CHARACTERISTICS OF A RABIES VIRUS STRAIN ISOLATED FROM THE BRAIN OF *DESMODUS ROTUNDUS*[1]

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This article describes certain immunogenic differences between a rabies virus strain (DR19) isolated from Desmodus rotundus *and the challenge virus standard (CVS), as revealed by serum-neutralization and cross-protection tests A description is also given of the virulence of DR19 in guinea pigs, mice, hamsters, and cattle when administered via different routes of inoculation. The use of strain DR19 is recommended to test the immunity conferred by rabies vaccines employed to protect cattle.*

Introduction

The WHO Expert Committee on Rabies recommends that "No vaccine should be approved for use in the field unless an adequately designed experiment demonstrates a duration of immunity of at least one year in the species of animal for which the vaccine is to be used" (*1*).

In Canada, where bovine rabies is transmitted by fox bite, a rabid fox salivary gland suspension has been used to evaluate the protection which a given vaccine confers on cattle (*2*). However, from Mexico to northern Argentina bovine rabies is transmitted principally by the bite of the common vampire bat, *Desmodus rotundus* (*3*). In order to evaluate the protection provided by rabies vaccines for cattle in these regions, virus isolated directly from such bats should be used for the challenge. Nonetheless, it is very difficult to fulfill this requirement; it would be almost impossible to obtain enough infected bats to prepare an adequate quantity of sufficiently virulent material for the challenge.

The Pan American Zoonoses Center has made a special effort within its research program to evaluate the immunogenicity of different rabies vaccines for cattle (*4, 5*). For this purpose a rabies virus strain (DR19) isolated from *D. rotundus* in Brazil[3] was used to challenge vaccinated animals. This strain has been maintained through a limited number of mouse passages in order to conserve its original characteristics as much as possible. This paper is concerned with the immunogenicity and virulence of strain DR19 for different animal species.

Materials and Methods

Animals

Three- to four-week-old white mice and hamsters, 300-400 guinea pigs, and cattle between 1 to 4 years old were used in the tests. The cattle were either bred at the Center's Farm Annex or purchased locally. The Annex is located in an area considered free from urban and wildlife rabies.

Rabies Virus Strains

From the time strain DR19 was first isolated from the brain of a *D. rotundus*, it had

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undergone 18 intracerebral (IC) passages in mice. The challenge virus strain (CVS) was obtained through the World Health Organization and used as a standard strain after 27 mouse brain passages. Also, the virus strain Apipé-1 was used after one mouse brain passage following its isolation from a *D. rotundus* captured in 1970 on Apipé Island in the Province of Corrientes, Argentina.

Each of these strains was inoculated intracerebrally into different groups of 100 to 200 three- to four-week-old mice. Brains taken from those animals which showed evidence of prostration were used to prepare a 20 per cent suspension (weight/volume) of brain material, which was centrifuged at 1,000 rpm for 10 minutes. One ml portions of the resulting supernatant material were put into ampoules and stored at −70°C. This became the "working lot." Another portion of the supernatant material was placed in ampoules and lyophilized. This became the "seed lot."

Virulence

Strain DR19 was titrated by IC inoculation in mice and by intramuscular (IM) inoculation in both mice and guinea pigs. For mice, the IC dose was 0.03 ml and the IM dose 0.1 ml; for guinea pigs, the IM dose was 0.3 ml. Cattle were inoculated with 2 ml of various dilutions of the suspension in the masseter muscle. Efforts were made to isolate virus from the saliva of those animals which became ill after inoculation. Seller's (*6*) and fluorescent antibody (*7*) techniques for diagnosing rabies were used to verify the cause of death.

Immune Sera

Equal volumes of sera were prepared as follows:

ERA-I: Sera from 16 cattle bled 30 days after immunization with primary pig kidney tissue culture ERA vaccine (*8*).

ERA-II: Sera from eight cattle immunized with ERA vaccine plus a booster 30 days after the initial vaccination. These animals were bled 8 days after they received the booster.

SBM-I: Sera from 10 cattle immunized with suckling mouse brain (SMB) vaccine adsorbed on aluminum hydroxide (*9*) and bled 30 days after vaccination.

SMB-II: Sera from the same 10 cattle (SMB-I) bled 200 days after vaccination.

HEP-I: Sera from 10 cattle immunized with HEP-Flury vaccine (*10*) and bled 30 days after vaccination.

HEP-II: Sera from the same cattle (HEP-I) bled 200 days after vaccination.

Serum-Virus Neutralization

These serum pools were tested against strains DR19 and CVS by the serum-neutralization (SN) test (*11*).

Vaccines

The following vaccines were used in the study: SMB-CVS, which is a SMB-type vaccine (*12*) produced with CVS; SMB-DR19, a SMB-type vaccine produced with DR19; and Ref-CPZ-1, which is a reference vaccine prepared at the Center. Using the NIH potency test (*13*), the antigenic value of the Ref-CPZ-1 vaccine was found equivalent to 0.66 of that of the reference NIH vaccine (lot 173).

The potency of the other two vaccines was similarly determined, using Ref-CPZ-1 as the reference vaccine and challenging immunized mice with CVS and DR19. The 50 per cent effective dose (ED_{50}) was found by determining the dilution at which a vaccine protected 50 per cent of the vaccinated mice, as has been previously described (*14*).

Results

Table 1 shows the titers obtained with strains CVS, DR19, and Apipé-1 when inoculated IC in adult mice and IM in guinea pigs. For each strain it was observed that the IM route was less effective in producing infection in mice than the IC route. The difference between titers found for these two routes varied with the virus strain used, falling within the range of log 2.5-log 4.0. Strains DR19 and

TABLE 1–Comparison of titers obtained with three strains of rabies virus in laboratory animals.

Strain	Adult mice		Guinea pigs
	IC, LD_{50}/0.03 ml	IM, LD_{50}/0.1 ml	IM, LD_{50}/0.3 ml
CVS	$10^{7.0}$	$10^{3.0}$	$10^{1.7}$
DR19	$10^{6.4}$	$10^{3.4}$	$10^{4.9}$
Apipé-1	$10^{4.5}$	$10^{2.0}$	$10^{2.8}$

Apipé-1 were more effective than CVS when inoculated IM in guinea pigs.

Table 2 shows the incubation periods observed in mice, guinea pigs, and hamsters inoculated with DR19 by the IC and IM routes.

Brain smear impressions taken from mice that became prostrate or died after inoculation with DR19 showed few Negri bodies after treatment with Seller's stain. Those observed were of reduced size with little variation in shape. On the other hand, considerable amounts of antigen were detected with the fluorescent antibody technique.

TABLE 2–Incubation periods of DR19 virus in laboratory animals.

Species	IC (days)	IM (days)
Adult mice	6-8	7-9
Guinea pigs	not tested	10-18
Hamsters	7	9

Table 3 shows the results obtained after IM inoculation of 33 cattle with DR19. Twenty-five of the animals (76 per cent) died of rabies. Twenty-three of these had incubation periods varying between 10 and 32 days. In two cases illness was observed after 120 days. The most effective challenge virus dilutions were $10^{-1.3}$ and $10^{-1.7}$. Cattle of all age groups showed a mortality of 75-100 per cent, except in one trial where the mortality was 40 per cent. The challenge virus dilution used in this trial was $10^{-2.0}$.

The presence of DR19 in saliva could only be proven in two of four inoculated hamsters (Table 4). It was not possible to isolate virus from the saliva of guinea pigs, nor from cattle which had died of rabies.

The extent to which DR19 and CVS were neutralized by sera from cattle immunized with the various vaccines is shown in Table 5. This table compares the titers obtained when sera were mixed with each of the two strains. It shows that the neutralizing capacity of the sera was consistently lower with DR19 than with CVS. (One serum which had a low titer with CVS was negative with DR19.)

Table 6 shows the cross-protection results indicated by the NIH test, using SMB-type vaccines prepared with DR19 and CVS. Vaccine made with DR19 had an antigenic value 15

TABLE 3–Mortality produced in cattle by a 2 ml dose of DR19 virus injected into the masseter muscle.

Age of cattle (years)	Dilution used	Deaths/all cattle innoculated	Mortality (%)	Incubation period (days)
1	$10^{-1.7}$	5/5	100	14-30
1	10^{-2}	4/5	80	15-160
1	10^{-2}	2/5	40	20-26
2-4	$10^{-1.3}$	6/8	75	16-32
2-4	10^{-2}	3/4	75	16-123
2-3	$10^{-1.3}$	5/6	83	10-25

TABLE 4–Elimination of virus via the saliva of animals infected with DR19.

Species	Number of animals infected	Number of animals with virus in their saliva
Guinea pigs	10	0
Cattle	13	0
Hamsters	4	2

TABLE 5–Comparison of seroneutralization titers* of sera from cattle immunized against CVS and DR19 with a variety of vaccines.

Sera†	Strain	
	CVS (29 DL_{50})	DR19 (31 DL_{50})
ERA I	45	10
ERA II	840	41
SMB I	211	29
SMB II	31	18
HEP I	75	8
HEP II	4	2

*Expressed as the reciprocal of the serum dilution that protected 50 per cent of the mice vaccinated.
†See text, p. 77.

times lower than vaccine prepared with CVS when both were challenged with the latter virus. When DR19 was used as the challenge virus, both vaccines showed similar antigenic values. All vaccines, including Ref-CPZ-1, offered better protection when challenged with DR19 than with CVS. The challenge LD_{50} used in the case of each strain was almost the same, 79 for CVS and 74 for DR19.

Discussion

It appears that the rabies virus strain DR19 retains some original characteristics of a street virus in spite of repeated mouse passages. A major difference between this strain and CVS fixed virus lies in the marked ability of DR19 to infect laboratory animals by the IM route; in this respect it is quite comparable to the recently isolated Apipé-1 strain. For CVS to infect guinea pigs by the IM route, $10^{5.3}$ mouse $ICLD_{50}$ were necessary, while only $10^{1.5}$ mouse $ICLD_{50}$ of DR19 and $10^{1.7}$ mouse $ICLD_{50}$ of Apipé-1 were sufficient to infect guinea pigs by this route.

Isolation of the virus from the saliva of animals inoculated with DR19 could be further evidence that this strain retains street virus characteristics. Excretion of virus by the salivary route was seen in hamsters; according to Reagan *et al.* (*15*), this species is more susceptible to rabies virus than are mice.

Attention is drawn to the results of the seroneutralization (SN) test using sera of cattle immunized with different rabies vaccines (ERA, HEP, and SMB). The titers obtained with DR19 were lower than those obtained with CVS in spite of the fact that the mouse LD_{50} used were almost the same in each case (31 for DR19 and 29 for CVS). Although DR19 anti-serum was not included in the SN test, it was evident that sera of cattle immunized with different vaccines prepared with strains originally isolated from dogs were more effective in neutralizing CVS than DR19.

TABLE 6–Potency of SMB-type vaccine prepared with CVS or DR19, challenged with CVS or DR19 virus in the NIH test.

Vaccine	Challenge virus strain used in the NIH test			
	CVS (79 LD_{50})		DR19 (74 LD_{50})	
	ED_{50}*	AV†	ED_{50}*	AV†
SMB-CVS, 2.5 %	200	22	>625	5
SMB-DR19, 5 %	14	1.5	>625	5
Ref-CPZ-1, 10 %	9	1	>125	1

*Median effective dose, expressed as the reciprocal of the vaccine dilution that protected 50 % of the mice vaccinated.
†Antigenic value.

The results of NIH potency tests (Table 6) showed that less vaccine was needed to protect 50 per cent of the vaccinated mice (ED_{50}) against DR19 than against CVS. This may be explained as follows: the challenge virus acts as a booster for previously immunized animals and thus produces a secondary response. Although this response may begin three days after the booster is given, it does not reach its peak until at least eight days later. The CVS strain, which has a short incubation period, would produce illness before a full secondary response is obtained. On the other hand, the incubation period of DR19 would be ending at about the time that the secondary response is reaching its peak. As a result, the vaccines would afford better protection against DR19 than against CVS.

Gallia (*16*) obtained results somewhat similar to ours in SN and cross-protection tests, even though his techniques were different. Gallia used a constant dilution of sera and vaccine against varying dilutions of challenge virus. In our study the challenge virus was constant and varying dilutions of sera and vaccine were used. The Bolívar strain used by Gallia was similar to DR19 in that both came from a sylvatic rabies cycle in vampire bats. Likewise, the Pasteur virus used by Gallia and the CVS strain used by us came from a canine rabies cycle. All four strains show the same characteristics (identifying them as rabies virus) when tested with hyperimmune serum. There are, however, notable differences in the pathogenicity of these strains; this paper presents data on some immunological differences between DR19 and CVS.

Since vaccines for cattle in Latin America are produced specifically to protect cattle against rabies transmitted by vampire bats, it is apparent that DR19 or some similar strain would be more appropriate for challenging the immunity of vaccinated cattle than strains isolated from dogs and foxes. This concept may be further extended to include the use of DR19 or similar strains for laboratory potency tests of rabies vaccines for cattle. This Center is already using DR19 for this purpose and suggests that it would be useful for other laboratories to carry out similar procedures.

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SUMMARY

A strain of rabies virus (DR19) isolated from the brain of a vampire bat (*D. rotundus*) and maintained through a limited number of mouse passages has retained some of the properties of a street virus. These include excretion of the virus in hamster saliva; greater infectivity than CVS for laboratory animals inoculated by the IM route; and a consistently high degree of infectivity for cattle inoculated by the IM route.

On the other hand, the seroneutralization and cross-protection tests have shown some differences between DR19 and CVS. The paper points out advantages of using a strain taken from vampire bats in testing the potency of rabies vaccines to be used for protecting cattle in Latin America.

REFERENCES

(*1*) World Health Organization. *WHO Expert Committee on Rabies, Fifth Report.* Technical Report Series No. 321, Geneva, 1966.

(*2*) Abelseth, M. K. "Vacunas antirrábicas producidas en cultivo de tejidos." In *Primer Seminario Internacional sobre Rabia para las Amé-*

ricas. Pan American Zoonoses Center, Buenos Aires, Argentina. *Scientific Publication PAHO 169,* pp. 286-294, 1969.

(*3*) Acha, P. N. "Epidemiología de la rabia bovina paralítica y de la rabia del murciélago." In *Primer Seminario Internacional sobre Rabia para las Américas.* Pan American Zoonoses Center, Buenos Aires, Argentina. *Scientific Publication PAHO 169,* pp. 103-132, 1969.

(*4*) Atanasiu, P. *et al.* "Etudes sur L'immunité antirabique des bovins vaccines. I. Comparison des niveaux d'anticorps antirabiques neutralisents obtenus sur les bovins à l'aide de divers vaccins, au cours d'une année." *Ann Inst Pasteur (Paris) 114:* 339-348, 1968.

(*5*) Fuenzalida, E. *et al.* "Rabies Immunity in Vaccinated Cattle." In *Proc Ann Meet USAHA 73,* October 1969.

(*6*) Sellers, T. F. "A New Method for Staining Negri Bodies of Rabies." *Am J Public Health 17:* 1080-1081, 1927.

(*7*) Goldwasser, R. A. and R. E. Kissling. "Fluorescent Antibody Staining of Street and Fixed Rabies Virus Antigens." *Proc Soc Exp Biol Med 98:* 219-223, 1958.

(*8*) Abelseth, M. K. "An Attenuated Rabies Vaccine for Domestic Animals Produced in Tissue Culture." *Canad Vet J 5:* 279-286, 1964.

(*9*) Fuenzalida, E. and G. F. Fábrega. "Vaccin antirabique pour bovins." *Bull Office Int Epizoot 67:* 443-449, 1967.

(*10*) Koprowski, H., J. Black, and D. J. Nelson. "Studies on Chick-Embryo-Adapted Rabies Virus. VI. Further Changes in Pathogenic Properties Following Prolonged Cultivation in Developing Chick Embryo." *J Immun 72:* 94-106, 1954.

(*11*) Atanasiu, P. *et al.* "Rabies Neutralizing Antibody Response to Different Schedules of Serum and Vaccine Inoculations in Non-Exposed Persons." *Bull WHO 14:* 593-611, 1956.

(*12*) Fuenzalida, E. and R. Palacios. "Un método mejorado en la preparación de la vacuna antirrábica." *Boletín del Instituto Bacteriológico de Chile 8:*3-10, 1955.

(*13*) Seligmann, E. B. "Potency-Test Requirements of the United States National Institutes of Health (NIH)." In *Laboratory Techniques in Rabies, 2nd ed.* World Health Organization, Monograph Series No. 23, pp. 145-151, Geneva, 1966.

(*14*) Sikes, R. K. and O. P. Larghi. "Purified Rabies Vaccine: Development and Comparison of Potency and Safety with Two Human Rabies Vaccines." *J Immun 99:* 545-553, 1967.

(*15*) Reagan, R. L., W. C. Day, and A. L. Brueckner. "Rabies Street Virus Strains in Syrian Hamster and in Swiss Albino Mouse." *Proc Soc Exp Biol 81:* 654-656, 1952.

(*16*) Gallia, F. "Diferencias inmunológicas entre el virus fijo Pasteur y los virus de rabia paralítica venezolana." *Boletín del Instituto de Investigaciones Veterinarias (Maracay, Venezuela) 3:* 371-390, 1956.