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Neurovirulence of yellow fever 17DD vaccine virus to rhesus monkeys

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Abstract

The yellow fever 17D virus is attenuated and used for human vaccination. Two of its substrains, 17D-204 and 17DD, are used for vaccine production. One of the remarkable properties of this vaccine is limited viral replication in the host but with significant dissemination of the viral mass, yielding a robust and long-lived neutralizing antibody response. The vaccine has excellent records of efficacy and safety and is cheap, used as a single dose, and there are well-established production methodology and quality control procedures which include the monkey neurovirulence test (MNTV). The present study aims at a better understanding of YF 17DD virus attenuation and immunogenicity in the MNVT with special emphasis on viremia, seroconversion, clinical and histological lesions scores, and their intrinsic variability across the tests. Several MNVTs were performed using the secondary seed lot virus 17DD 102/84 totaling 49 rhesus monkeys. Viremia was never higher than the accepted limits established in international requirements, and high levels of neutralizing antibodies were observed in all animals. None of the animals showed visceral lesions. We found that the clinical scores for the same virus varied widely across the tests. There was a direct correlation between the clinical scores in animals with clinical signs of encephalitis and a higher degree of central nervous system (CNS) histological lesions, with an increase of lesions in areas of the CNS such as the *substantia nigra*, *nucleus caudatus*, *intumescentia cervicalis*, and *intumescentia ventralis*. The histological scores were shown to be less prone to individual variations and had a more homogeneous value distribution among the tests. Since 17DD 102/84 seed virus has been used for human vaccine production and immunization for 16 years with the vaccine being safe and efficacious, it demonstrates that the observed variations across the MNVTs do not influence its effect on humans.

Keywords: Yellow fever vaccine; 17DD vaccine virus; Monkey neurovirulence test

Introduction

The YF 17D virus, including the two main substrains in use nowadays, 17D-204 and 17DD, is one of the most successful vaccines developed to date. There is a well-defined and efficient production methodology and strict quality control including monkey neurovirulence test (MNTV); it induces long-lasting immunity, is cheap, and is used as a single dose. Since 1945, the use of YF 17D virus has been estimated to be over 200 million doses with an excellent record of safety. Only 21 cases of postvaccinal encephalitis have been recorded with incidence predominantly in very young infants (WHO, 1993; Monath, 1999). More recently, cases of severe adverse events associated with the 17D vaccine have been reported (Chan et al., 2001; Martin et al., 2001; Vasconcelos et al., 2001). Earlier, Jennings et al. (1994) found that virus recovered from one fatal case contained two amino acid changes in the envelope glycoprotein gene and had an increased neurovirulence in animals. However, virus isolates from two other fatal cases demonstrated genetic stability and attenuated phenotype (Galler et al., 2001), suggesting that some peculiarities of the health status of a host could be responsible for such rare events.

The MNVT is used for control of attenuation of YF vaccine viruses (Levenbook et al., 1987; WHO, 1998). Any

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Table 1
Viremia mean peak titers in monkeys inoculated with YF 17DD 102/84 virus

Test[a]	Mean peak titers $\log_{10}$ PFU/mL (SD)	Range
B	1.66(0.48)	1.22–2.51
C	1.93(0.40)	1.40–2.48
D	2.11(0.24)	1.70–2.51
E	2.14(0.32)	1.70–2.70
F	1.30(0.40)	0.90–1.50
For comparison:		
YF 17D-204[b]	2.63(0.40)	1.80–3.00
YF 17D-JE[c]	2.10(0.20)	1.30–2.30
YF 17D-den-2[d]	1.34(0.40)	0.70–1.50

[a] There was no detectable viremia in test A.
[b] YF 17D-204 (Monath et al., 2000).
[c] YF 17D-chimeric japanese encephalitis (Monath et al., 2000).
[d] YF 17D-chimeric dengue type 2 (Guirakhoo et al., 2000).

new virus seed or new vaccine(s) lots must be tested for monkey neurovirulence before its administration to humans. In this MNVT a group of monkeys is required to be used with a well-known vaccine virus that will serve as the upper limits for the parameters observed, such as viremia, seroconversion, type, duration, and magnitude of clinical signs and the histological scores derived from several areas of the central nervous system (CNS).

The history of YF 17DD virus in Brazil has been well documented (Fox and Penna, 1943; Post et al., 2001). This strain was brought in Brazil as vaccine NY41, which corresponded to subculture 230 of the 17DD substrain. Seed lot 17DD was received from a low passage of this substrain, 17DD EPlow, and represents one of its subcultures, used for vaccine production (secondary seed lot). The 17DD EPlow virus remained as the main substrain for vaccine production given its attenuation for humans (Fox et al., 1942) and nonhuman primates (Fox and Penna, 1943) as well as its immunogenicity (Fox and Cabral, 1943; Fox et al., 1948).

We have used the YF 17DD 102/84 virus as reference in several MNVTs to examine the attenuation of experimental YF vaccine viruses. Here we describe the neurovirulence of the YF 17DD 102/84 virus for rhesus monkeys observed in these tests.

Results

Behavior of YF 17DD 102/84 in the neurovirulence test in monkeys

Viremia

Table 1 shows that mean viremia values were in the range of 1.30 to 2.14 $\log_{10}$ PFU/mL with minimal and maximal individual titers of 0.90 to 2.70 $\log_{10}$ plaque-forming units (PFU)/mL. In test F, three animals were inoculated and bled for nine consecutive days postinoculation (p.i.) for viremia analysis: peak viremia occurred on the fourth and fifth days p.i. Only 2 of 49 monkeys displayed viremia on the sixth day but 25 showed viremia on the second and fourth days p.i. In general, the viremic period was between days 2 and 5 p.i., with an average peak titer of 1.30 $\log_{10}$ PFU/mL.

The definition put forward by WHO (1998) of viscerotropism of 17D virus limits the amount of circulating virus to below 500 mouse LD_{50}/0.03 mL for all (10 of 10) sera and $\geq$100 LD_{50}/0.03 mL in 1 of 10 monkey sera at 1:10 dilution. In this context, the highest mean peak titer observed in all tests (B–F) was 2.14 $\log_{10}$ PFU/mL that corresponds to 1.12 LD_{50}/0.03 mL, consequently well below the established limits. For comparison, this range of titers is similar to those observed for YF 17D-204 virus or recombinants thereof expressing the prM/E genes of Japanese encephalitis or dengue type viruses (Monath et al., 2000, 2002; Guirakhoo et al., 2000, 2002).

The neutralizing antibody titer showed a statistically significant linear correlation with the amount of circulating virus (0.76; $P = 0.004$). Table 2 shows the extremely variable antibody titers measured for 12 monkeys with peak

Table 2
Correlation of viremia, neutralizing antibody response, clinical and histological scores in monkeys inoculated with YF 17DD 102/84 virus

Animals	Titer ($\geq$2.30 $\log_{10}$ PFU/mL)	Neutralizing antibody titers	Clinical score	Combined histological score
01	2.51	1280	0.00	0.97
Q1	2.44	37153	0.57	0.82
Q46	2.48	38019	0.34	1.33
R17	2.30	12589	0.27	0.61
R63	2.40	49564	0.37	1.47
R65	2.48	51501	0.20	0.88
S12	2.40	93415	0.70	1.81
S17	2.51	64565	0.27	1.34
S27	2.70	218408	0.10	0.53
S44	2.35	35930	0.17	1.22
S57	2.36	13400	0.00	0.42
S61	2.35	239	0.00	1.09

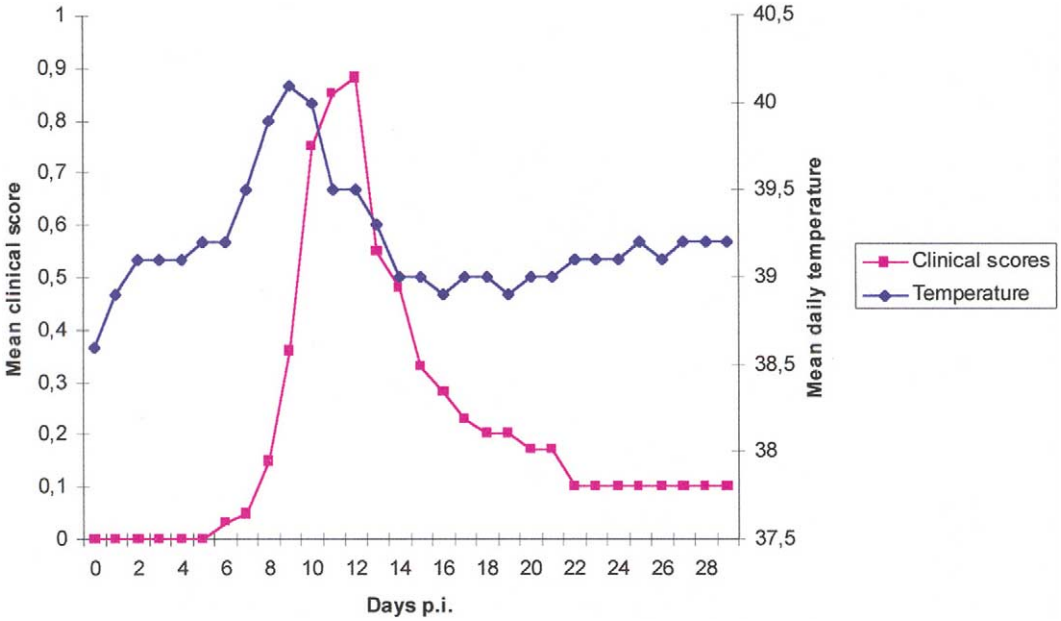

Fig. 1. Febrile reactions (°C) and mean daily clinical scores in monkeys inoculated with YF 17DD 102/84 virus.

viremia titers equal or above 2.30 $\log_{10}$ PFU/mL, suggesting that even with limited viral replication (as evidenced by circulating virus) there is enough viral antigen for eliciting an effective neutralizing antibody response. It is noteworthy that cynomolgus monkeys inoculated intracerebrally (i.c.) had measurable neutralizing antibody titers 6 years after receiving the 17D virus (M.Simões, A. Travassos da Rosa, and R. Galler, unpublished data). In the case of humans inoculated by the subcutaneous route, as recommended by WHO (1998), the viremia titers are limited and nondetectable in the majority of the vaccinees (Camacho et al., unpublished data). However the efficacy is high (>90%); immunity is long-lasting, and antibodies may persist for over 30 years (Poland et al., 1981). Therefore revaccination is recommended every 10 years (WHO, 1993).

Animals with higher viremia (≥2.30 $\log_{10}$ PFU/mL; n = 12; Table 2) show a tendency of an early onset of fever (7.4 days) as compared to animals with lower viremia (<2.30 $\log_{10}$ PFU/mL; n = 37; 8.4 days). This difference is statistically significant (t = 1.74; P = 0.04). The duration of fever does not differ significantly between the two groups (3.5 versus 3.7 days; Kolmogorov–Smirnov test, P = 0.98). It is of interest that 3 of 12 animals, which had the highest viremias, did not display clinical manifestations (Table 2), whereas the other 9 monkeys had clinical scores ranging from 0.10 to 0.70. The same holds for the combined histological score as the 12 animals that presented higher viremia (≥2.30 $\log_{10}$ PFU/mL) presented a combined score of 1.04 (SD = 0.41). The other group (n = 37) with lower viremia (<2.30 $\log_{10}$ PFU/mL) had a combined histological score of 1.11 (SD = 0.36). These differences were not statistically significant (t = 0.53, P = 0.60).

Immunogenicity

All animals developed measurable levels of neutralizing antibodies, evaluated by the plaque reduction test with values ranging from 239 to 218,408 (Table 2).

Fever

The data for rectal temperature of all 49 monkeys are displayed in Fig. 1 as a graph and represented by the average temperature of the number of monkeys per group per day. There is a tendency for the animals to have fever between days 8 and 11 p.i.

Clinical scores

Fig. 1 shows daily mean clinical scores. Clinical signs were observed from the 6th day p.i. until the 16th day. From this day on, some animals did not improve and remained with clinical signs until the end of the observation period (30 days). Sixteen monkeys developed some signs of encephalitis with clinical scores of 3 or more for at least 1 day (Table 3), whereas only five of them had at least 1 day of higher viremia (≥2.30 $\log_{10}$ PFU/mL). Clinical signs were more prevalent after viremia waned and it overlaps with the development of fever. The most severe signs usually took place after defervescence. The clinical scores varied from 0 to 2.67, resulting in an average score of 0.32 (SD = 0.56). Two monkeys with the highest scores died. The coefficient of variability for a sample is defined by the standard deviation over the average (SD/x), which must be between 0.2 and 0.3 (Armitage and Berry, 1994). For the tests above this relation is 1.75, an extremely high value indicating variable data and rendering it difficult to apply any statistical analysis. This variability is evident in all experiments in which

Table 3
Clinical and histological scores of animals with clinical signs of encephalitis

Monkey	Clinical score	Histological scores		
		Discriminator areas	Target area	Combined
19	2.67	1.14	2.50	1.82
Q11	0.57	0.64	1.00	0.82
Q33	0.94	0.53	1.00	0.76
9	0.74	0.72	2.00	1.36
R6	2.60	1.25	2.00	1.62
R17	0.27	0.73	0.50	0.61
R37	0.24	1.09	2.00	1.54
R63	0.37	0.94	2.00	1.47
R67	0.34	1.00	2.00	1.50
S12	0.70	1.62	2.00	1.81
S17	0.27	0.68	2.00	1.34
S20	0.50	1.37	2.00	1.69
S37	0.37	0.54	2.00	1.27
S15	0.87	0.60	1.00	0.80
S44	0.17	0.44	2.00	1.22
S67	0.35	0.64	2.00	1.32
Mean (SD)	0.75(0.77)	—	—	1.31(0.38)

there is always at least one animal that extrapolates the group average.

Histological lesions: scores and distribution within different areas of the CNS

All 49 animals developed histological lesions in the CNS. The same was demonstrated for other 17D viruses (Fox and Penna, 1943; Nathanson et al., 1966; Levenbook et al., 1987; Marchevsky et al., 1995). The combined histological scores in our study varied from 0.34 to 1.82 (mean = 1.09; SD = 0.37). The coefficient of variability (SD/x) is 0.33, allowing the application of statistical tests as recommended by internationally accepted guidelines (WHO, 1998).

The different anatomical structures of the CNS are classified as target, discriminatory, and spared areas (Levenbook et al., 1987), depending on the degree of tissue involvement. The spared areas have indeed minimal lesions as compared to the target and discriminator areas. Among the spared areas mostly affected the *nucleus ruber* showed grade 1 in 12 structures of 98 observed. Among the discriminator areas, the most affected were the *nucleus caudatus*, *globus pallidus*, and *putamen*, in which 45, 29, and 38 structures (of 98 examined) presented grade 2. The target area (*substantia nigra*) showed grade 2 in 55 of 98 hemisections.

Histological analysis of animals with clinical encephalitis

Tables 3 and 4 show the correlation between the observation of clinical encephalitis and the extent of histological lesions in the target and discriminator areas. The average combined histological score for the group with encephalitis was 1.31 (SD = 0.38), whereas the group without signs of encephalitis showed an average score of 0.98 (SD = 0.30). The use of one-sided *t* test on these averages demonstrated that they are significantly different ($P = 0.04$). Moreover the use of linear regression analysis also suggested that this correlation is real (Pearson coefficient = 0.41; $P = 0.01$).

Table 5, which was derived from Tables 3 and 4, shows that animals from the group with clinical encephalitis do not differ from animals without when we considered the total number of grades 1 and 2 in the different structures of the target and discriminatory areas. However, if we consider the percentage of structures in both areas that displayed grades 1 and 2, it is apparent that certain structures are more relevant. We observed that animals with clinical signs of encephalitis, as compared to the group without, had increased grade 2 lesions in the target (*substantia nigra*) and some discriminator areas (*nucleus caudatus*, *intumescentia cervicalis*, and *ventralis*). These are probably the areas most likely to be involved in clinical manifestations in animals inoculated i.c. with YF 17DD 102/84 virus.

Discussion

Monkeys inoculated subcutaneously with wild-type YF virus developed viremia between days 2 and 6 p.i. with

Table 4
Clinical and histological scores of animals without clinical signs of encephalitis

Monkey	Clinical score	Histological scores		
		Discriminator areas	Target area	Combined
01	0.00	0.45	1.50	0.97
02	0.00	0.55	1.00	0.77
03	0.00	0.83	2.00	1.41
04	0.00	0.41	2.00	1.20
05	0.00	1.36	2.00	1.18
16	0.00	0.50	1.00	1.00
17	0.00	0.57	1.00	0.78
18	0.17	0.86	1.50	1.18
20	0.00	0.39	1.00	0.70
Q30	0.30	0.69	2.00	1.34
Q46	0.34	0.67	2.00	1.33
R1	0.03	0.57	2.00	1.28
R7	0.00	0.09	1.00	0.54
R24	0.03	0.35	1.50	0.92
R25	0.20	0.63	2.00	1.31
Q28	1.20	0.76	1.00	0.88
R65	0.20	0.76	1.00	0.88
R31	0.14	0.63	2.00	1.31
R39	0.17	0.58	1.00	0.80
R66	0.47	1.09	1.50	1.30
S27	0.10	0.07	1.00	0.53
S28	0.24	0.54	2.00	1.27
S57	0.00	0.35	0.50	0.42
S61	0.00	0.69	1.50	1.09
Mean (SD)	0.11(0.23)	—	—	0.98 (0.30)

Table 5
Comparison of the frequency of histological lesions in the CNS of animals with or without clinical signs of encephalitis

Monkeys/CNS structure	*Substantia nigra*	*N. caudatus*	*G. pallidus*	*Putamen*	*N. ant./med. thalami.*	*N. ventrolat. thalami*	*Intumescentia cervicalis.*	*Intumescentia ventralis*
With signs of encephalitis ($n = 16$)								
Grade 2	71.8	65.6	43.7	40.6	31.2	18.7	25.0	31.2
Grade 1	21.8	25.0	50	53.1	46.8	50.0	37.5	40.6
Without signs of encephalitis[a] ($n = 24$)								
Grade 2	52.0	37.5	31.2	33.3	20.8	8.3	4.1	6.2
Grade 1	45.8	50.0	66.6	58.3	47.9	39.5	47.9	35.4

[a] The six animals from test A and three animals from test F did not show any clinical signs and were not included.

titers near 8.0 $\log_{10}$ PFU/mL (Monath et al., 1981). We have recently inoculated 14 rhesus monkeys by the subcutaneous route with YF 17DD 102/84 virus, and 4 of the animals showed viremia with the highest titer only 2.43 $\log_{10}$ PFU/mL (data not shown). YF 17DD 102/84 virus inoculated i.c. penetrated the blood-brain barrier and was found in the bloodstream of most rhesus monkeys. The highest peak titers of peripheral viremias observed for 49 rhesus monkeys inoculated i.c. was 2.70 $\log_{10}$ PFU/mL. Galler et al. (2001) have shown that monkeys inoculated intrahepatically with 17DD 102/84 virus also displayed limited viremia (highest peak titer of 1.98 $\log_{10}$ PFU/mL). Altogether, these observations suggest that YF virus attenuation implies in its limited capacity of replication in monkeys, regardless of the inoculation route.

We have examined histologically several extraneural organs of all rhesus monkeys from all six tests. Those organs included liver, heart, kidneys, spleen, and adrenals since they are often involved in YF pathogenesis and other nonrelated organs (tongue, esophagus, lungs, stomach, pancreas, gut, and tonsils). All of them showed no lesions (data not shown). The liver of the monkeys from test F was tested by virus isolation and immunostaining, and no viral antigen was detected. Clinical chemistry analysis of serum aminotransferases (AST/ALT) and several other markers, as well as hematological analysis with total and differential white blood cell counts, did not suggest any alteration in the condition of the inoculated animals (Galler et al., 2001). These data further support the attenuation of YF 17DD 102/84 virus.

Recently, Vasconcelos et al. (2001), Martin et al. (2001), and Chan et al. (2001) described a total of seven human cases with multiple organ failure, including liver and kidneys, after vaccination with 17D virus. These are very rare manifestations and constitute the first cases of fatal adverse events of this nature amid the estimated 400 million vaccinees to date. Previous adverse reactions to YF 17D vaccination involved exclusively the central nervous system (Fox et al., 1942; WHO, 1993; Monath, 1999). Galler et al. (2001) have shown that virus derived from two of the cases had identical genomic sequences as 17DD 102/84 seed lot virus from which the vaccine batches had been derived. Further inoculation of rhesus monkeys through the intracerebral and intrahepatic routes failed to reveal any phenotypic changes, suggesting that some as yet unknown host factors were involved in the exquisite sensitivity to the vaccine. It may be relevant that usually one animal in each group of 10 develops more severe forms of encephalitis following i.c. inoculation of YF 17DD 102/84 virus. The higher frequency in the MNVT may be due to the intracerebral route of inoculation.

It would be of interest to see whether two main substrains of YF 17D virus, used for vaccine production and human immunization, the 17DD and the 17D-204 viruses, differ in their attenuation in the MNVTs as genetic differences have been noted between them (Monath et al., 1983; Ryman et al., 1997; Galler et al., 1998). Data on YF 17D-204 virus neurovirulence for rhesus monkeys are available from the literature (Levenbook et al., 1987; Monath et al., 2000). Since these tests with 17D-204 viruses were carried out following the WHO guidelines, they are comparable to ours. The comparison between both substrains of YF 17D virus was limited to the combined histological scores since they are less prone to individual variations. We have used meta-analysis to compare both substrains. This is an important statistical tool that allows the comparison of different studies (Hedges and Olkin, 1985). In the 95% confidence interval, animals inoculated with YF 17DD 102/84 ($n = 49$) had values ranging from 0.98 to 1.20 with an average of 1.09, whereas the animals inoculated with YF 17D-204 ($n = 106$) displayed values from 0.40 to 0.90 (mean 0.65). As the average for virus YF 17DD 102/84 lies outside the 95% confidence interval established for YF 17D-204 virus and vice versa, there is a suggestion that the YF 17DD 102/84 virus presents a higher degree of neurovirulence in the MNVT than the 17D-204 virus. The molecular basis for this difference remains undetermined as these viruses differ at several positions in the genome (Galler et al., 1998, 2001; Pugachev et al., 2002). Since both strains have been used for decades in human immunization with similar efficacy and safety, the observed differences, genetic or phenotypic, are not relevant concerning human vaccination.

Materials and methods

Virus

Yellow fever secondary seed lot (YFV 17DD 102/84) was used for vaccine production at Bio-Manguinhos from 1984 through 2001 with an estimate of 373 million doses produced. It was prepared with a titer of 6.47 $\log_{10}$ PFU/mL. The virus contained in the original vial was reconstituted in 0.5 mL of sterile water as specified by the manufacturer (Bio-Manguinhos, Rio de Janeiro, Brazil). All animals received approximately 25,000 LD_{50} (mouse 50% lethal dose, MLD_{50}) according to the WHO guidelines that recommend doses between 5000 and 50,000 LD_{50}. The MLD_{50} was established for YF 17DD vaccine virus at Bio-Manguinhos by inoculating groups of mice with different dilutions of the virus and estimating the 50% mortality (Reed and Munch). The ratio between MLD_{50} and plaque forming units in Vero cells culture is 0.70 $\log_{10}$, meaning that the PFU titer is five times higher. The viral inocula were back titrated by plaque assay on Vero cells.

Cells

Vero cells (ATCC, CCL 81) were maintained in Medium 199 with Earle's salts (E199), buffered with sodium bicarbonate, and supplemented with 10% fetal bovine serum (FBS) and antibiotics.

Inoculation of monkeys

A total of 49 captive-bred healthy rhesus monkeys (*Macaca mulatta*), 37 male and 12 female, weighing 1440 to 10,300 g, were obtained from the Primatology Division (CECAL/FIOCRUZ, Rio de Janeiro/Brazil). All monkeys were shown to be free of YF neutralizing antibodies by plaque reduction neutralization assay before inoculation. Monkeys for the study were combined from six groups, designated as A to F (Table 1). Each animal was kept in a separate cage under controlled environmental conditions (temperature 20–22°C, relative humidity ~60%, and 12 h of artificial light and 12 h of darkness). Animals were fed twice daily with monkey chow supplemented with fresh fruits and allowed water ad libitum.

Monkeys were anesthetized by intramuscular injection of ketamine hydrochloride (20 mg/kg body weight) and inoculated with 0.25 mL of viral suspension by the intracerebral route into the right frontal cortex.

Viremia

Blood samples for the determination of viremia were collected on days 2, 4, and 6 after inoculation in tests A through E and from 1 through 9 in test F. Serial dilutions (1:3, 1:30, 1:300) of each monkey serum were titrated by plaque assay on Vero cell monolayers (10^5 cells/cm^2) with 3.5% carboxymethylcellulose as overlay in six-well plates. One hundred microliters of virus suspension was inoculated per well with four wells per dilution.

Seroconversion

Antibody titers were determined by a 50% plaque reduction test on Vero cells. The plaque reduction neutralization test (PRNT) was conducted in serial twofold dilutions starting at 1:16 in six-well tissue culture plates, with 30 PFU/well. A positive monkey serum sample with yellow fever antibody content calibrated by a WHO International Reference Preparation was included in each set of test. Dilutions of both virus and samples were performed in 199 medium containing 5% fetal bovine serum. After incubation at room temperature for 1 h, 50 μL of a suspension of Vero cells in 199 medium in a density of 1.6×10^5 cells was added to each well and the plates were incubated at 37°C in 5% CO_2 atmosphere for 3 h. The medium was discarded and replaced with 199 medium with 5% fetal bovine serum containing 3.5% carboxymethyl cellulose. After incubation at 37°C in 5% CO_2 atmosphere for 7 days, the monolayers were fixed with formalin and stained with crystal violet and plaques were counted. The $\log_{10}$ dilution of the test and standard serum which reduced the plaque numbers by 50% relative to the virus control was determined by linear regression. The mean antibody titer at the 50% end point of the standard serum was then calculated and added to the $\log_{10}$ end point for each sample to provide the final value in $\log_{10}$ mIU/mL.

Clinical observation

Monkeys were observed for 30 days and rectal temperature was recorded. Temperature equal to or greater than 40.0°C was considered elevated. Records of clinical observation were obtained using the WHO (1998) recommended scores: grade 1 = rough coat, not eating; grade 2 = high-pitched voice, inactive, slow moving; grade 3 = shaky movements, tremors, uncoordinated movement, limb weakness; grade 4 = inability to stand, limb paralysis, or death. A monkey that died received the score "4" from the day of death until day 30.

Necropsy and histological examination

All animals were submitted to full necropsy at the end of the observation period. They were euthanized by exsanguination under deep anaesthesia. Serum samples were collected to determine anti-YFV antibodies by plaque-reduction neutralization assay.

Five levels of the brain and six levels of each of the lumbar and cervical enlargements were examined. Brain levels included block I, the corpus striatum at the level of

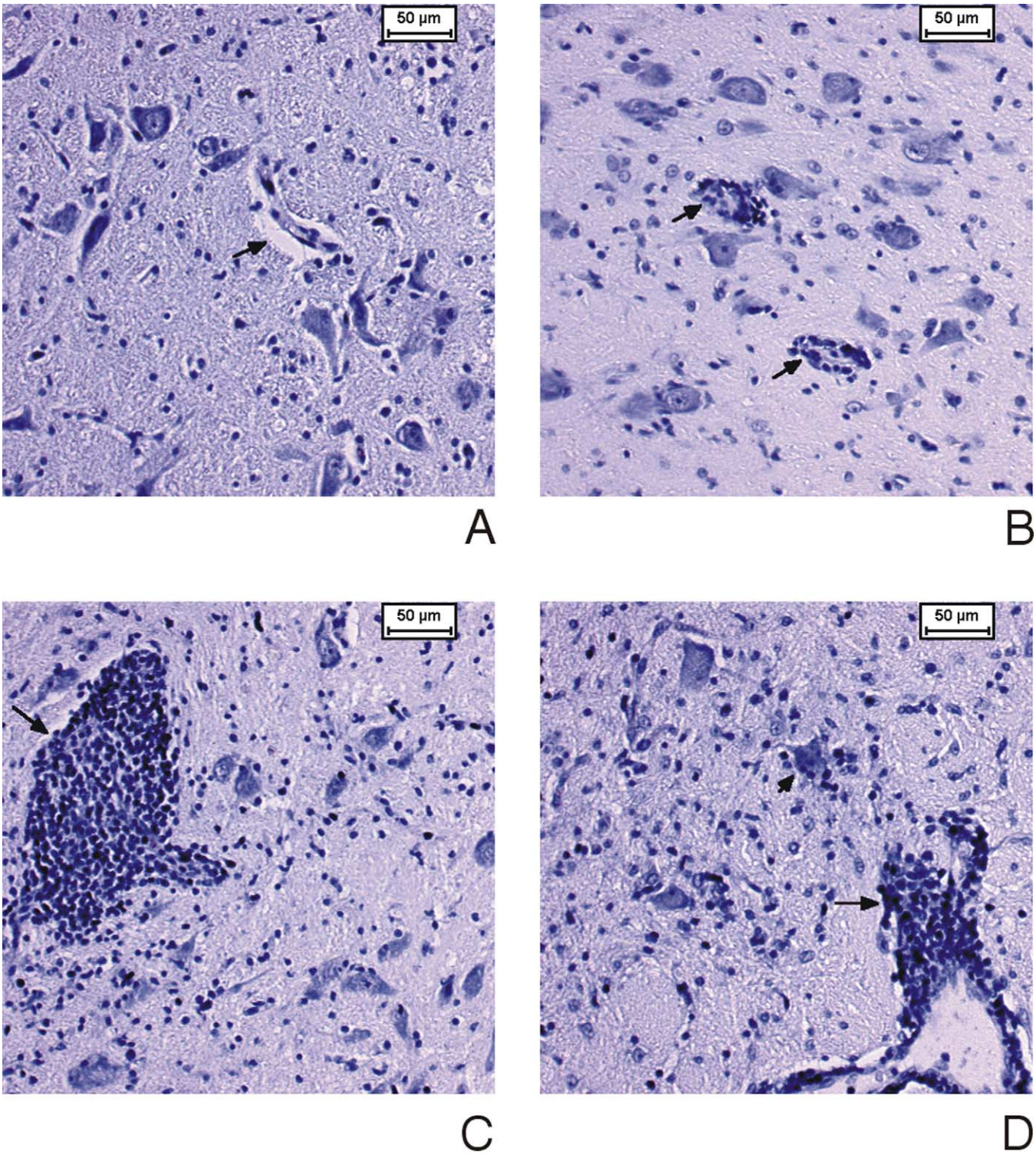

Fig. 2. Histological lesions in the *substantia nigra* of rhesus monkeys inoculated with YF 17DD 102/84 virus. All panels are ×160 magnifications. All sections are 6 micra and stained with gallocyanin. (A) Grade 0: normal neurons without evidence of inflammation or perivascular infiltration (arrow). (B) Grade 1: no neuronal involvement and minimal perivascular cuff (arrows) with few layers of inflammatory cells. (C) Grade 2: perivascular cuffs (arrow) with densely packed layers of mononuclear inflammatory cells and an increase in the number of glial cells over the affected area. (D) Grade 3: multiple focal infiltrates and perivascular cuff (arrow), reduction in the size and number of neuronal cells, and evidence of neuronophagia (arrowhead).

the optic chiasma; block II, the thalamus at the level of the mamillary bodies; block III, the mesencephalon at the level of the superior colliculi; block IV, the pons and cerebellum at the level of the anterior olives border; block V, the medulla oblongata at the middle of the inferior olives (WHO, 1998).

Numerical scores were given to each hemisection of the cord and to structures in each hemisection of the brain. Lesions were scored according to the following grading system: 1, (minimal), one to three small, focal inflammatory infiltrates, a few neurons may be changed or lost; 2, (moderate), more extensive focal inflammatory infiltrates, neuronal changes, or loss affects no more than one-third of neurons; 3, (severe), neuronal changes or loss of 33–90% of neurons, with moderate focal or diffuse inflammatory infiltration; 4, (overwhelming), more than 90% of neurons are changed or lost, with variable, but frequently severe, inflammatory infiltration. Fig. 2 shows a panel of histological sections representative of grades 0–3 in the *substantia nigra* of rhesus monkeys inoculated i.c. with YF17DD 102/84 virus.

Three separate scores were calculated for each monkey: discriminator areas only, target areas only, and discriminator plus target areas (Levenbook et al., 1987). The target area is the *substantia nigra*, whereas the discriminator areas include the *caudate nucleus, globus pallidus, putamen*, anterior and medial thalamic nucleus, lateral thalamic nucleus, and cervical and lumbar enlargements. Both discriminator and combined group mean scores were be taken into account for decision making on vaccine acceptability (WHO, 1998).

Statistical analysis

Means and standard deviations were calculated for the onset and duration of fever, viremia peak titers, seroconversion, and clinical and combined histological scores. Variables were compared for animals with high and low levels of viremia and for animals with and without clinical encephalitis. Student *t*-test was used for comparing means. When there was a suggestion that data were asymetrical or variances were not homogeneous, data transformation was carried out. If this procedure was not successful, nonparametric statistical tests were performed (Kolmogorov–Smimov test). Differences were considered as statistically significant if *P* value was 0.05 or less. *P* values between 0.06 and 0.10 were considered as borderline. Pearson correlation coefficient was used to evaluate the presence of linear correlation between continuous variables. Meta-analysis technique was used for combining findings from different experiments carried out under the same conditions.

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