis had only minor effects on HSV type 2. Most perinatal infections undoubtedly are caused by HSV type 2, which infects the maternal genital tract far more often than type 1. Consequently, the practical impact of Credé prophylaxis on herpetic perinatal infection will probably be small.

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LITERATURE CITED

1. Bobo, C. B., B. Antine, and J. P. Manos. 1970. Neonatal herpes simplex infection limited to the cornea. Arch. Ophthalmol. **84**:697-698.
2. Fiala, M., A. Chow, and L. B. Guze. 1972. Susceptibility of herpesviruses to cytosine arabinoside: standardization of susceptibility test procedures and relative resistance of herpes simplex type 2 strains. Antimicrob. Ag. Chemother. **1**:354-357.
3. Forbes, G. B., and G. M. Forbes. 1971. Silver nitrate and the eyes of the newborn. Amer. J. Dis. Child. **121**:1-3.
4. Hagler, W. S., P. V. Walters, and A. J. Nahmias. 1969. Ocular involvement in neonatal herpes simplex virus infection. Arch. Ophthalmol. **82**:169-176.
5. Lowry, O., N. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265-275.
6. Nahmias, A. J., W. R. Dowdle, W. E. Josey, Z. M. Naib, L. M. Painter, and C. Luce. 1969. Newborn infection with *Herpesvirus hominis* types 1 and 2. J. Pediat. **75**: 1194-1203.
7. Nahmias, A. J., W. E. Josey, Z. M. Naib, M. G. Freeman, R. J. Fernandez, and J. H. Wheeler. 1971. Perinatal risk associated with maternal genital herpes simplex virus infection. Amer. J. Obstet. Gynecol. **110**:825-837.
8. Person, D. A., P. J. Sheridan, and E. C. Herrman. 1970. Sensitivity of types 1 and 2 herpes simplex virus to 5-iodo-2-deoxyuridine and 9-beta-D-arabinofuranosyladenine. Infect. Immunity **2**:815-820.
9. Plummer, G., J. L. Waner, A. Phuangsabc, and C. R. Goodheart. 1970. Type 1 and type 2 herpes simplex viruses: serological and biological differences. J. Virol. **5**:51-59.
10. Sery, T. W., and F. P. Furgiuele. 1961. The inactivation of herpes simplex virus by chemical agents. Amer. J. Ophthalmol. **51**:42-57.
11. Thygeson, P., and W. Stone, Jr. 1942. Epidemiology of inclusion conjunctivitis. Arch. Ophthalmol. **27**:91-112.
12. Torphy, D. E., C. G. Ray, R. McAlister, and J. N. H. Du. 1970. Herpes simplex virus infection in infants: a spectrum of disease. J. Pediat. **76**:405-408.