Antiviral Research, 2 (1982) 53–67
Elsevier Biomedical Press

PATHOLOGICAL FINDINGS OF ADENINE ARABINOSIDE (ARA-A) AND CYTARABINE (ARA-C) IN THE TREATMENT OF HERPES SIMPLEX ENCEPHALITIS IN RABBIT MODEL

H.K. NARANG

Regional Public Health Laboratory, Newcastle General Hospital, Westgate Road, Newcastle upon Tyne NE4 6BE, U.K.

(Received 1 April 1981; accepted 29 December 1981)

The injection of herpes simplex virus type 1 (HSV-1) into the vitreous body of the eye in the 18 day old albino rabbits consistently induced herpes encephalitis with 90% survival. In the untreated rabbits the lesions follow a defined anatomical pathway producing a progressive disease not dissimilar to the natural human disease in that HSV travels slowly by cell-to-cell infection of neuroglia.

The effects of adenine arabinoside (ara-A) and cytarabine (ara-C) on HSV encephalitis in rabbit model were studied by starting the treatment on 4th day post-inoculation of HSV. Deaths due to toxic side effects were caused by ara-A and ara-C in 30% and 50% of animals respectively, compared with 10% in untreated animals. Neurological signs, such as head jerking, ataxia and frequent epileptiform fits, occurred in ara-A, ara-C and untreated rabbits. Comparative histological studies of optic nerves and brains showed that ara-A and ara-C had no beneficial effect, but surprisingly enhanced the disease.

herpes simplex virus encephalitis adenine arabinoside cytarabine

INTRODUCTION

Herpes simplex virus (HSV) is probably the commonest cause of sporadic fatal encephalitis in man [11, 12]. Although herpes encephalitis has been the subject of much active research, since the early 1960's particular attention has been focussed on the use of chemotherapeutic agents for the treatment of systemic HSV infections. To date, only a small number of drugs potentially of therapeutic value have emerged, and amongst these adenine arabinoside appears to be the most promising and has been tried on humans [24]. However, antiviral agents have toxic side effects and are as yet not fully understood. Lack of knowledge concerning the therapeutic value and toxic effects of these drugs has been largely due to the paucity of suitable animal models for this condition: most studies have relied on mortality and mean survival times both in humans [24] and in experimental animals [1, 3].

An animal model for progressive herpes encephalitis has been developed [14, 15]. This involves injecting virus into the eyes of 18 day old rabbits which results in specific

0166-3542/82/0000–0000/$02.75

progressive lesions of the optic nerves, chiasma and brain, with characteristic neurological signs. Histological and ultrastructural findings are remarkably reproducible after any inoculation [14, 15]. HSV remains demonstrable both in the optic nerves and the brain up to 64 days post-inoculation [14].

As the extent of damage is known at any single day in the encephalitic rabbit model, the present investigation intends to evaluate the antiviral activity of two drugs, adenine arabinoside (ara-A) and cytarabine (ara-C), against HSV type 1 (HSV-1).

MATERIALS AND METHODS

Virus HSV-1 was isolated and characterised in the Newcastle Public Health Laboratory from the brain of a patient who died of herpes encephalitis [16]. The virus was cultured and passed over 30 times in Bristol HeLa cells and the tissue culture fluid was used for inoculation and for plaque assay of infectivity. A known strain of HSV-1 (Houton) was also used [14–16] in the present study.

Rabbits

Twenty-two litters of New Zealand albino rabbits, totalling 120 animals, were used. The rabbits were divided into three groups: 50 were treated with ara-A; 50 with ara-C; and 20 were left untreated. Another group of 12 rabbits of comparable age were left uninoculated, but were given daily either 6 mg/kg of ara-A or 20 mg/kg of ara-C over a period of 5–10 days to determine the toxicity of the drugs.

The rabbits, when 18 days old, were anaesthetised with ether and, after topical application of procaine to the conjunctiva, were injected with 20 μl of either of the two strains of HSV with a titre of 10^{-3} plaque-forming units (p.f.u)/ml. The inoculation was made through the sclera into the vitreous body of the right eye.

The drugs

Both drugs used were commercial preparations; adenine arabinoside (ara-A) was supplied by Park Davis and Co. and cytarabine (ara-C) was supplied by Upjohn Ltd.

Treatment

The rabbits were divided into groups of 10 for different treatment regimes (Table 1). In all cases, treatment was started on the 4th day after inoculation and drugs were administered intravenously or intramuscularly, either as one dose daily of 6 mg/kg (ara-A) and 20 mg/kg (ara-C) or in two doses in half amounts each day, or one full dose of 6 mg/kg (ara-A) and 20 mg/kg (ara-C) on alternate days (Table 1).

Histology and electron microscopy

Groups of animals were killed on 5, 10 and 15 days at 1, 2, 3, 6 and 9 months post-treatment, together with matched controls. After administration of sodium pentabarbital containing heparin, the whole body was perfused through the left ventricle with 1–3

litres of 4% glutaraldehyde in cacodylate buffer (pH 7.2). Immediately after the perfusion for electron microscopy, the eyes, together with the optic nerves and chiasma, were removed and further fixed before embedding as described previously [14].

For histological sectioning the brains were fixed in 10% formaldehyde solution, processed and embedded in paraffin wax. The brains of the animals which died during treatment were also fixed for histological examination. Statistical data were analysed by the χ^2 test and any calculated expected values of less than 5 were checked for significance with the Fisher Exact Test.

RESULTS

Toxicity of drugs in normal rabbits

In the same dosage range of ara-A and ara-C employed for the treatment over a period of 5–10 days in the normal control rabbits, there was no clinical evidence of toxicity. Examination of blood elements showed reduction of white-cell count in ara-C group. These changes were reversed within a few days of stopping the treatment and the animals remained healthy thereafter.

Inoculated animals

For the first 3 days the treated animals in all of the regimes appeared healthier than the control group. The untreated rabbits developed characteristic neurological signs [14, 16], such as head jerking, ataxia and occasional epileptic fits between the 6th and 8th days post-inoculation. In some of the rabbits these symptoms lasted for 1 or 2 days, while these signs appeared in all the ara-C-treated rabbits 2–3 days later than in the non-treated controls, but with the same frequency as in the untreated group. These neurological signs were observed in only about 50% of the rabbits treated with ara-A and also with less frequency. The animals treated with ara-C became anaemic within a few days of treatment. The depression of neutrophil polymorphonuclear leucocytes and platelets was particularly marked by the 4th and 5th days of treatment. Also from the 2nd day of treatment of these animals, hair and somatic growth was retarded. An appreciable proportion in both the treated groups died between the 8th and 11th days post-inoculation; that is over 50% among the daily treated and 30% among alternate days with ara-C. Corresponding figures for ara-A were 30% and 10%, while the mortality rate was under 10% in untreated controls. The survival rate was higher when the dose was lower or when it was given on alternate days (Table 1).

Macroscopically the optic nerves on the 5th and 10th days post-infection showed no differences in any of the group. By the 15th day, however, gross atrophy of the right optic nerve was observed in the treated animals. By the 90th day, atrophy of the right optic nerve had progressed futher (Fig. 1b) and had occurred also to a limited extent in the left optic nerve. By comparison, the untreated rabbits did not show any gross

TABLE 1

Treatment regime

No. of rabbits[a]	Duration of treatment (days)	Antiviral drugs	
		Ara-A	Ara-C
10 (3)	5	6 mg/kg daily	–
10 (6)	5	–	20 mg/kg
10 (2)	5	3 mg/kg twice daily	–
10 (5)	5	–	10 mg/kg twice daily
10 (1)	10	6 mg/kg alternate day	–
10 (3)	10	–	20 mg/kg alternate day
10 (1)	5	3 mg/kg daily	–
10 (2)	5	–	10 mg/kg daily
10 (0)	15	3 mg/kg daily for 5 days, no treatment for 5 days, and then 3 mg/kg for futher 5 days	–
10 (1)	15	–	10 mg/kg for 5 days, no treatment for 5 days, then 10 mg/kg for 5 days

[a] Figures in parentheses refer to the number of rabbits that died during treatment.

atrophy of the right optic nerve before the 30th day and at 9 months it was less severe than in the treated animals.

Histological examination

Examination of semi-serial 1 μm toluidine blue sections of the whole length of the optic nerves and chiasmas on the 5th day revealed that the lesion did not extend beyond the chiasma in the treated animals and there was very little infiltration by macrophages compared with the untreated animals. From the 10th day post-treatment onwards the picture was reversed (Fig. 2a, b) and with further intervals of time. The lesions appeared more active and spongy in the treated groups (Figs. 2b and 3a, b). Most of the astrocytes and myelinated axons had been lost and large extracellular spaces had appeared. Many of these spaces had been filled by large numbers of macrophages which were full of fat droplets (Fig. 3b). Many of the astrocytes and oligodendrocytes showed chromatin changes (Fig. 3b), and virus particles were seen in these cells by electron microscopy (see Fig. 10). Cuffing of the blood vessels in these animals was not as marked as in the controls.

From 30 and more days after treatment sections cut near the entry zone and behind the atrophied segment (Fig. 1b, arrow) in both the ara-A- and ara-C-treated rabbits showed that in the medial side of the right optic nerve only thick-walled blood vessels

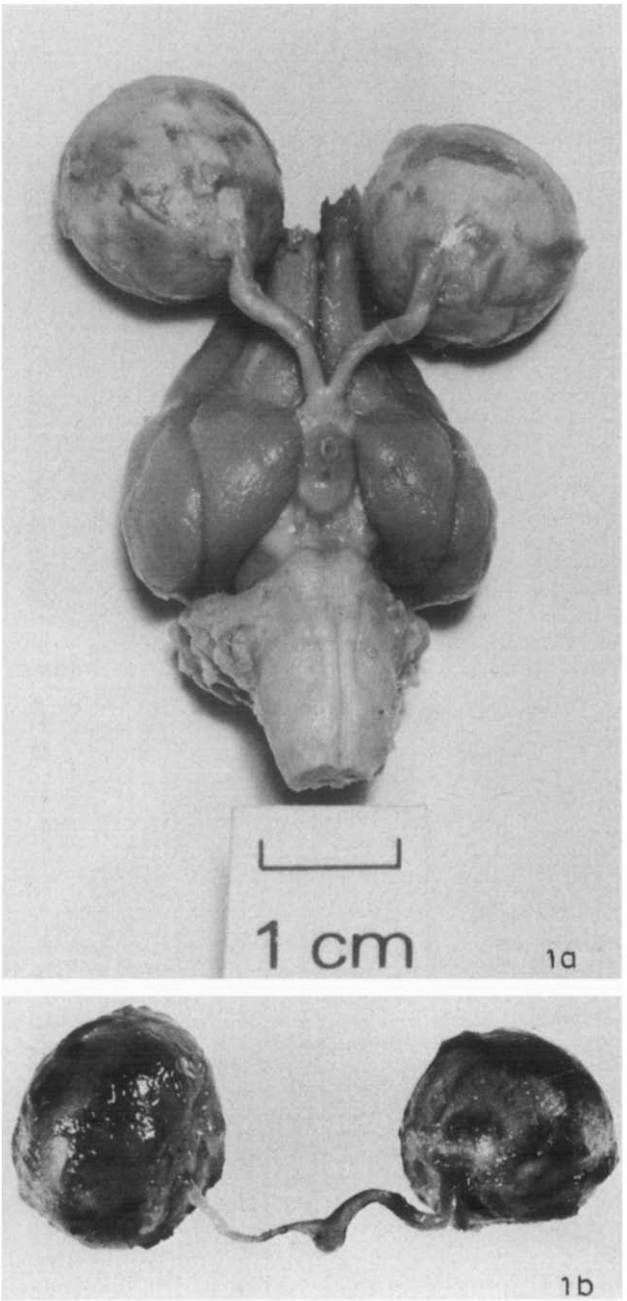


Fig 1. a) Ventral view of brain from a 3 month old normal rabbit showing right and left optic nerves with intact eyes. b) Photograph of right and left optic nerves from rabbit 90 days post-treatment with ara-A.

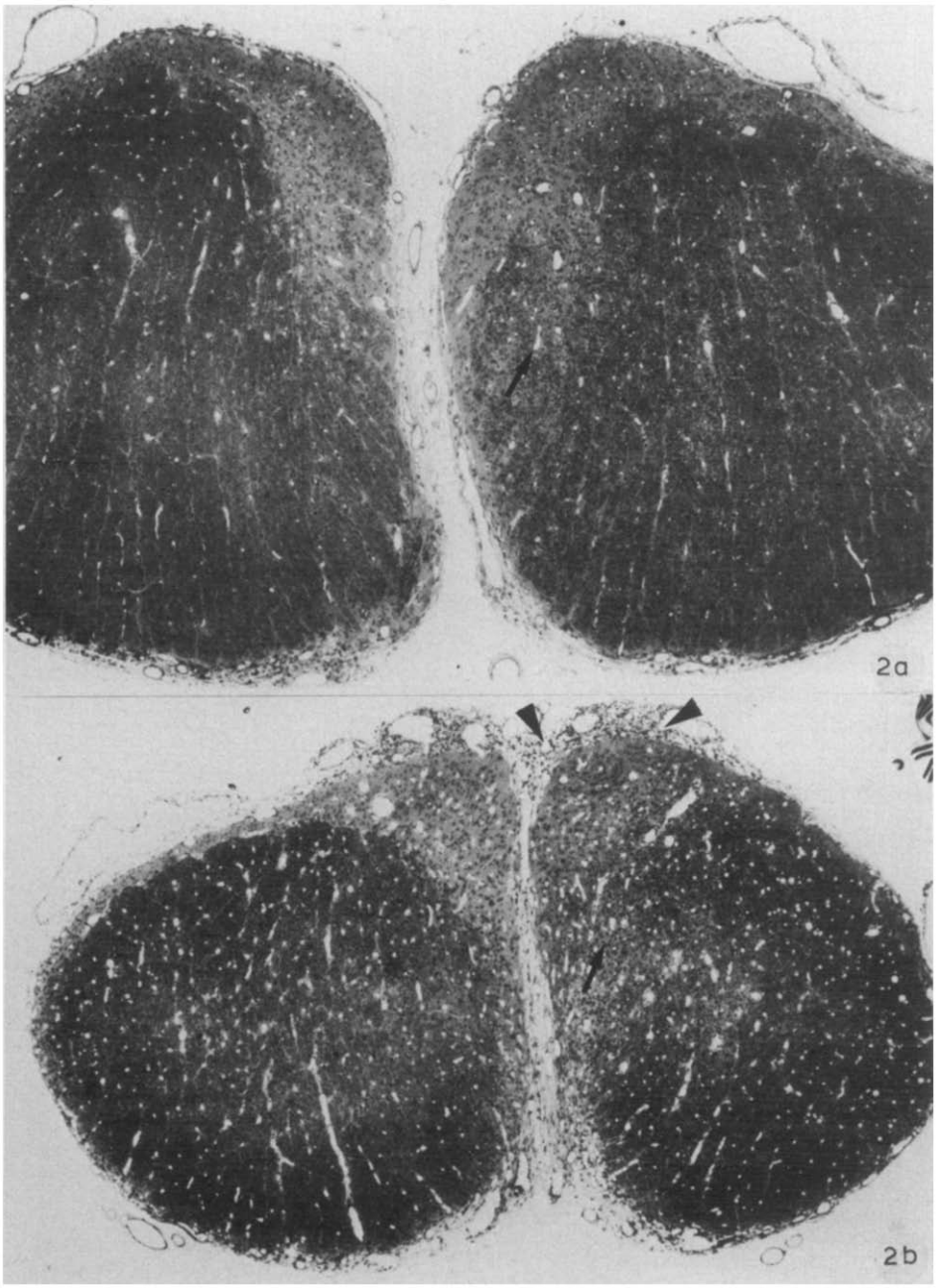


Fig. 2. a) Cross-section of right and left optic nerves, just before optic chiasma from untreated 10 days post-inoculated rabbit. Note lesions in the right optic nerve (arrow) while left is normal. ×160. b) Ten days post-treatment (ara-C) right and left optic nerves showing lesion in the right optic nerve (arrow). Note marked involvement of meningeal layers (arrow heads). ×100.

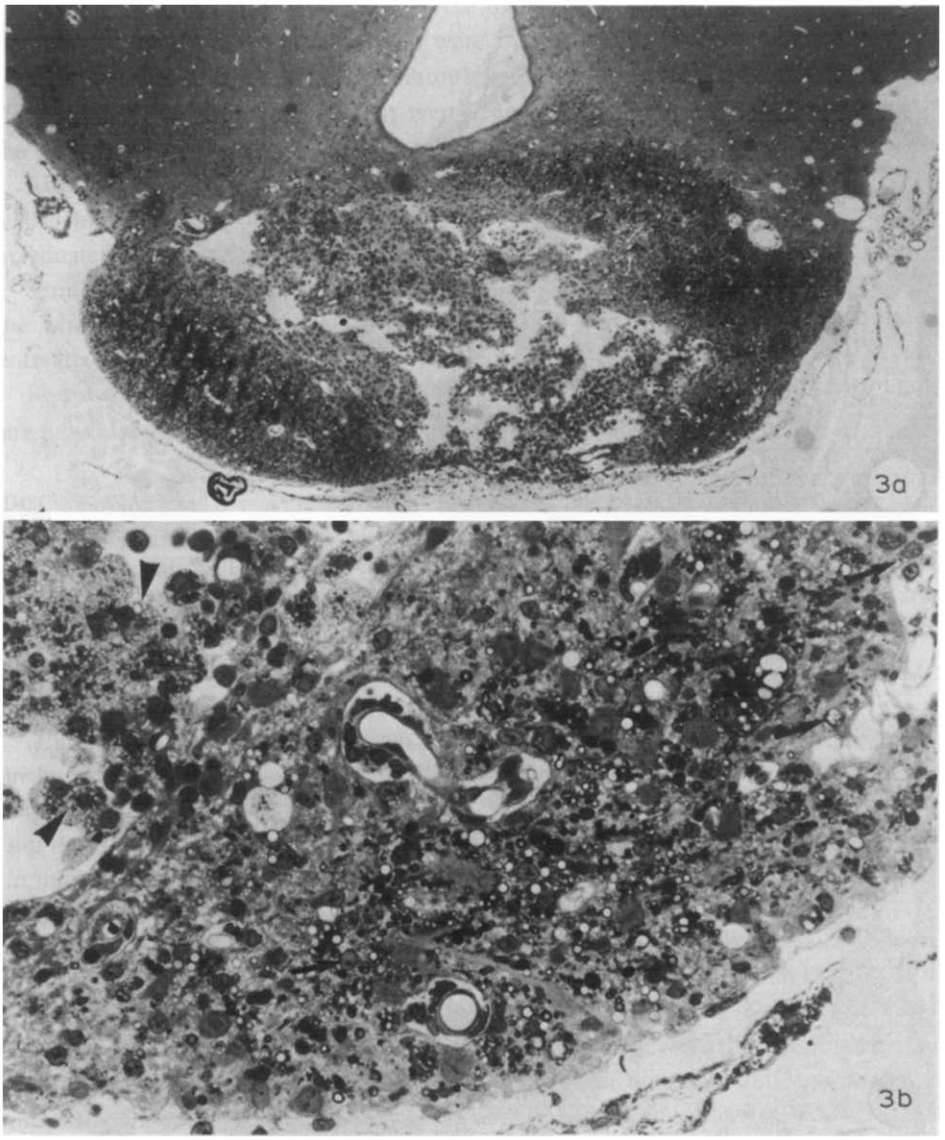
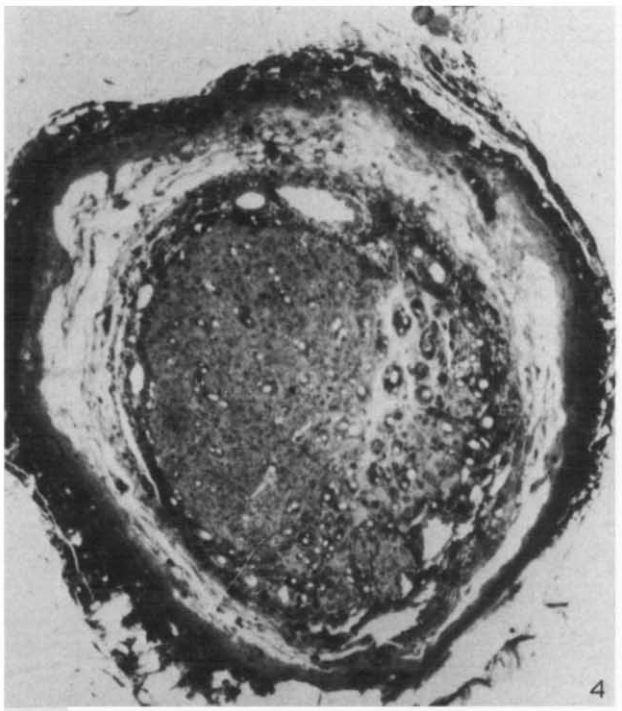
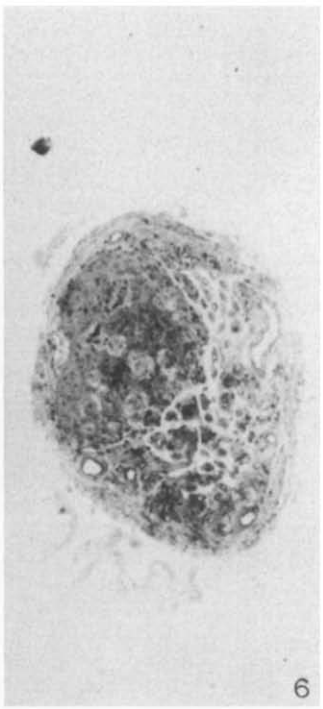
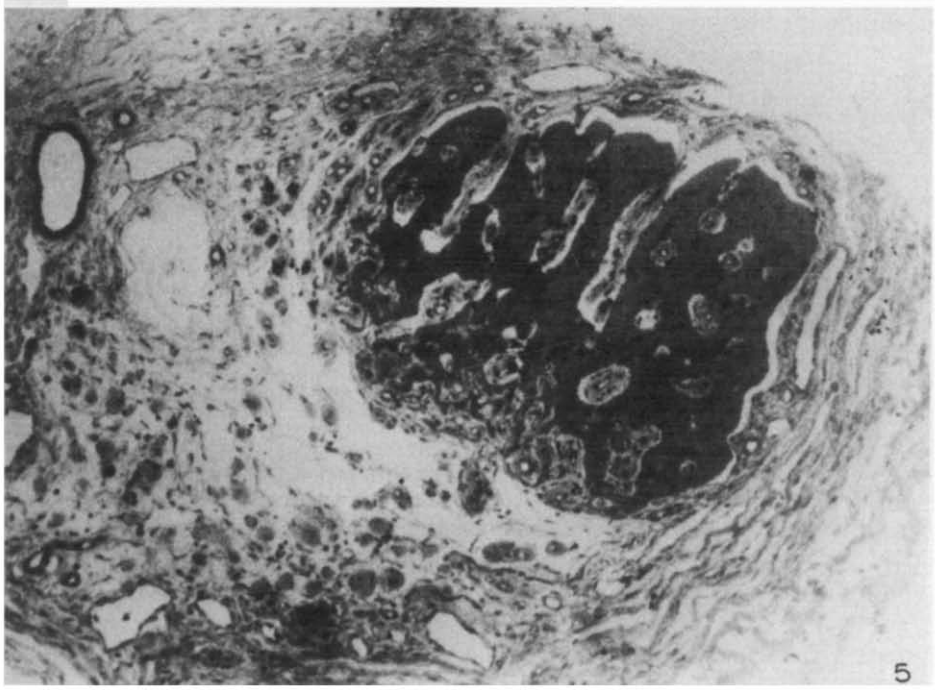


Fig. 3. a) Cross-section from the middle of chiasma from 15 days post-treatment (ara-C) rabbit. Note the marked lesion with large extracellular spaces. X120. (For comparison with age match control refer to Fig. 3a, b, Neuropathol. Appl. Neurobiol., 1978, 4, 461.) b) Higher magnification of the lesion in Fig. 3. Note chromatin changes in some of the nuclei (arrows) and infiltration of macrophages particularly those in the extracellular spaces contain large number of fat droplets (arrow heads). X250.

and some extracellular space appeared (Figs. 4 and 7). The lateral half of the optic nerve appeared to contain a few blood vessels and only a small proportion of astrocytes, oligodendrocytes and fewer thinly myelinated axons.


4


6


5

The cross-sections of the segments of the optic nerves which showed marked atrophy (Fig. 5) contained blood vessels which were thickened by endothelial hyperplasia. The extracellular spaces were filled by fibroblasts, collagen and lipid-laden macrophages. Only a few thinly myelinated axons were seen in this area, and some of these were in the process of being wrapped and remyelinated by neuroglial cells.

Ahead of this atrophied segment, the medial side of the optic nerve contained thick-walled blood vessels with a very small lumen (Figs. 6 and 8). On the opposite side few myelinated axons were present.

Semi-serial sections of the left optic nerve showed a diffuse involvement of almost the whole length with no significant difference between the control groups and the untreated animals (Fig. 9a, b).

Statistical analysis of survival of ara-A- and ara-C-treated animals to the control group are presented in Tables 2 and 3.

DISCUSSION

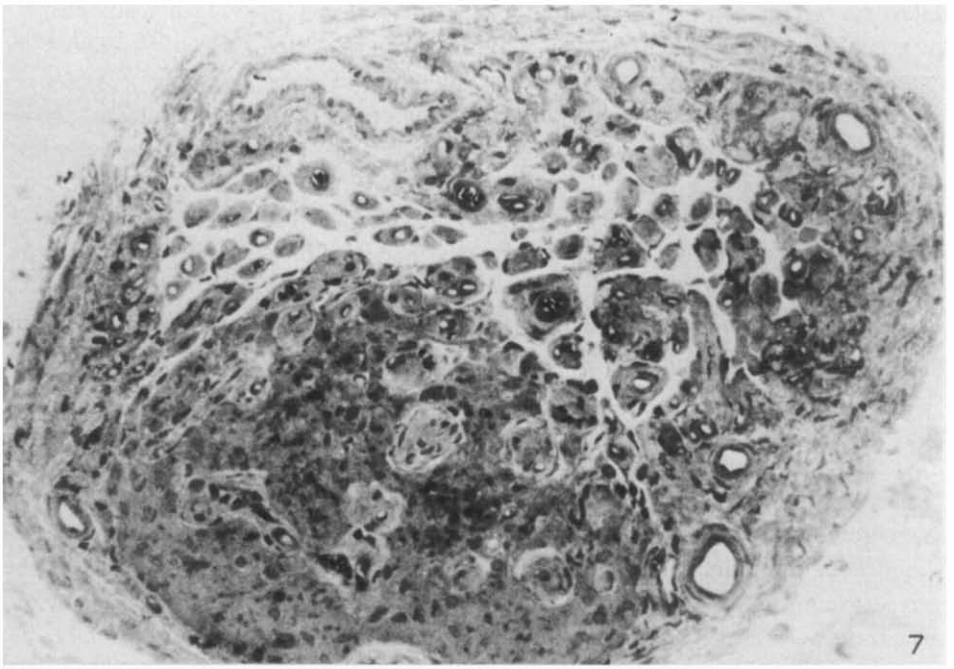
The injection of HSV-1 into the vitreous body of the eye in rabbits consistently induces chronic herpes encephalitis with only 10% mortality. Consequently, this is a useful model for the evaluation of currently available viricidal drugs as the animals can be observed over a long period of time. Assessment of the drugs performance is made by observing the general health of the animal and the pathological findings.

Chow et al. [4, 5] and Juel-Jensen [7] have reported no significant toxic side effects in humans receiving low-dose treatment with ara-C. On the contrary, Talley and Vaitkevicias [22] observed, in systemic treatment of humans, megaloblastosis and marrow suppression, producing leucopenia, anaemia and thrombocytopenia. Toxicity studies in mice, rats and rabbits [2, 9] have shown that the drugs are well tolerated in moderate doses except for a traumatic reaction at the injected site. It was also found in the present series of experiments that normal rabbits tolerated the drugs without major permanent toxic effects, while the effect was completely different in rabbits with underlying disease. The results of the present investigations, based on a large number of rabbits, clearly suggest that ara-C is more toxic than ara-A to the animals with the underlying disease. This is further supported by the fact that, with lower doses, mortality in both groups of treated rabbits decreases. Although in humans serious adverse effects of adenine arabinoside on bone marrow, liver and renal function have not been observed, Whitley et al. [24] frequently noticed reversible white-cell reductions among treated patients. Ara-A might be more toxic in the young developing rabbit than in man.

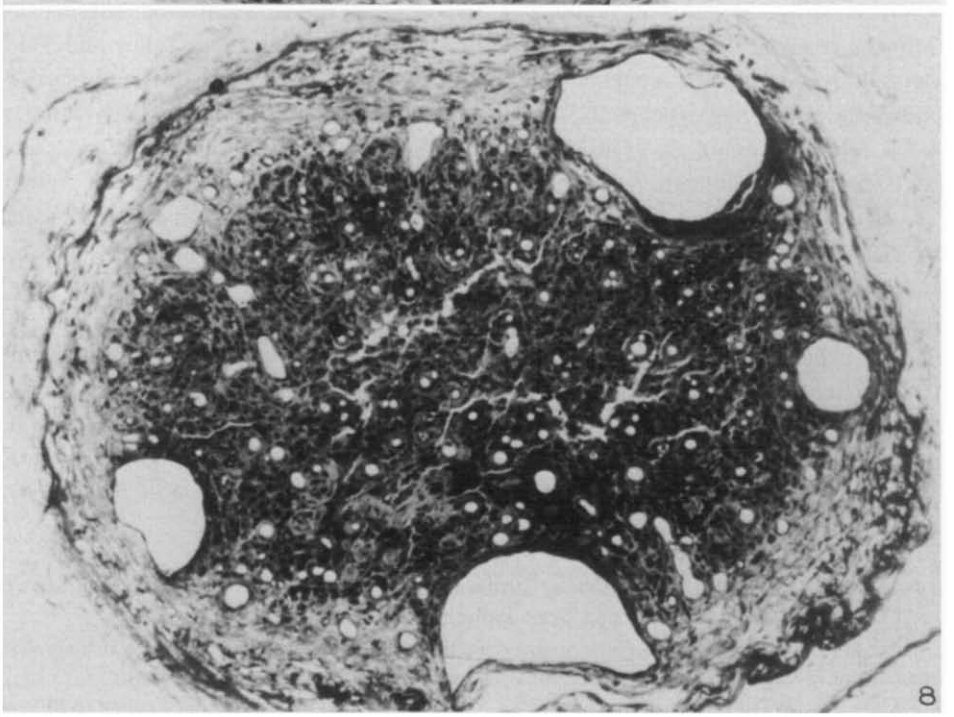
Fig. 4. Cross-section of right optic nerve of 30 days post-treatment rabbit (ara-A) showing marked spread of the lesion from the medial side. Note involvement of meningeal layers. ×160.

Fig. 5. Cross-section from same animal of the right optic nerve through the segment which showed marked atrophy. Note involvement of meningeal layers with unfiltrated marophages. ×250.

Fig. 6. Cross-section from same animal of the right optic nerve just past the atrophied region. ×100.



7



8

Both these drugs seem to have some effect on the course of the disease. The animals appeared healthier during the first 2–3 days and, in those treated with ara-A, neurological signs occurred later than in the control group. At this early stage of treatment, there was less infiltration by macrophages in the nervous tissue. However, in the long term, in the treated animals the picture was reversed: there was much more damage to the central nervous system.

The delay of the macrophage infiltration observed in the present experiments could be related to known immunosuppressive effects of ara-C [13] rather than inhibition of HSV, as the virus particles were seen in thin sections cut from treated animals.

In vitro studies by Rapp [18] showed that ara-C concentration of 0.01–0.10 μg/ml caused 13.4–90% of inhibition of varicella-zoster infection as measured by inhibition of plaque-forming cells. It is known that ara-C maintains a steady state of equilibrium between cerebrospinal fluid and plasma [23]. The treatment in this investigation with different schedules shows that activity of both the drugs against the infecting agent is minimal, even when the dosage of the drug was sufficient to produce high mortality. Efficacy can apply only to the dosage regimen which is non-toxigenic to the host. Percy and Hatch [17] also questioned the validity of cytosine arabinoside in their study, where they used the drug in near toxic doses (12.5 or 25 mg/kg/day for 5 days) in experimental infection with HSV-2 in newborn rats. Sloan et al. [21] investigated the antiviral activity of ara-A in mice inoculated intracerebrally with herpes simplex by various dosage levels (50–500 mg/kg/day), treatment schedules and routes of drug administration. As their experiments were based on mean survival time (14–21 days), the authors did not study the toxic effect of the drug, but concluded that ara-A did not completely suppress virus multiplication in the mouse brain. The results of the present study with previous evidence suggests that other regimens using low or high dosage would be less likely to have a beneficial effect.

The literature is full of contradictory reports. Evaluation of published claims of efficacy for the drugs is difficult because of variations in drug regimes, the timing of initiation of therapy, and the lack of quantitative measurement of the extent of disease. Proper controls are mandatory, because the course of this disease, if untreated, is highly variable and sometimes self-limiting. In the rabbit herpes encephalitis model, where many of the conditions can be controlled and results in the untreated group of animals predicted, it appears that the drugs have no beneficial effect but, on the other hand, surprisingly enhance the disease. It would be unwise to suggest that this happens only in rabbits.

In a different study, using ara-A therapy of biopsy-proven type 1 herpes simplex

---

Fig. 7. Cross-section of the right optic nerve of 30 days post-treatment (ara-C) rabbit. Note multilayered blood vessels and loss of myelinated fibres as compared to ara-A-treated rabbits (Fig. 4). ×200.

Fig. 8. Cross-section from same animal of right optic nerve just past the atrophied region. ×200.

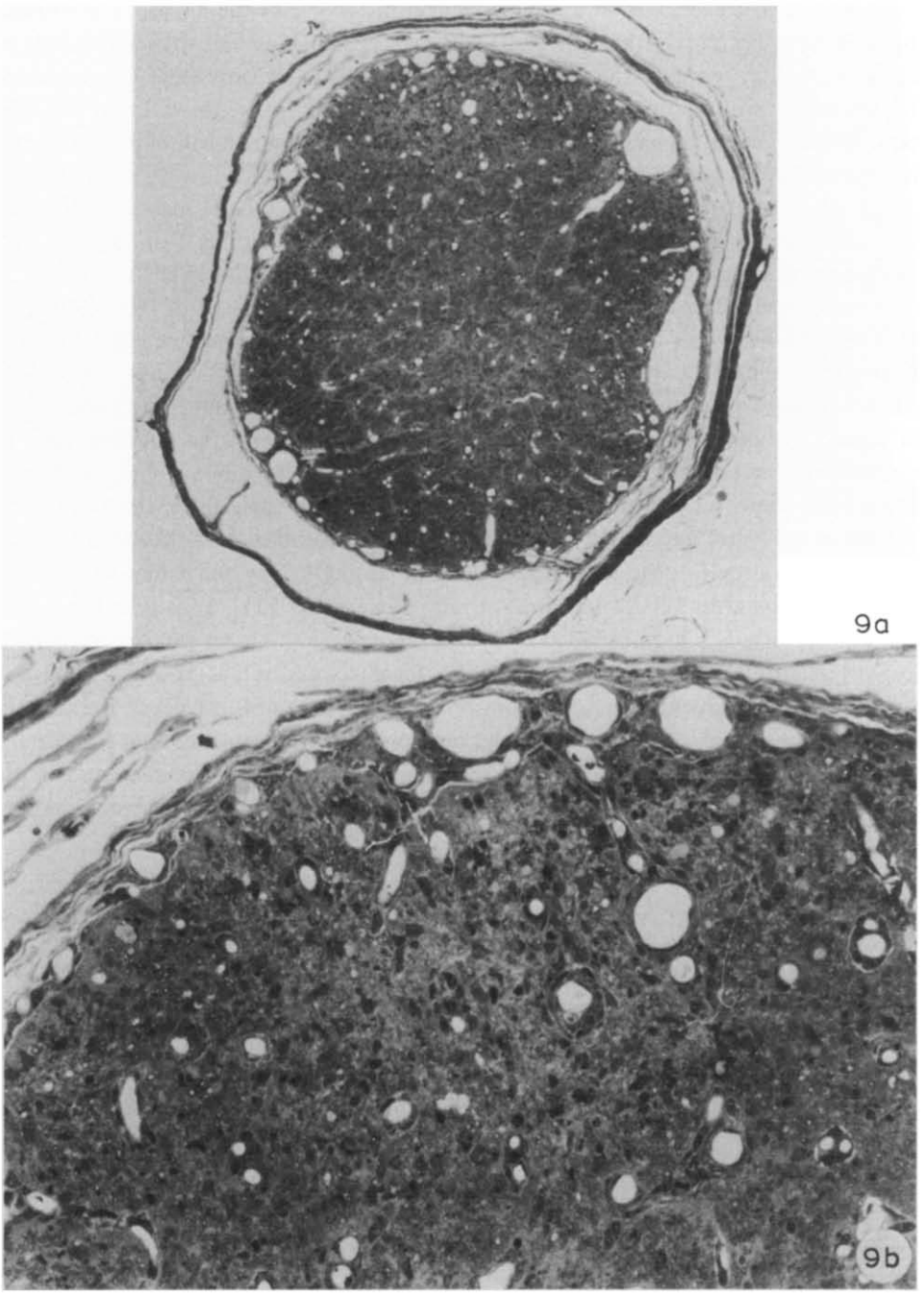


Fig. 9. a) Cross-section of left optic nerve of 30 days post-treatment (ara-A) rabbit showing no infiltration of macrophages through the meningeal layers. ×100. b) Higher magnification of 9a showing very little damage as compared with the right optic nerve. ×250. (For comparison with age match control refer to Fig. 4, Neuropathol. Appl. Neurobiol., 1978, 4, 461.)

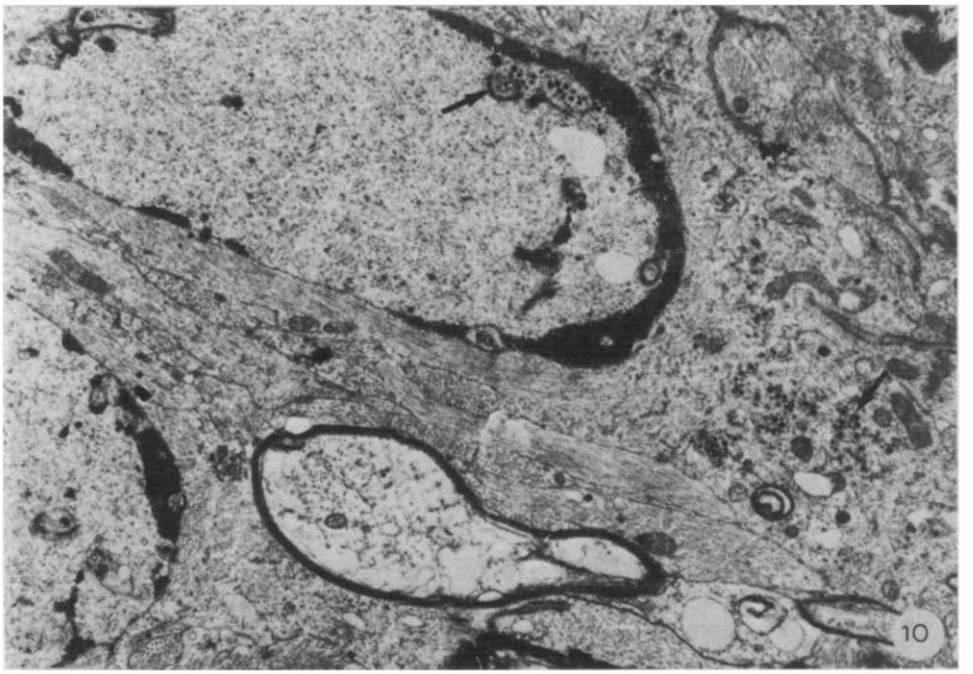


Fig. 10. Electron micrograph of a neuroglial cell from 16 days post-treatment rabbit showing herpes virus particles (arrows) ×11,400.

TABLE 2

Overall results of χ^2 analysis

Groups compared	χ^2	*P* value
Control vs. ara-A	0.38	> 0.5
Control vs. ara-C	8.39	< 0.001
Control vs. ara-A + ara-C	4.189	0.05–0.02
Ara-A vs. ara-C	5.482	0.02–0.01

TABLE 3

Results of χ^2 analysis with high and low dose

Groups compared	χ^2	*P* value
Control vs. ara-A	1.56	0.5–0.2
Control vs. ara-C high dose	9.231	< 0.001
Control vs. ara-A	0.2182	> 0.5
Control vs. ara-C low dose	1.1764	0.5–0.2

encephalitis, Whitley and the Collaborative Study Group [24] showed a good therapeutic index provided that the drug was given early in the course of infection and, unless given before the advent of coma, the therapy appeared to be futile. In these experiments the treatment is started when the infection is confined to the right eye and optic nerve and has not yet reached the brain [15, 16] but even this early treatment does not seem to have benefitted the animals. It is possible that, due to the low solubility of ara-A and because of the blood-brain barrier, the concentration required for the antiviral activity was not achieved in the optic nerve or brain.

Ara-A selectivity inhibits the synthesis of nuclear DNA [20]. Significant blood levels result after parenteral administration and this is due in part to enzymatic conversion to a more soluble metabolite hypoxanthine arabinoside (ara-Hx), and therefore ara-A cleared rapidly from mouse blood, appearing in the urine as ara-Hx [2]. Therefore, one would not expect a different metabolic pathway in rabbits. This metabolite (ara-Hx) has been shown to be as active an inhibitor of DNA viruses as ara-A [19]. However, it has been demonstrated that ara-A could break down by oxidation into free hypoxanthine [6] or phosphorylated to the 5′-mono, di- and triphosphates [10]. Although it has been shown that 5′-monophosphate has a significant effect on experimental herpes simplex keralitis [8], the role of most of the intermediates with the antiviral activity of ara-A is not yet known.

Treatment by direct intracerebral (i.c.) injection was not considered for two reasons. Firstly, the infection on the 4th day of treatment is confined to the optic nerves and, secondly, Cameron and Adams [3] have shown that i.c. treatment of mice with ara-A often produces head nodding with rolling features usually associated with hydrocephalus, which might have been associated with multiple i.c. injections rather than the drug. There is also a high mortality rate in the i.c.-treated group as compared to intraperitoneal.

REFERENCES

1 Adams, D.H. and Cameron, K.R. (1977) Lancet 2, 760.
2 Brink, J.J. and Le Page, G.A. (1964) Can. Res. 24, 1042.
3 Cameron, K.R. and Adams, D.H. (1979) J. Infect. Dis. 140, 921.
4 Chow, A.W., Foerster, J. and Hryniuk, W. (1970) Antimicrob. Agents Chemother. 1, 214.
5 Chow, A.W., Ronald, A., Fiala, M., Hryniuk, W., Weil, M.L., Gerne, J. St. and Guze, L.B. (1973) Antimicrob. Agents Chemother. 3, 412.
6 Cohen, S.S. (1966) Progress in Nucleic Acid Research and Molecular Biology, Vol. 5 (Academic Press, New York) p.1.
7 Juel-Jensen, B.E. (1970) Oxford Med. School Gaz. 22, 56.
8 Kaufman, H.E. and Varnell, E.D. (1976) Antimicrob. Agents Chemother. 10, 885.
9 Kurtz, S.M., Fisken, R.A., Kaump, D.H. and Schardein, J.L. (1968) Antimicrob. Agents Chemother. 8, 180.
10 Le Page, G.A. (1970) Can. J. Biochem. 48, 75.
11 MacCallum, F.O. and Pattison, J.R. (1977) J. Antimicrob. Chemother. 3 (Suppl.) 5.
12 Meyer, H.M., Johnson, R.T., Crawford, I.P., Dascomb, H.E. and Rogers, N.G. (1960) Am. J. Med. 29, 334.

13 Mitchell, M.S., Wade, M.E. and DeConti, R.C. (1969) Ann. Intern. Med. 70, 535.
14 Narang, H.K. (1980) J. Neurol. Sci. 45, 109.
15 Narang, H.K. and Codd, A.A. (1978) Neuropathol. Appl. Neurobiol. 4, 137.
16 Narang, H.K. and Codd, A.A. (1978) Neuropathol. Appl. Neurobiol. 4, 457.
17 Percy, D.H. and Hatch, L.A. (1975) J. Infect. Dis. 132, 256.
18 Rapp, F. (1964) J. Immunol 93, 643.
19 Schabel, F.M. (1968) Chemotherapy 13, 321.
20 Shipman, C., Jr., Smith, S.H. and Drach, J.C. (1972) Proc. Natl. Acad. Sci. U.S.A. 69, 1753.
21 Sloan, B.J., Miller F.A., Ehrlich, J., McLean, I.W. and Machamer H.E. (1968) Antimicrob. Agents Chemother. 161.
22 Talley, R.W. and Vaitkevicias, V.K. (1963) Blood 21, 352.
23 Wang Ho, D.H. and Frei, E. (1971) J. Infect. Dis. 133, Suppl. A, 192.
24 Whitley, R.J., Soong, S.J., Durbin, R., Galasco, L.J., Chi'en, L.T., Alford, C.A. and the Collaborative Study Group (1977) N. Engl. J. Med. 297, 289.