Inhibition of Rabies Virus in vitro by the Ammonium-5-Tungsto-2-Antimoniate

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SUMMARY

In vitro multiplication of rabies virus was inhibited by a condensed mineral ion, ammonium-5-tungsto-2-antimoniate (HPA 23). The inhibitory effect was evaluated by two different methods, plaque reduction and one step virus growth. Plaquing showed 50% inhibition with 4.5 µg/ml of HPA 23 and complete inhibition with 12.5 µg/ml. A reduction of two logs in virus yield was obtained in BHK₂₁C₁₃S cells in suspension treated with 50 µg/ml of HPA 23. Inhibition also occurred when treatment with HPA 23 was started 18 to 24 h after infection in the plaque assay but no effect was seen when HPA 23 was added 48 h after virus inoculation. All these inhibitory effects of HPA 23 on rabies virus multiplication were observed at non-cytotoxic doses. Therefore HPA 23 contrasts with other antiviral drugs which do not inhibit rabies virus multiplication without affecting the viability of cells.

Rabies virus is only poorly sensitive to antiviral drugs. In vitro activity of some antibiotics like actinomycin D and some nucleoside analogues have been reported (Harmon & Janis, 1976; Flamand et al. 1977) but inhibition of virus multiplication may be due to cellular toxicity rather than to a specific antiviral action. Ammonium-5-tungsto-2-antimoniate heteropolyanion (HPA 23) is a new antiviral compound which has shown significant activity in vitro against many RNA and DNA viruses at concentrations well below cellular toxic levels (Jasmin et al. 1975; Werner et al. 1976). Furthermore, HPA 23 also had an in vivo protective effect in mice against infection initiated by RNA viruses, namely murine leukaemia and sarcoma viruses (Jasmin et al. 1974, 1975), lymphocytic choriomeningitis virus (Streissle, 1975) encephalomyocarditis virus and vesicular stomatitis virus (Werner et al. 1976). We report here on the specific in vitro inhibition of rabies virus multiplication by HPA 23 at non-cytotoxic doses.

The Flury high egg passage (HEP) strain of rabies virus was used for plaque assay and the Pasteur strain for the one-step growth experiments. The plaque assay described by Sedwick & Wiktor (1967) was used for titration of rabies virus. BHK₂₁C₁₃S cells were suspended at 10⁶/ml in complete MEM containing 2% foetal calf serum and 0.3% agarose and seeded on plastic Petri dishes (60 × 15 mm) previously coated with 0.6% agarose solution. Virus dilutions were added in a vol. of 0.1 ml and the cultures incubated at 35 °C under 5% CO₂. After 6 days incubation, plaques were stained with a 1/10000 neutral red solution in 0.6% agarose and counted. The results are expressed as p.f.u./ml.

Experiments with a single cycle of virus multiplication were performed in BHK₂₁C₁₃S cells grown in suspension in complete MEM medium containing 1% foetal calf serum. The medium was removed on the following day by centrifugation, cells from the pellet were infected with approx. 10 p.f.u./cell and incubated for 2 h at 37 °C. The cell concentration was adjusted to 10⁶/ml in a medium containing 0.4% BSA (Sigma) and incubated with stirring at 35 °C. Samples were harvested at different times and intra-cellular virus was
titrated as described previously (Tsiang & Atanasiu, 1971) in vivo by intracerebral inoculation of mice and in vitro by the plaque assay method. 5-Tungsto-2-antimoniate was prepared according to the procedure previously described (Jasmin et al. 1974). Fresh solutions in culture medium were made for each experiment.

The effect of HPA 23 on rabies virus was assayed by incubating the latter with 1 mg/ml of HPA 23 during 30 min at 35 °C in MEM medium supplemented with 1% foetal calf serum. The residual infectivity was titrated by the plaque assay method. Only HPA 23 concentrations known to be non-toxic for BHK21C13S cells, and thus not inhibiting plaque formation, were used.

No cytotoxic effect of HPA 23 was observed when cells were maintained in agarose medium for 5 days at 35 °C and stained with neutral red if the medium contained 100 μg/ml or less of the compound. A 50% reduction of cell survival was obtained with 800 μg/ml. Thus it was assumed that the effect of HPA 23 on plaque assay was specific. Addition of HPA 23 immediately after absorption of the virus resulted in a reduction of plaque number. The 50% inhibitory dose was 4.5 μg/ml and a complete inhibition was obtained with 15 μg/ml (Fig. 1). When doses of 10 μg/ml of HPA 23 were added to plates infected with dilutions of virus, plaque counts were significantly reduced (from 48- to 330-folds in different experiments). Timing of the action of HPA 23, determined by the plaque reduction, showed for both 5 and 10 μg/ml a maximum inhibitory effect from 18 to 24 h. No protective effect was recorded 48 h after addition of HPA 23. Indeed, an enhancement of plaque numbers was observed in plates treated 72 h after inoculation. HPA 23 (5 to 10 μg/ml) added 24 h before virus inoculation and remaining for the entire experiment has a complete inhibitory effect.

Incubation of virus in presence of HPA 23 resulted in mean titres of 10^{4.4} p.f.u./ml for HPA 23 treated particles and 10^{5.9} p.f.u. for untreated virus control.

Inhibition of virus production was assayed in one-step growth curves in cell suspensions. Only a partial inhibition occurred with 20 μg/ml of HPA 23, either measured by plaque titration or mouse inoculation (Fig. 2a). With higher HPA 23 concentrations (50 μg/ml) a reduction of 2 logs in virus yield was obtained with both titration methods (Fig. 2b).

The data reported in this paper demonstrate a strong inhibitory effect of HPA 23 on fixed rabies virus growth in vitro. As shown with other viruses inhibited by HPA 23, this compound does not have a direct effect on the infectivity virus particles and it was active after the virus absorption period. In the plaque assay where plaque formation requires multiple cycles of virus replication, HPA 23 was active as late as 24 h after virus inoculation, a period which corresponds to a complete cycle of virus replication and probably to the initiation of infection of cells surrounding the initial focus of infection. Rabies virus is only poorly sensitive to antiviral drugs; it is insensitive to inhibitors of DNA synthesis like fluorodeoxyuridine and mitomycin (Hamparian, et al. 1963).

It has been reported that nucleoside analogue such as cytosine arabinoside, adenine arabinoside and 6-azauridine inhibit the replication of rabies virus in vitro (Harmon & Janis, 1976), but in these experiments a very low multiplicity of infection was used (3 x 10^{-3} p.f.u./cell) and the virus in the control group was low 24 h after virus inoculation and did not attain maximal values until after 3 days of replication. As pointed out by the authors, considerable alterations in the morphology of the cells from the drug control monolayers were noticed. Similarly, it has been reported that high doses of actinomycin D and cytosine arabinoside inhibit rabies virus replication by affecting the host cell in such a way that it is no longer able to support normal virus production (Flamand, et al. 1977).

Therefore, HPA 23 represents a unique drug which inhibits rabies virus multiplication at
Fig. 1. Inhibition of rabies by different HPA 23 concentrations. Fifty per cent inhibition of plaque forming (IPF50) was evaluated for different HPA 23 concentrations: 2.5, 5, 10, 20 and 40 μg/ml. HPA was added after inoculation of the virus. All the dishes were inoculated with a virus concentration adjusted to approx. 50 p.f.u./dish.

Fig. 2. Recovery of virus from infected cell suspensions after treatment with HPA 23. Samples were taken at different times from infected cell suspensions containing (a) 20 or (b) 50 μg/ml of HPA 23, or from infected control cells. Virus infectivity was titrated in vitro in a plaque system in BHK21 cells and in vivo by intracranial mouse inoculation. Plaque titration: ■ control; ◦ — ◦, with HPA 23 Mouse titration: — , control; — — ◦, with HPA 23.

Doses which are not cytotoxic. The 50 % inhibitory dose of plaque formation is well below the toxic level. The absence of cellular toxicity of HPA 23 at antiviral doses has also been demonstrated in other virus cell systems where HPA 23 inhibited virus replication. Another polyanionic drug, silicotungstate, known to be inhibitory for some RNA viruses and particularly for vesicular stomatitis virus (Raynaud et al. 1971; Raybaud et al. 1972) was inactive in vitro (unpublished results). The difference in action on vesicular stomatitis and rabies viruses may involve different mechanisms of inhibition for polyanionic inhibitors.

HPA 23 has been shown to be a strong inhibitor of retrotranscriptase of murine tumour viruses (Chermann et al. 1975) by acting at the binding site of the enzyme with the template primer. It is also active on cellular DNA polymerase (Ablashi et al. 1977). However, the mechanism of action of HPA 23 is not yet known and it is impossible to decide whether the
specific inhibiting effect of HPA 23 on virus and cellular polymerases is responsible for its antiviral properties.

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