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Rabies vaccines and interferon

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SUMMARY

Samples of Fermi, Semple, modified Semple, Duck embryo and tissue culture rabies vaccine were inoculated by different routes and in different doses into rabbits, mice and hamsters. The vaccines induced neither detectable interferon nor immediate protection against lethal challenge with CVS rabies virus.

Under similar conditions, high but transient levels of interferon were induced in control animals of the same species with the polynucleotide complex Poly I.C. Hamsters but not mice were protected by Poly I.C.-induced interferon.

No autointerference by vaccine with challenge virus was established. Vaccine-induced protection in mice was directly related to immune response.

INTRODUCTION

Rabies virus, like most other viruses, both induces and is sensitive to interferon. Abundant interferon appears just before death in the brains of hamsters and mice infected with fixed rabies strains and detectable amounts of interferon are found in their blood and other organs (Stewart & Sulkin, 1966; Karakuyumchan & Bektenerova, 1968; G. S. Turner, unpublished results).

Resistance to superinfection mediated by interference or interferon has been induced in cell cultures by live rabies virus (Kaplan, Wecker, Forsek & Koprowski, 1960; Wiktor, Fernandes & Koprowski, 1964; Fernandes, Wiktor & Koprowski, 1964; Selimov, Chuprikova, Kalinina & Sharova, 1965; Depoux, 1965; Yoshino, Taniguchi & Arai, 1966; Barroeta & Atanasiu, 1969). Both live and inactivated rabies virus inhibit the development of Rous sarcomas in fowls by an interferon-like mechanism (Kravchenko, Voronin & Kosmiadi, 1967; Desai, 1970).

Brief protection, mediated by interferon or 'interferon-like' mechanisms, occurs in rabbits or hamsters, challenged with rabies virus after inoculation with the viruses of vaccinia, bovine parainfluenza and Newcastle disease (Levaditi, Nicolau & Schoen, 1926; Vieuchange, 1967; Fayaz, Afshar & Bahmanyar, 1970; Atanasiu, Barroeta, Tsiang & Favre, 1970). High levels of interferon induced in rabbits by the polynucleotide complex (Poly I.C.) protected them for 24 hr. against lethal infection with rabies street virus (Fenje & Postic, 1970, 1971; Janis & Habel, 1970; Postic & Fenje, 1971).

These data add credibility to earlier speculation that similar, non-immune mechanisms might be involved in post-exposure protection by rabies vaccines, although little direct evidence supports these conjectures (Schindler, 1963; Stewart

& Sulkin, 1966; Habel, 1966*a*). In the present study five commonly used rabies vaccines were examined for their capacity to induce interferon in rabbits, hamsters or mice. Resistance to challenge with rabies virus, antibody formation and interferon induction was tested in groups of animals given vaccine by different routes and in different doses. Poly I.C., which induces abundant interferon and protects some animals against lethal challenge was used as control material.

MATERIALS AND METHODS

Vaccines

(i) Conventional Semple type rabies vaccine prepared from infected rabbit brain and inactivated with phenol was obtained from current stocks at the Lister Institute. It had a potency index > 6·0 estimated by the method of Habel (1966*b*).

(ii) Semple vaccine modified by fluorocarbon treatment (Turner & Kaplan, 1967; Kaplan & Turner, 1968) with a potency of 4·8 was also prepared at the Lister Institute.

(iii) Duck Embryo vaccine (DEV) (Eli Lilly & Co.) had a potency of 4·0.

(iv) Tissue Culture vaccine (TCV) (Rabiffa, Institute Merieux, Lyon was a veterinary product with a potency > 6·0.

(v) Fermi type vaccine (Institute of Sera and Vaccines, UFA, USSR) had a potency of > 6·0 and contained $10^{2·7}$ mouse LD 50 of residual live virus.

Viruses

The Lister Institute and WR strains of vaccinia virus were reconstituted for use from freeze-dried stocks kept at 0 to 4° C. 'Standard challenge virus' (CVS) fixed rabies virus was kept at −160° C. as a 10% suspension of infected mouse brain. Samples when thawed were used immediately and not refrozen.

Polyinosinic-polycytidylic acid

Poly I.C. solution 1 mg./ml. (Microbiological Associates, Bethesda, Md, USA) was kept at 0–4° C.

Animals

New Zealand red rabbits, golden hamsters and T.O. mice were used with initial weights of 1–2 kg., 73 g. and 11–13 g. respectively.

Interference tests

Serial tenfold dilutions of CVS rabies virus were prepared in either undiluted vaccine or in buffer. Five mice were inoculated with each dilution either intramuscularly (0·25 ml.) or intracerebrally (0·03 ml.).

Interferon induction tests

The vaccines were tested in the animals by different routes, doses and numbers of inoculations.

Rabbits. Groups of two to four rabbits were inoculated daily for 14 days, with

subcutaneous (sc) 0·2 ml. doses of Semple vaccine, diluted on a weight basis to correspond with an average human dose (2·0 ml./63 kg.). Modified Semple, DEV and TCV vaccines were administered similarly. Further groups of rabbits received undiluted vaccines subcutaneously either as 14 × 2 ml. daily doses, 6 × 2 ml. doses during 14 days or 1 × 2 ml. dose on each of days 0 and 14. All these rabbits were bled before, during and after immunization on days 0, 2, 4, 7, 10, 14 and 21. Within a group, sera from each day's bleedings were pooled and tested for rabies-neutralizing antibody and interferon.

In other experiments groups of two to four rabbits were bled and inoculated intravenously with 1·0 ml. of modified Semple, DEV or TCV vaccines or Poly I.C. (1 mg./kg.). All were bled 4 and 24 hr. later and their sera were tested for interferon.

Mice. Groups of mice were inoculated with 6 × 0·25 ml. doses of either Semple or Fermi vaccines. Inoculations were given intraperitoneally on days 0, 2, 4, 7, 9 and 11 of two successive weeks (Habel, 1966*b*). On each of these days and on the 14th day, ten mice were challenged intracerebrally with 90 LD 50 of CVS rabies. Ten unchallenged mice were killed at the same time and serum pools and pooled brain tissue extracts (10 %, w/v) were tested for interferon and antibody.

Maximum serum interferon titres are found in mice 2–4 hr. after inoculation with NDV or Poly I.C. (Atanasiu *et al.* 1970; Buckler, du Buy, Johnson & Baron, 1971). Suitable numbers of mice were inoculated with either Semple or tissue culture vaccine. Control mice were inoculated with similar amounts of normal rabbit brain suspension, tissue culture fluid, buffer, or with Poly I.C. (10 μg./g. i.p.). Two hours later mice from each series were inoculated with serial dilutions of CVS rabies, either intramuscularly (0·25 ml.) or intracerebrally (0·02 ml.); five mice were used per dilution. Five unchallenged mice from each group were killed before, then 2 and 24 hr. after vaccine or Poly I.C. treatment; their pooled sera and 10 % brain extracts were tested for interferon.

Poly I.C. (30 μg. in 0·03 ml.) was inoculated intracerebrally into mice in attempts to induce more interferon *in situ*. Groups of these animals were tested for resistance to rabies challenge and for serum and brain interferon titres as described above.

Hamsters. Suitable numbers of this species were inoculated intraperitoneally with undiluted Semple or TCV vaccines (0·2 ml.) or with Poly I.C. (1 mg./kg.). Three doses were given, the first 24 hr. before, the second coincident with, and the final one 24 hr. after challenge. Interferon was estimated in serum or brain extracts of unchallenged animals killed 4 hr. after each dose. Hamsters are highly susceptible to CVS inoculated intramuscularly (Atanasiu *et al.* 1970), and groups of ten treated and ten control animals were challenged by this route with 0·5 ml. CVS calculated to contain 5–50 LD 50.

Interferon assays

Serum or tissue extracts from mice or hamsters were tested for interferon (IF) by applying suitable dilutions to cell cultures prepared from embryos of the respective species (Gifford, 1963). Samples of rabbit origin were tested in the rabbit kidney cell line (RK 13) (Field, Tytell, Lampson & Hilleman, 1967).

Monolayer cell cultures were grown in plastic dishes in Eagle's minimum essential medium (MEM) containing 5% foetal bovine serum and 1% glutamine for RK 13 cells and 10% calf serum for mouse or hamster embryo cells.

Since longer incubation times did not significantly increase interferon titres in this system, six cultures were incubated for 5 hr. at 37° with each dilution of each sample (Subrahmanyan & Mims, 1966). Test material was removed and replaced by maintenance medium (Eagle's MEM + 1% calf serum) and treated and control cells were infected with 50–100 plaque forming units (p.f.u.) of vaccinia virus. Cultures were incubated for 40–48 hr. at 37° C. without agar overlay in 5% CO_2 in air; the monolayers were then stained and plaques were counted.

Interferon titres are expressed as the reciprocal of the dilution reducing vaccinia plaque production by 50% (Wagner, 1961). The method was controlled with internal reference material prepared from the sera of animals treated with Poly I.C.; the sensitivity and reproducibility of rabbit interferon assays was also verified with an international reference preparation (Research Reference Reagents Branch NIH, Bethesda, Md), and that of the mouse assays with material kindly supplied by Dr C. Bradish (Microbiological Research Establishment, Porton). Both standards contained a nominal 1000 international units of interferon. Material reacting positively was identified as interferon by its stability at pH 2·0, resistance to heat (65°) and to nuclease treatment. Further criteria were species but not virus specificity and susceptibility to tryptic digestion (Wagner, Levy & Smith, 1968).

Antibody assays

Rabies antibody was estimated by serum neutralization tests in mice by the method of Atanasiu (1966).

RESULTS

Interferon induction

In rabbits

Rabies vaccines inoculated subcutaneously produced rabies-neutralizing antibody, but no circulating interferon was detected in more than 100 serum samples taken at different times during the several immunization series. No detectable interferon was induced by the rabies vaccines administered intravenously although control animals receiving intravenous Poly I.C. (1 mg./kg.) always responded with serum interferon titres that exceeded 10^3 after 2–4 hr. and declined during the next 24 hr. (Table 1). Our sample of CVS rabies did not regularly kill rabbits by intramuscular injection, and challenge 2 hr. after intravenous administration of vaccine or Poly I.C. was unsatisfactory. In most instances however the mortality in vaccine-treated rabbits exceeded that in controls.

In mice

No interferon was detectable in the sera or brain extracts of mice inoculated intraperitoneally 2 or 24 hr. previously with rabies vaccines. Titres of serum interferon exceeding 10^3 were induced in control mice 2 hr. after inoculation with suit-

Table 1. *Serum interferon in rabbits injected intravenously with rabies vaccine or Poly I.C.* (1·0 *ml.*)

Vaccine	Interferon titres after 0 hr.	2 hr.	24 hr.
Semple*	—	—	—
Areton treated Semple	< 5	< 5	< 5
Duck embryo	< 5	< 5	< 5
Tissue culture	< 5	< 5	< 5
Poly I.C. (1 mg./ml.)	< 5	1500	15

* Rabbits died within a few minutes of injection even when the dose was reduced four-fold.

able doses of Poly I.C.; much smaller amounts were present in their brains. Treated and control mice did not differ significantly in their susceptibility either to intracerebral or to intramuscular challenge with rabies virus. Median lethal end points determined by titration of challenge virus in both groups were similar, indicating that mice were unprotected by interferon even against minimal challenge doses. Intracerebral inoculation of Poly I.C. induced slightly higher and more persistent titres of brain interferon, but very little circulating interferon (Cathala & Baron, 1970). Again no animals resisted rabies challenge administered by either route.

Serum and brain extracts taken from mice during a six-dose course of Semple vaccine contained no detectable interferon; protection by vaccine appeared to be related entirely to circulating antibody which appeared in increasing amounts after the 4th day after inoculation. Antibody levels in the brain tissue of immunized mice were minimal (Fig. 1). Similar results were obtained when the experiments were repeated with Fermi vaccine containing $10^{2\cdot7}$ LD 50 of residual live virus.

In hamsters

The rabies vaccines tested in hamsters neither induced interferon nor conferred short term protection. Poly I.C. induced substantial amounts of circulating interferon in hamsters but interferon titres in brain extracts were much lower. In both sites, however, interferon increased after the second and third dose of Poly I.C. Fifty per cent of the animals treated with Poly I.C. survived a challenge which killed 90 % of the controls (Table 2).

Auto-interference

When CVS rabies was diluted in either vaccine or buffer and titrated in mice, similar median lethal end-points were obtained. The rabies vaccines did not demonstrably inhibit the replication of homologous live virus in mice whether intracerebral or intramuscular routes of inoculation were used, suggesting that direct interference is an unlikely mode of action for protection by vaccine (Koprowski, Black & Nelsen, 1954; Mitchell, Everest & Anderson, 1971).

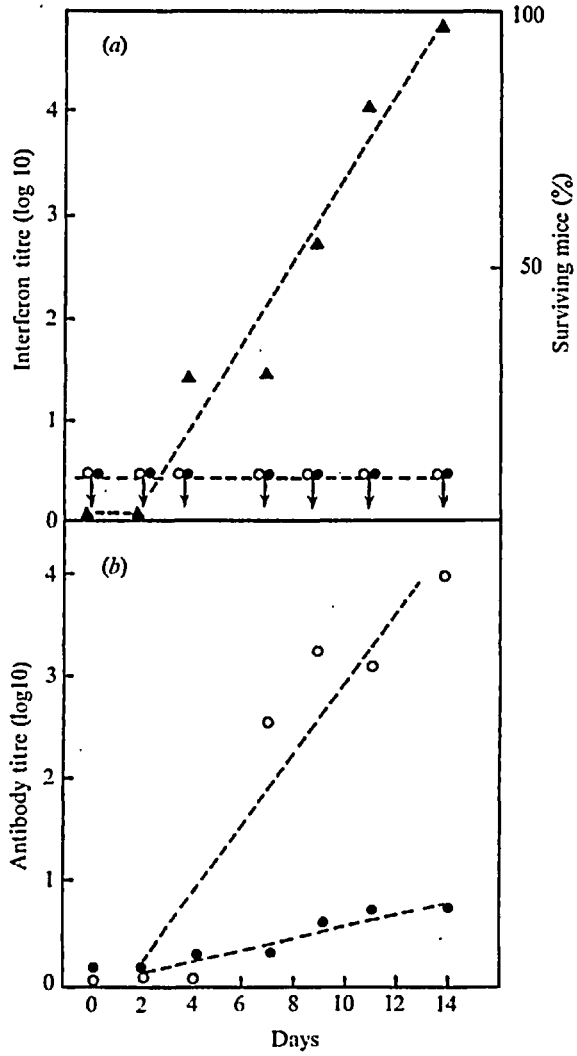

Fig. 1. Protection, antibody response and interferon in mice immunized with rabies vaccine. Groups of 20 mice were inoculated with 0·25 ml. of Semple vaccine diluted 1/10 on days 0, 2, 4, 7, 9 and 11; 10 mice challenged intracerebrally with 90 LD 50 CVS and 10 sampled for interferon or antibody on days 0, 2, 4, 7, 9, 11, 14. (*a*) Surviving mice ▲- - ▲; interferon in brains ●- -●; interferon in serum ○- -○. (*b*) Neutralizing antibody in brains ●- -●; neutralizing antibody in serum ○- -○.

DISCUSSION

The rabies vaccines were tested under conditions and in animals in which interferon is readily induced by Poly I.C. None of the vaccines tested, however, induced either detectable interferon or immediate protection in any of the animals. The virus content of several of the vaccines was similar to that of Newcastle disease virus used for interferon induction by Atanasiu *et al.* (1970). The vaccines were completely or partially inactivated by phenol, β-propiolactone or UV irradiation; the latter method at least is compatible with the retention of interferon-inducing properties. Although other live or killed viruses induce interferon in animals,

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Table 2. *Effect of rabies vaccines and Poly I.C. on interferon induction and protection against rabies in hamsters*

Vaccine or inducer*	Interferon† in Serum			Brain			Dead/ challenged
Semple	< 5	< 5	< 5	< 5	< 5	< 5	8/10
Tissue culture	< 5	< 5	< 5	< 5	< 5	< 5	10/10
Poly I.C.	60	87	360	< 5	17	22	5/10
Control	—	< 5	—	—	< 5	—	9/10

* Vaccine or inducer (0·2 ml. ip) given 24 hr. before, at the same time as and 24 hr. after challenge.

† Interferon titres in the sera and brain extracts of pairs of unchallenged hamsters 4 hr. after each dose.

detectable interferon induction by rabies apparently occurs only when large amounts of infective virus are present and is usually highest in the brain just before death (Matsumoto, 1970; Stewart & Sulkin, 1966; Karakuyumchan & Bektenerova, 1968). The live virus in Fermi vaccine apparently neither replicates sufficiently after peripheral inoculation nor is intrinsically enough to induce interferon.

Mice have been protected against several other viral encephalitides by interferon (Field *et al.* 1967; Baron, Buckler, Friedman & McCluskey, 1966; Finter, 1966; Haahr, 1971). Despite the presence of abundant circulating interferon induced by different methods most workers have failed to significantly protect mice against rabies (Baron & Habel, 1967; Atanasiu *et al.* 1970; Soave, 1968; Finter, 1967; Fayaz, Afshar & Bahmanyar, 1970; Hilleman, 1970). Mice are good indicators of the immune response to rabies vaccines but why they are unsatisfactory for testing interferon-mediated protection against rabies is obscure. Little interferon penetrates the central nervous system of mice (Subrahmanyan & Mims, 1966; Finter, 1967). The present results confirm that peak titres of interferon induced in mouse brain are less than 2% of those in their serum, a value only slightly improved by injecting the inducer intracerebrally.

Hamsters, on the contrary, were significantly protected by Poly I.C., despite low titres of brain interferon. Fenje & Postic (1970) also showed that a single dose of Poly I.C. protected rabbits against street virus infection for up to 24 hr., by which time interferon titres in this species have also declined to low values (Cathala & Baron, 1970), (Table 1). The time-limited vulnerability of rabies virus to interferon in some species and the poor penetration of interferon into the central nervous system perhaps indicates that its activity against rabies is exerted in some extraneural cell site. These findings suggest that current concepts of rabies pathogenesis may need reappraisal (Johnson, 1971).

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