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TREATMENT OF RABIES IN MICE AND FOXES WITH ANTIVIRAL COMPOUNDS

F. BUSSEREAU*, M. PICARD[1], J. BLANCOU[2], P. SUREAU

Unité Rage Recherche, Institut Pasteur, 75724 Paris Cédex 15, [1]Ministère de l'Agriculture, Services Vétérinaires, L.C.R.V., 94704 Maisons-Alfort Cédex, and [2]Ministère de l'Agriculture, Direction de la Qualité, Services Vétérinaires, C.N.E.R.P.A.S., 54220 Malzéville, France

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Summary. — Thirty four chemical compounds were injected into rabies infected mice by intramuscular (i.m.) route. Twenty four compounds such as well known therapeutic agents: amantadine, lipacids, phenol compounds, didemnin-B, procaine, nucleosides analogues (ribavirin, tiazofurin, pyrazofurin) had no effect. Two compounds had a slight effect not justifying to consider them as possible therapeutic agents: selenazofurin and an analogue of ribavirin (RTA). Eight heteropolyanions (HPA), which have a related chemical structure, were efficient providing 100% protection. Nineteen compounds were injected into rabies infected mice by the intracerebral (i.c.) route. Fourteen compounds such as ribavirin, RTA, selenazofurin, tiazofurin and 9 HPA compounds had no effect. Five other HPA compounds (HPA 23-39-46-51-56) were efficient preventing the development of clinical infection in some mice. Whatever was the treatment route, treated surviving mice developed rabies neutralizing antibodies. No proof of viral multiplication was found in their brains. As some HPA compounds did produce a therapeutic effect in mice, two of them HPA 23 and HPA 39 were administered to rabies-infected foxes. In foxes the compounds prolongued the mean survival time and increased the number of survivors. These data suggest that chemotherapy might be worthwhile when vaccination was impossible.

Key words: antiviral therapy, foxes, mice, rabies virus

Introduction

Rabies in man today can be prevented on the days following exposure by vaccination. However, once clinical signs develop, rabies is invariably fatal. Even intensive treatment using interferon had been ineffective. Our aim was to attempt curative treatment of rabies testing 40 compounds. They were

* To whom correspondence should be addressed to

choosen either because they were known to act as virus inhibitors or because they possessed anti-tumour activity (Bussereau *et al.*, 1983*a*). The mouse was choosen as animal model. In order to mimic natural exposure and clinical outcome as much as possible, a street strain of RV isolated from rabid foxes was employed. To approximate the conditions of treatment of humans, two injection routes were used and treatment had started either early or late in the incubation period. Some trials were carried out on foxes. The foxes monitored a model of fully susceptible animal since rabies is consistently fatal in foxes, but this is not always the cause in mice. Treatment of foxes started early after virus inoculation administered by two routes. The results obtained on mice and foxes are the subject of this report.

Materials and Methods

Rabies virus. A fixed strain corresponding to the Challenge Virus Strain CVS/cell was used. This strain was described in detail elsewhere (Bussereau *et al.*, 1982*a*). The viral suspension frozen at −70 °C and thawed many times, did not lose infectivity and had a titre of 2×10^7 PFU/ /ml. The plaquing procedure was previously described (Bussereau *et al.*, 1982*b*).

Two types of street strains were used. The GS strain was prepared from salivary glands of rabid foxes as previously described (Blancou *et al.*, 1979). Five AH/mouse strains were prepared from Ammon's horns of five naturally infected foxes. Each material was homogenized in distilled water containing 2% horse serum. After 10 min centrifugation at $500 \times g$ the supernatant was recovered to constitute the virus stock (AH strain). The AH strain was passaged in 50 mice by intracerebral route (i.c.). This was carried out for two reasons: to adapt the virus to mice, i.e. for reproducible mortality, and to obtain virus homogeneous enough avoiding blending from different foxes. The entire brains of dying mice were removed, homogenized and centrifuged under the same conditions as the virus stock. Then a 40% brain suspension was prepared corresponding to the highest concentration which could be injected without causing immediate death (= AH/ /mouse strain). The 5 strains had the same characteristics after inoculation in mice and were used on the day they had been prepared because storage at −70 °C lead to a significant decreas of their infectivity.

Animal studies. Three- to four-week-old male outbred mice were provided from by IFFA-CREDO (69210 Saint Germain sur l'Arbresle, France). The OF_1 mice were housed under conventional conditions with unrestricted access to food and water. They were infected between 3 (17−20 g) and 6 weeks (30−35 g) of age. Ten mice were treated or included into virus control group and five were used for toxicity control. The two types of street strains were inoculated into the hind leg muscle (i.m. = 0.1 ml). The challenge in treated surviving mice was carried out by i.c. inoculation with 0.03 ml of the CVS/cell strain.

Red foxes were captured in rabies free areas in France and raised as described elsewhere (George *et al.*, 1980). They were used between 1 and 2 years of age (4−5 kg). Before these experiments their sera were checked for the absence of rabies antibodies. The GS virus strain was inoculated into a temporal muscle (i.m. = 1 ml). For each experiment the maximal number of available foxes was used. The challenge was carried out by i.m. inoculation deep into a temporal muscle, with 1 ml of the CVS strain.

Chemicals and treatments. Compounds assessed in this study were listed and their usual abbreviations were given in Table 1. All the compounds were water soluble. They were dissolved in sterile physiological saline solution just before use. As the aim of the work was to find a curative treatment for rabies, LD_{50} toxicity values were determined only if no acute or subacute toxicity values were previously available, i.e. for HPA treatment of foxes.

Detection of RV. Brains were removed from surviving, from dying or dead animals. Smears were made for the detection of the N (nucleocapsid) antigen and such detection was confirmed by a fluorescenr antibody test (FAT) using anti-nucleocapsid conjugare (P. Perrin, Unité Rage).

Individual mouse brains were homogenized. The presence or absence of infectious virus (IV) was determined in mice by i.c. inoculation

Detection of RV antibodies. Blood was drawn from the jugular vein of surviving foxes or by bleeding of surviving mice. The blood samples were left 1 hr at 37 °C and overnight at 4 °C; serum was collected after centrifugation (500 × g, 10 min) and stored at −20 °C. Titration of rabies neutralizing antibodies (NA) was performed by the fluorescent focus reduction test (Sureau et al., 1982).

Statistical analysis. For comparisons of mice groups (treated = r, untreated = s) which had survivors (treated = a, untreated = c) at the end of the experiment, an adjusted Chi square test for a 2 × 2 comparative trial was carried out:

$X^2 = (N - 1)(ad - bc)^2/rmns$;

$a + b = r$; $a + c = m$; $b + d = n$; $c + d = s$; $m + n$ and $r + s = N$.

Upton (1982) who considered 22 alternative tests, has recommended such adjusted χ^2 test taking in account the sample sizes (m, n, r, s $\geqq$ 5).

Table 1. Abbreviations for the 40 antiviral compounds

Amantadine	= 1 − aminotricyclo (3.3.1.1.^{3,7}) decane	
C_4 Cu	= copper butyrate	
C_4 Co	= butyryl collagene	
C_4 Co Cu	= butyryl collagene, copper salt	
C_4 Co Cu Mn	= butyryl collagene, copper salt, manganese salt	
C_4 Co Cu Zn	= butyryl glycine zinc salt	
C_8 G	= caprylyl glycine	
C_8 G Lys	= caprylyl glycine, lysine salt	
CHOP	= oxidation product of chlorogenic acid	
(S)-DHPA	= (S)-9-(2,3)-dihydroxypropyl) adenine	
2,5-DHPOP	= 2.5-dihydroxyphenylacetic acid	
3,4-DHPOP	= 3,4-dihydroxyphenylacetic acid	
Didemnin-B	= peptide: C_{57} H_8 N_7 O_{15}	
GALOP	= gallic acid	
GENOP	= gentisinic acid	
HPA 23	= $(Na\ Sb\ W_{21}\ O_{86})^{18-}$	ammonium salt
HPA 32	= $(Sb\ W_{11}\ O_{38}\ H)^{6-}$	sodium salt
HPA 39	= $(K\ S_9\ W_{21}\ O_{86})^{18-}$	potassium salt
HPA 40	= $(Sb_9\ W_{21}\ O_{86})^{19-}$	ammonium salt
HPA 44	= $(K\ As_4\ W_{40}\ O_{140})^{27-}$	sodium salt
HPA 45	= $(Pa\ As_4\ W_{40}\ O_{140})^{26-}$	sodium salt
HPA 46	= $(K\ Sb_9\ W_{21}\ O_{86})^{18-}$	ammonium salt
HPA 47	= $(Na\ Sb_9\ W_{21}\ O_{86})^{18-}$	sodium salt
HPA 51	= $(Na\ Sb_9\ W_{21}\ O_{86}\ (Fe^{III}\ (H_2O)_2\ O)_3)^{14-}$	sodium salt
HPA 52	= $(Na\ Sb_9\ W_{21}\ O_{86}(V^{IV}O\ (H_2O)_2O)_3)^{16-}$	sodium salt
HPA 53	= $(Ca\ Sb_9\ W_{21}\ O_{86})^{17-}$	ammonium salt
HPA 56	= $(Sr\ Sb_9\ W_{21}\ O_{86})^{17-}$	ammonium salt
HPA 57	= $(Na\ Sb_9,\ W_{21},\ O_{86},\ Co_x)^{18-2x}$	sodium salt
HPA 59	= $(P_4\ W_{14}\ O_{58})^{12-}$	potassium salt
HYKOP	= hydrocaffeic acid	
KOP	= oxidation product of caffeic acid	
3 MQ	= 5, 7, 3′, 4′ tetryhydroxy-3-methoxy flavone	
NORADROP	= L-noradrenaline	
Procaine	= $C_{13}H_{20}O_2N_2$	
Pyrazofurin	= 3-β-D-ribofuranoxyl-4-hydroxyýyrazole-5-carboxamide	
PYROP	= pyrogallol	
Ribavirin	= 1-β-D-ribofuranosyl-1, 2, 4-triazole-3-carboxamide	
RTA	= analog of ribavirin: 2′, 3′, 5′ - triacetate	
Selenazofurin	= 2-β-D-ribofuranosylselenazole-4-carboxamide	
Tiazofurin	= 2-β-D-ribofuranoxylthiazole-4-carboxamide	

Table 2. Intramuscular administration of 9 compounds in rabies infected mice

Compound	Dose mg/kg/day	Toxicity control[a] day 1 to 5	Survival after treatment[b]					Virus control[c]
			day 1	day 1 to 2	day 1 to 3	day 1 to 4	day 1 to 5	
Amantadine[e]	30	5/5	1/10	0/10	0/10	0/10	1/10	1/10
	67	5/5			2/10			4/10
Didemnin-B[e]	0.19	5/5	0/10	2/10	2/10	3/10		1/10
	0.39	5/5	2/10	1/10	1/10			4/10
HPA 39[g]	177	5/5	0/10	4/10	5/10[d]	7/10[d]	10/10[d]	1/10
	216	5/5	9/10[d]	9/10[d]	10/10[d]	9/10[d]		5/10
Procaine[e]	118	5/5	2/10	1/10	1/10	2/10	0/10	1/10
	267	5/5			1/10			4/10
Ribavirin[e]	59	5/5	0/10	0/10	0/10	0/10	1/10	1/10
	100	5/5	6/10	7/10	6/10	5/9		5/10
RTA[f]	50	5/5	4/10	5/10	6/10			5/10
	100	5/5	8/10	10/10[d]	5/10			5/10
(S-)-DHPA[e]	118	5/5	0/10	1/10	0/10	1/10	1/10	1/10
	150	5/5			8/10	6/10		5/10
Selenazofurin[f]	100	5/5	2/10	6/10	5/10	7/10[d]		3/10
	143	5/5		10/10[d]	10/10[d]			4/10
	286	5/5		10/10[d]	10/10[d]			4/10
Tiazofurin[e]	800	5/5	6/10	4/10	4/10	2/10		3/10
	900	5/5	6/10	8/10				5/10

(a) No. survivors/No. of treated uninfected.
(b) No. survivors/No. of infected then treated.
(c) No. survivors/No. of infected.
(d) Significantly different from virus control $\leq P\ 0.05$.
(e) 6 compounds with no effect (6/24).
(f) 2 compounds with a slight effect (2/2).
(g) 1 compound effective compound (1/8).

Results

Characterization of rabies virus (RV)

The characteristics of street strain in mice can be summarized as follows: 1) After i.c. inoculation (0.03 ml) of undiluted virus suspension death occurred from day 8 up to day 13, while after i.m. (0.1 ml) inoculation the first dead mice appeared on day 13 and the last one on day 27 post-infection (p.i.). 2) Undiluted suspension given by i.m. route caused 80% mortality while given by i.c. route even diluted 10^5 fold it still caused 100% mortality. 3) Susceptibility of mice inoculated by i.m. route decreased linearly by age: out of 4 weeks old mice 80% were killed, out of 7 weeks ones 50% but all 13 weeks old mice survived. The fact that a street strain even at the highest concentration killed less than 100% of young mice inoculated by i.m. route is commonly reported with RV. This just means that even young mice are not fully susceptible to peripheral RV administration. In fact, the i.m. route was preferred to reproduce natural exposure and to give the mice the opportunity to develop an immune response against infection. As in this work young mice were used, a fully immune response may not be obtained. Due to the fact that surviving infected mice developed rabies NA we concluded that 100% of mice were infected.

The characteristics of the CVS/cell strain can be summarized as follows: 1) After inoculation by i.c. or i.m. routes the first dead animals appeared on day 6 and the last on day 13; 2) to obtain 100% mortality by i.m. route the suspension should be undiluted while given by i.c. route even diluted 10^4 fold it still caused 100% mortality; 3) the lethal dose was the same whatever was the age of mice. The fact that laboratory strains are more adapted to mice is commonly reported with RV. Two reasons lead us to prefer the CVS/cell strain for challenge: challenge was performed in older mice, survivors of treatment and a CVS strain are used for potency testing of the rabies vaccines.

Infection of mice

The street strain virus was inoculated directly into the hind leg muscle (10^2LD_{50}/i.m.). It is generally accepted that virus multiplication occurs at the bite site and that only a part of the virus progeny reaches to the peripheral nerves. The rabies virus (RV) travels centripetally to spinal ganglia and then to the CNS. There is no viraemia. At the onset of clinical symptoms the virus is found throughout the brain. After day 3, in our hands, an accumulation of newly synthesized viral N protein was observed. After reaching the CNS, RV spreads centrifugally from the CNS. Various symptoms like ruffled fur and increased excitability appear on day 10 and increase rapidly in severity. Loss of weight is due to animal paralysis, as the mouse is not able to take in the food (liquid and solid). Once paralysis on the hind leg has appeared, its progress is inexorable (hind quarters and entire body) leading to coma. Death ensures on the second to the third day after onset of the symptoms, i.e. on day 13. As freshly prepared virus was

used in each experiment, no prior titrations could be made. Under these conditions the mortality varied from 50 up to 100% in the different experiments.

Early treatment of mice by i.m. route

We aimed to determine whether or not compounds were effective by i.m. administration if given near to the site of primary infection. Thirty four compounds were assayed. In preliminary experiments, various concentrations of each compound were injected once a day for 5 consecutive days. We concluded that a concentration was not toxic when none of the mice changed behaviour or died in a group, within the 2 weeks period. Subsequently 2 to 4 nontoxic concentrations were used for treatment. For each concentration 6 groups of mice were infected at day 0. One group was used as infection control. In 5 groups the drug was first administered after 24 hr at day 1 near to the virus injection site into the same hind leg. Then 4 animal groups received a second injection at day 2, 3 groups a third one at day 3, 2 group a fourth one at day 4 and the last group received a fifth injection at day 5 i.e. each drug concentration was inoculated 1, 2, 3, 4 or 5 times at 24 hr intervals. One group of uninfected mice received 5 regular doses and was used as control of nontoxicity (Table 2).

Among the 40 compounds available 26 had different chemical structure (Table 1). The results obtained can be classified into 2 groups: (i) 24 compounds had no effect, i.e. no mice survived or mice survived similarly as in ;the control group. Lipacids (such as C_4Cu, C_4Co, C_4CoCu, $C_4CoCuMn$, $C_4CoCuZn$, C_8G, C_8G Lys) phenol compounds (such as 2,5-DHPOP, 3,4--DHPOP, GALOP, GENOP, HYKOP, KOP, NORADROP, PYROP), 3 MQ and pyrazofurin belong to this group. Experimental results with 6 other compounds which belong to this group are shown on Table 2. Amantadine, didemnin-B, procaine, ribavirin, (S)-DHPA and tiazofurin were taken as examples, since they are well known antiviral drugs. All treated surviving mice developed rabies NA as the survivors from the infection control. (ii) two compounds had a slight therapeutic effect i.e. survivors might be observed in larger numbers than in the control. These two compounds, RTA and selanozofurin are well known antiviral agents (Table 2). Since no correlation was found between the effective dose and the percentage of survival after treatment these compounds were not considered effective in rabies therapy. All the surviving mice had NA but only some of them were protected to challenge.

Of the 40 compounds, 14 had a related chemical structure (Table 1) corresponding to heteropolyanions — HPA. For one of the compounds given by i.p. route the acute toxicity in adult mice was reported to be 750 mg/kg and the lowest dose causing a significant weight loss in normal mice was found to be 250 mg/kg/i.p. (Werner *et al.*, 1976). Therefore, all HPA were administered once a day by i.m. inoculation during 5 consecutive days to determine the maximum concentration at which all the mice survived. HPA-23-32-39-40-46-51-53-57 were less toxic (420 mg/kg). For HPA 47-52-

Table 3. Effect of intracerebral HPA 39 treatment in rabies infected mice

Treatment Dose: 0.01 mg/mouse/day	Survival(a) No. of survivors/No. treated	FAT(b)	IV(b)	NA(b)
day 1 to 3	3/10	—	—	++
day 2 to 4	1/10	—	—	++
day 3 to 5	2/10	—	—	++
day 4 to 6	2/10	—	—	++
	= 8/40(c)			
virus control	0/10			
	0/10			
	= 0/20			

(a) Calculated on day 35.
(b) Determined as described in Materials and Methods.
— = absence in the brain of viral N protein by FAT or of infectious virus (IV)
++ = presence of rabies neutralizing antibodies (NA).
(c) Significantly different from virus control. $P < 0.05$

-56-59, 200 mg/kg was the maximum dose. HPA-44 and HPA-45 were the most toxic (150 mg/kg) and therefore not used by i.m. route. To compare the efficacy of these compounds, eight of them available in sufficient amount were administered up to 216 mg/kg: HPA-23-32-39-46-52-56-59. All of them had a chemotherapeutic effect, i.e. survivors were found. It was also possible to find such conditions of treatment which resulted in 100% survival. HPA-39 was choosen as prototype compound because in a previous report it had the best antiviral activity against rabies production in vitro (Bussereau *et al.*, 1983*a*). Results with HPA-39 are presented Table 2.

Three parameters in the treatment were modified to determine the best conditions for HPA-39: the inoculation site of the compound, the onset of treatment, and the interval between two doses. The concentration of 200 mg/kg was used. When treatment consisted of a single dose only either on day 1, 2 or 3 on the same side as the virus, the best result was obtained soon after infection, i.e. on day 1. When the treatment course consisted of two doses, on days 1 and 2 or on days 2 and 4 or on days 3 and 4 given on the same side the best result was obtained when first injection was given 24 hr post-infection, i.e. days 1 and 2. When the treatment course consisted of four doses on days 1, 2, 3 and 4 all were injected either on the same side to the same or opposite hind leg as the virus or 2 on one side and 2 on another. The best result was obtained with 4 injections given to the same side as the virus while 4 injections to other side gave the worst result. Alternative treatment gave an intermediate result (not shown).

The present study demonstrates that by i.m. HPA had the best efficacy when injected early after infection, near the site of virus administration.

Table 4. Effect of intramuscular HPA 39 treatment in rabies infected mice

Treatment Dose: 150 mg/kg/day	Survival[a] No. of survivors/No. treated	FAT[b]	NA[b]
day 1 to 3	4/10[c]	−	+
day 5 to 9	0/5		
day 6 to 10	0/5		
day 7 to 11	0/5		
day 8 to 12	2/5	−	+
day 9 to 13	1/5	−	+
day 10 to 14	0/5		
day 11 to 15	0/5		
day 12 to 15	0/10		
day 13 to 15	2/10	−	+
	= 5/55[d]		
Virus control	0/10		
	0/10		
	= 0/20		

[a] Calculated at day 35.
[b] Determined as described in Materials and Methods
− = absence in the brain of viral N protein as detected by FAT
+ = presence of rabies neutralizing antibodies (NA)
[c] Significantly different from virus control. $P < 0.05$
[d] Not significant. $P > 0.05$

Early treatment of mice by i.c. route

To determine whether HPA-39 was effective when administered by i.c. route, it was inoculated to the brain at the beginning of infection. To minimize trauma due to this type of inoculation, injections were restricted to a total of 3 at one day intervals. As the dose of 0.1 mg per mouse was toxic, a nontoxic dose of 0.01 mg/mouse was used. Six groups of mice were infected by i.m. route at day 0. Two groups were used as controls. In 4 groups HPA-39 was first administered i.c. on day 1, or day 2, or day 3, or day 4. Then the mice received two additional doses at 24 hr intervals. The results are presented in Table 3. Surviving mice were found. These mice had rabies NA. No sign of infection was observed in the brain of these mice, as neither N protein was visible and nor infectious virus was recovered. As 20% survival was a significant result, the present investigation demonstrates that HPA-39 was also potent while injected by i.c. route early after infection, i.e. when the virus reached the CNS.

Late treatment of mice by i.m. route

We aimed to determine whether HPA-39 was still effective when given by i.m. route late in the infection. Twelve groups of mice were infected by

i.m. route at day 0. Two groups were used as infection controls. In nine groups, treatment with HPA-39 (150 mg/kg) was given on the same side as virus and started at the mid point of incubation period. Seven groups received 5 doses either from day 5 to day 9 or from day 6 to day 10, or from day 7 to day 11, or from day 8 to day 12, or from day 9 to day 13, or from day 10 to day 14 and finally from day 11 to day 15. Obviously, mice which died before completing treatment with the 5 doses received a lower dose, thus 4 doses those treated from day 12 to 15 or 3 doses those treated from day 13 to 15. The results are presented in Table 4. A control was also made to verify the efficacy of the choosen dose: 150 mg/kg if given 3 times starting at day 1; a significant 40% survival was obtained. After late treatment at the same dose 5 survivors were found out 55 treated mice. As the difference in survival between treated and untreated infected mice was not significant it was concluded that late treatment by i.m. route with HPA-39 was not valuable.

Late treatment of mice by i.c. route

To find out whether the compounds were effective if given by i.c. route they were administered near to the site of infected cells. Two facts incited us to use this route: in humans rabies is detected when the virus occurs in the CNS and some compounds do not pass through the blood-brain barrier. Therefore, it seemed reasonable to consider i.c. treatment at the middle of incubation period. The 14 HPA compounds were used as well as RTA, and selenazofurin which showed a slight effect by i.m. route. Three other compounds which had no effect by i.m. route were used: ribavirin, (S)-DHPA and tiazofurin. Nontoxic dose of 0.01 mg/mice was used for all compounds. As this was a very small dose ($\simeq$0.5 mg/kg) it was not possible to use a smaller one. Twenty two groups of mice were employed. Twenty one groups were infected i.m. at day 0. One group was non-infected and kept as challenge control. The treatment started on day 5 and was repeated twice at days 6 and 7. The results are presented in Table 5. Survivors were obtained in larger numbers than in the control only with HPA compounds such as HPA-23-39-46-51 and 56. The surviving mice were challenged. With HPA-39-46 and 56 some mice resisted challenge, i.e. were protected against rabies virus. Although the challenge was carried out through the i.c. route, no virus multiplication was observed in the brain, i.e. no accumulation of N protein was noticed. In another experiment, brains of treated survivors were tested. No accumulation of N protein was seen and no infectious virus was recovered. The sera of survivors had rabies NA. These data confirm that some of these compounds might be effective acting directly on infected brain cells.

Experimental treatment of foxes

In foxes the disease is usually paralytic, but in some instances (1% of rabid foxes) furious forms occurred. Development of rabies was described previously (Blancou, 1979). The first symptoms were noticed from day 12 to

Table 5. Intracerebral administration of 19 compounds in rabies infected mice

Compound 0.01 mg/mouse/day	IC Treatment da 5 to 7 Survival after treatment[a]	IC Challenge[b] Survival after challenge[c]	FAT[d]
	No. of survivors/No. treated		
HPA 23	3/10[f]	2/3	—
HPA 23	1/10	0/1	
HPA 39	4/10[e]	4/4	—
HPA 40	0/10		
HPA 44	1/10	0/1	
HPA 45	0/10		
HPA 46	6/10[e]	2/6	—
HPA 47	1/10	0/1	
HPA 51	3/10[f]	0/3	
HPA 52	1/10	0/1	
HPA 53	0/10		
HPA 56	3/10[f]	3/3	—
HPA 57	0/10		
HPA 59	1/10	0/1	
(S)-DHPA	1/10	0/1	
Ribavirin	0/10		
RTA	0/10		
Selenazofurin	1/10	0/1	
Tiazofurin	0/10		
Control of virus toxicity in treatment	0/10 1/10	0/1	
Control of virus toxicity in challenge	10/10	0/10	

[a] Calculated at day 35.
[b] Treated surviving mice were challenged at day 35.
[c] Survival was calculated 25 days after challenge., *i.e.* day 60 after rabies infection.
[d] Determined as described in Materials and Methods — = absence in brain of viral N protein.
[e] Significantly different from virus control $P < 0.05$
[f] Significantly different from virus control $P = 0.08$

day 18, and deaths from day 15 fo day 25, after inoculation of 3000 LD_{50} RV by i.m. route. As foxes are very sensitive to RV given into temporal muscle, a low dose of 40 LD_{50} i.m./fox also caused 100% mortality. At that concentration the onset of the disease was delayed.

As (S)-DHPA was reported to be effective on rabies infected mice by oral route (Sodja and Holý, 1980) this compound was assayed. Foxes were infected by the i.m. route with low and high virus doses. For each virus concentration two g roups of animals were used: one as control of virus effect and the other as the treated group. This group was treated per os the same day with the nontoxic dose of 30 mg/kg. As shown on Table 6 such treat-

Table 6. Effect of (S)-DHPA administered to rabies infected foxes

	No therapy	Therapy per os(c) Dose: 30 mg/kg
Infected foxes(d)		
40 LD_{50}		
survival(a)	0/4	0/4
death(b)	36 – 43 – 67 – 68	**40 – 78 – 85 – 90**
9000 LD_{50}		
survival(a)	0/1	0/3
death(b)	18	15 – 18 – 20
Uninfected foxes		
survival(a)	5/5	5/5

(a) No. of survivors/No. treated or not
(b) given on days post infection
(c) treatment given the same day as RV
(d) deaths confirmed by FAT: presence in the brain of viral N protein

ment delayed the time of death if the virus was given in a low dose. This result was observed in foxes; in mice, whatever the compound, such treated animals did not survive.

As HPA compounds gave the best result in mice, we used HPA-23 and HPA-39. They were toxic at lower doses given i.m. to non-infected mice. In mice HPA-23 treatment at the dose of 216 mg/kg for 5 days caused skin necrosis when the drug was each time administered near the infection site. These lesions, however, were well tolerated by mice and they disappeared within a week. In foxes even a single dose of 50 mg/kg caused severe lesions which were not well tolerated. In addition, changes in behaviour were also noticed. Therefore, the acute toxicity value was determined by a single injection of HPA-23 given 1/3 by subcutaneous (s.c.) and 2/3 by the intraperitoneal (i.p.) routes at doses of 100, 120 and 150 mg/kg. Some animals died within a few hrs to 3 days. These allowed us to determine the $LD_{50} = 138 \pm 12$ mg/kg. The same LD_{50} was observed with HPA-39. In a first series of experiments, HPA-23 was administered to rabies infected foxes at toxic doses, in order to obtain the maximal possible antiviral effect. Since HPA-23 is now synthesized by Rhône Poulenc Santé (France), thus a sufficient amount was available for such experiment. Foxes were infected by i.m. route with large doses of RV. Two groups of animals were treated, each with the doses of 120 and 150 mg/kg, respectively. In one group treatment started at the day of infection, in another at day 1 or day 2 p.i. (Table 7). Some foxes died rapidly as the result of treatment: 3/15 at 120 mg/kg (20%) and 7/10 at 150 mg/kg (70%). When HPA-23 was administered at the same time as the virus, 2 foxes survived. These 2 foxes were

Table 7. Effect of HPA administered against RV infection in foxes

	Drug toxicity (c)	Survival (d) to RV infection
3000 LD_{50}/IM		
no treatment	—	0/10 (16 – 17 – 17 – 17 – 19 – 19 – 20 – 20 – 21 – 22)(e)
HPA 23 treatment(a)		
120 mg/kg		
day 0	3/10	2/7 (21 – 24 – 26 – 28 – 31)(e)
day 2	0/5	0/5 (19 – 21 – 25 – 26 – 27)(e)
150 mg/kg day 0	4/5	0/1 (39)(e)
day 1	3/5	0/2 (26 – 30)(e)
9000 LD_{50}/IM		
no treatment		0/2 (18 – 20)(e)
HPA 39 treatment(b)		
50 mg/kg		
days 8 and 9		1/1

(a) given 1/3 by IP and 2/3 by s.c. routes
(b) given by i.m. route
(c) No. deaths immediately after treatment/No. treated
(d) No. survivors/No. treated or not treated
(e) death given in days post-infection

also protected: three months after challenge they had rabies NA. When HPA-23 was administered later after infection, the treatment delayed the time of death. In a second series of experiments HPA-39, which gave the best results in mice, was used. In order to confirm the effect of HPA-39, treatment was administered late during the incubation period: 3 days before the appearance of the first symptoms of two control animals (Table 7). The treated infected fox received two low doses (50 mg/kg) into the temporal muscle (i.m. injection) at 24 hr interval. While each single dose was given in 5 sites, severe lesions were noticed one week afterwards, including loss of one eye. Two months later the animal was still alive while the two controls were dead. The brain of the treated fox was examined. Neither accumulation of N viral protein nor the presence of infectious virus were detected. This survivor had rabies NA. This result was considered as significant because no fox ever had such antibodies, except vaccinated foxes.

Discussion

The search for antirabies compounds is still of extensive interest, as human rabies infection is detected too late and 100% fatal. In this study, therapeutic attempts have been made with 40 compounds (Table 1) on two

experimental models, mice and foxes. This report is more devoted to the results obtained in mice. Foxes have been used because in France this animal is considered for virus reservoir and virus vector.

The mice were infected by the i.m. route with a street strain of rabies in order to mimic natural exposure. This type of contamination results in the mice in paralysis and subsequent death. We focused our treatment protocol on the two main and distant sites of virus multiplication, i.e. muscle and nervous system.

To inhibit rabies multiplication at the virus penetration site, i.m. treatment started early after infection, i.e. on the day post-infection near to the site of virus inoculation. Thirty four compounds were tested. Most of them (24/34) were inactive. As most of these compounds were choosen according to their activity against viruses from different groups, this result confirms that host cell-virus interaction or molecular biology of each virus differ in various families. The inactive compounds correspond to the 7 lip-acids, including C8G (Alfalouji *et al.*, 1983), 9 different acids including oxidation products of some acids, including CHOP and KOP (Eichhorn *et al.*, 1982, 1984; Mentel *et al.*, 1983; Thiel *et al.*, 1983), didemnin-B which is a peptide-like (Reinhart *et al.*, 1981; Canonico *et al.*, 1982*a*) and pyrazofurin (Canonico *et al.*, 1982*b*). In the same group were 6 compounds which we previously tested in vitro on rabies infected cells. Tiazofurin (Kirsi *et al.*, 1983) and 3MQ (Ieven *et al.*, 1982; Van Hoof *et al.*, 1984), had no effect on the viral cycle (results not shown) in the contrary to amantadine (Douglas, 1982; Helenius *et al.*, 1982; Bektimirov et al., 1985), (S)-DHPA (De Clercq *et al.*, 1978; De Clercq, 1981) procaine and ribavirin (Sidwell *et al.*, 1972; Stephen *et al.*, 1977) which inhibited in vitro rhabdovirus multiplication (Bussereau and Genty, 1980; Bussereau and Perrin, 1982; Bussereau *et al.*, 1983*a*). It is not uncommon, however, that a drug exerts an antiviral effect in vitro but has little, if any activity against the virus in animal studies. This difference between in vitro and in vivo activities suggests that in vitro screening is not preliminary to clinical tests. The fact that (S)-DHPA had no therapeutic efficacy in the range from 50 to 150 mg/kg was not in agreement with the conclusions of Sodja and Holý (1980), who reported activity in a large dose range from 1 to 100 mg/kg. Two compounds (2/34) RTA (Stephen *et al.*, 1977; Koff *et al.*, 1983) and selenazofurin (Kirsi *et al.*, 1983) had a slight therapeutic effect. RTA, a lipophilic analogue of ribavirin was reported to act after being slowly hydrolyzed to ribavirin. Used at the same concentrations in rabies infected mice, RTA was more efficient than ribavirin itself. The second compound, selenazofurin, which is structurally and functionally related to ribavirin had no in vitro activity (results not shown) but an in vivo activity. Fourteen heteropolyanions -HPA- are structurally closely related (Michelon *et al.*, 1980) but only 8 of them were available in sufficient amount for i.m. testing. With these eight compounds (8/34): HPA-23,-32,-39,-46,-47,-52,-56 and-59, used in vivo, a dose-dependent therapeutic efficacy by the i.m. route was encountered with 100% survival in the highest dose range. The results obtained with one of them (HPA-23) are in good agreement with a previous report from one of us (Blancou *et*

al., 1982). According to this report when HPA-23 was injected by the i.m. route on the same day as the virus, survivors were found. The results presented here showed the activity of all HPA compounds (not only HPA-23) even when the drug was injected 24 hr after virus inoculation. In correlation with in vivo activity, 12 HPA compounds showed in vitro activity (Bussereau *et al.*, 1983*a*). HPA-44 and HPA-45 were not tested. In addition, the peritoneal route was also an effective one. By this route good efficacy was found with HPA-23 and HPA-39 (results not shown) but as this type of injection would not be a suitable route for human being this route was not further explored.

To inhibit rabies virus multiplication in the CNS i.c. treatment started at the middle of incubation period, i.e. five days p.i. or later. We observed in the brain of control rabies infected mice that virus multiplication started 3 days p.i. Nineteen compounds were tested. Ribavirin, RTA, selenazofurin, tiazofurin and (S)-DHPA were ineffective. Among the 14 HPA, five of them HAP-23-39-46-51 and -56 reduced the mortality. But by the i.c. route a variability in the number of survivors was observed from one experiment to another while by the i.m. route the experiments were consistently reproducible. This variability of i.c. treatment could be explained in part by variation of drug administration site. It might also be argued that direct brain inoculation may alter, at the injection site, the blood-brain barrier and allow drug leakage. Despite a relative low i.c. efficacy when treatment was started late in infection, the data by early i.m. treatment suggest that administration of HPA might be worthwhile when vaccine treatment is not possible.

The favourable effect of i.m. and i.c. treatments could be due to a rapid elimination of the infectious virus either with or without immune response. Surviving mice were tested for NA. Presence of rabies antibodies demonstrated that an immunological reaction could take place. In the case of i.m. route of the drug administration, no correlation was observed between the number of injections or the total dose injected and the antibody level. When i.c. treatment was administered the titre was 1000-fold higher as compared to i.m. treatment. This increase of the antibody response was unexpected. Whatever was the route of treatment after challenge only some mice were protected. No definit relation between immunogenic and protective potencies was found. This lack of correlation is regularly found in rabies-infected mice (Bussereau *et al.*, 1983*b*), and can be explained by the fact that in rabies cellular immune defences are also involved. In addition, viral multiplication could not be demonstrated in the brains of surviving mice, i.e. infectious virus was neither recovered from the brains nor the presence of N viral nucleoprotein was observed in the cells.

In the first set of experiments, HPA-39 was injected soon after infection at the very site of virus inoculation and was fully effective. Then in case of early treatment the drug was injected in a place distant from the multiplication site of virus inoculation (i.m., i.p. or i.c. treatments). This compound was still efficient but generally at a lower level. As already mentioned HPA-39 was also active by the i.c. route injected late during infection. As

virus was present in the brain of control mice since day 3 p.i., this result clearly indicates that HPA acts directly on infected neurons. We previously reported that HPA had no virucidal effect and in vitro inhibited molecular synthesis in infected cells (Bussereau and Ermine, 1983). As the efficient dose by the i.c. route was 400-fold less than by the i.m. route, it was concluded that the best efficacy for HPA was obtained by the i.c. route. Treatment efficacy by i.m. route decreased as the virus multiplies in the brain when given at mid-incubation period. This lack of efficacy might be due to a rapid elimination of the drug which consequently does not reach the infected area in sufficient concentration. No data concerning half life of HPA by i.m. route were reported but an estimation of less than 20 min by intravenous route in humans was published (Rosenbaum *et al.*, 1985).

The results obtained in this report encouraged us to study the effect of HPA-39-46-51-56 as well as the well known HPA-23 (Dormont *et al.*, 1982*a*, *b*; Kimberlin and Walker, 1983; Rosenbaum *et al.*, 1985) throughly to obtain 100% survival when rabies virus is already in the brain. It would be of interest to determine the effect of intravenous or/and intracerebral treatments given daily during a longer time period. In addition, an interesting question arises from the disappearance of the virus from neurons. Studies concerning these two points are planned at Institut Pasteur.

After the therapeutic results on mice comparable data were obtained on foxes. These experiments were considered as preliminary, because the number of animals per group was small. The restriction came from the number of available animals per year. HPA-23 and HPA-39 were effective as some foxes survived and were protected after challenge. This result was considered as significant not only because foxes from control group died, but because in numerous experiments no fox (0/300) ever survived to such a dose of virus. A therapeutic and pharmacological study of intravenous and/or intramuscular treatments of rabies infected foxes is planned at Malzeville.

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