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MOLECULAR ANALYSIS OF YELLOW FEVER VIRUS 17DD VACCINE STRAIN

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The Oswaldo Cruz Foundation produces most of the yellow fever (YF) vaccine prepared world-wide. As part of a broader approach to determine the genetic variability in YF 17D seeds and vaccines and its relevance to viral attenuation the 17DD virus was purified directly from chick embryo homogenates which is the source of virus used for vaccination of millions of people in Brazil and other countries for half a century. Neutralization and hemagglutination tests showed that the purified virus is similar to the original stock. Furthermore, radioimmune precipitation of ^{35}S-methionine-labeled viral proteins using mouse hyperimmune ascitic fluid revealed identical patterns for the purified 17DD virus and the YF 17D-204 strain except for the 17DD E protein which migrated slower on SDS-PAGE. This difference is likely to be due to N-linked glycosylation. Finally, comparison by northern blot nybridization of virion RNAs of purified 17DD with two other strains of YF 17D virus revealed only genome-sized molecules for all three viruses. These observations suggest that the vaccine phenotype is primarily associated with the accumulation of mutations.

Key words: yellow fever – vaccine – 17DD strain – envelope – RNA

The yellow fever virus (YF) belongs to the family Flaviviridae, a group of about 60 closely related viruses many of which cause serious human disease (Monath, 1986). The YF virus is transmitted by *Aedes* and *Haemagogus* mosquitos, being, therefore, arthropod-borne, and infects primarily primates in which viscero-tropism and neutotropism can be observed. It has caused epidemics for centuries in the Americas, Europe and Africa and presently still remains an important cause of morbidity and mortality in large parts of tropical areas of Africa and South America with its true incidence being underestimated (WHO, 1986).

The YF virus was first isolated from the blood of an african human patient named Asibi by monkey/monkey passage (Stokes et al., 1928). This Asibi strain gave rise to the YF 17D strain which is the only live attenuated flavivirus vaccine with proven safety and efficacy available to date. The 17D strain was obtained by serial passage of the Asibi strain in monkeys, cultures of embryonic mouse tissue and minced whole chick embryo with and without nervous tissue. It was observed that after 114 culture passages the virus was no longer able to produce in monkeys either the visceral lesions or fatal encephalitis although it retained the capacity to immunize these animals against subsequent challenges with virulent virus strains (Theiler & Smith, 1937a, b). The reason for the change in virulence is presently unknown and attempts to develop other avirulent strains by repeating the experiments have failed. Since 1937 when production of YF 17D virus for vaccination was scaled up (Smith et al., 1938) a number of different substrains of that virus were recognized. These substrains, usually originating at about the two hundreth subculture level (see Fox & Penna, 1943 for details) were serially passaged in parallel but independently, in tissue culture or chick embryos, and maintained to provide the virus source for the vaccines. Such vaccine viruses were shown to differ significantly

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regarding monkey neurovirulence (Fox & Penna, 1943) and some were related to encephalitis in man following vaccination (Fox et al., 1942). By the end of 1940 the maintenance of source virus in serial passages was replaced by the seed-lot system in which vaccine destined for human use was taken from large lots previously tested (Fox & Penna, 1943). Several of such YF 17D vaccine strains were shown to have some degree of genomic sequence variability as detected by RNA fingerprinting but there was no correlation of specific changes to the viral phenotype (Monath et al., 1983). In this approach only a small part of the viral genome is analysed and the use of recombinant DNA should prove more efficient in displaying the genetic variability. Indeed, the complete nucleotide sequencing of both YF 17D-204 and the Asibi strains revealed 68 nucleotide and 32 amino acid differences corresponding to 0.63% overall sequence divergence (Rice et al., 1985; Hahn et al., 1987).

In this regard it would be of interest to analyze other YF 17D vaccine strains with independent passage histories in order to study the extent of genetic variability in YF 17D seeds and vaccines. In addition more correlative data would be obtained to further highlight the nucleotide/amino acid sequence changes possibly related to virulence/attenuation and/or adaptation to growth in chicken embryo cells, that is, the substrate for YF 17D vaccine virus propagation. The obvious first candidate is the YF 17DD vaccine virus which has been used for 50 years at the Oswaldo Cruz Foundation (FIOCRUZ) for vaccine production and to vaccinate millions of people world-wide. Considering the large vaccine production it should be possible to purify and analyze the virus directly from the embryo homogenate, which constitutes the vaccine itself, instead of using viruses obtained from cultured cells without any relation to vaccine virus production and the vaccine phenotype; a procedure which might actually lead to the selection of YF virus genetic variants with undesirable traits as vaccines (Monath, 1985).

Here we describe the purification of YF 17DD virus from the human vaccine and the identification of the viral proteins and nucleic acid.

MATERIALS AND METHODS

Cells and viruses – Vero cells were maintained in M199 medium supplemented with 4% fetal calf serum. Primary cultures of chicken embryo fibroblasts (CEF) were propagated in MEM medium in 5% fetal calf serum. The YF virus strain 17DD was either purified from chicken embryo (CE) homogenates (vaccine) according to the procedure described below or constitute the supernatant of CE homogenate-contained 17DD virus passaged once in Vero cells (DD/V) or in CEF (DD/C). A brief description of YF 17DD passage history in Brazil/FIOCRUZ is depicted in Fig. 1. The FIOCRUZ 17D-213/86 seed is derived from the original ALV-free WHO 213/77 seed by one passage in embryonated chicken eggs. The FIOCRUZ 17D-213/86 seed was then propagated at low multiplicity in CEF culture without fetal calf serum and the viral stock is the supernatant of such cultures. It is the YF tissue culture vaccine virus previously monkey neurovirulence tested by others with satisfactory results (R. Marchevsky, personnal communication). The YF 17D-204 virus is derived from a twice plaque-purified stock on CEF with additional passages in mammalian cells for the amplification of the viral titers for RNA isolation and cDNA cloning (Rice et al., 1985). Such virus was further passaged four times in SW13 human cells. All viruses were aliquoted, titered on Vero cells and stored at $-70\ ^{o}C$.

YF 17DD virus purification – A four-step procedure for the purification of YF 17DD virus from chick embryo homogenates was developed. Each step in the procedure, originally based on a protocol for an alphavirus (Cabral, 1986), was tested in several ways and the protocol given below represents our current one for preparing larger amounts of YF 17DD vaccine virus. Frozen or fresh homogenates were furnished by Drs A. R. Nicolau & M. Cerqueira from the FIOCRUZ YF vaccine production unit (Biomanguinhos). The homogenates were diluted 1:1 with M199 medium without serum and centrifuged (6500 g) 30 min at $4\ ^{o}C$ to yield a supernatant to be treated with protamine sulfate (4.5 mg/ml). Precipitates were removed by centrifugation and the virus was precipitated by the addition of polyethylene glycol 6000 (Sigma) to 8% with gentle stirring for 2 h at $4\ ^{o}C$. Precipitates containing virus were collected by centrifugation at 2000 g for 15 min at $4\ ^{o}C$ and resupended by gentle

agitation on ice in one tenth of the initial embryo homogenate volume. The resuspension was spun at 2000 g for 15 min at 4 °C and the pellet again resuspended in M199 medium. A second centrifugation as above yielded the second supernatant (PEG2) which was combined to the first (PEG1), loaded on top of 20% sucrose cushions and spun for 2 h at 100,000 g. Sediments were resuspended at 1:100 of the initial volume in M199 medium without fetal calf serum, aloquoted and stored at −70 °C. Virus titrations, neutralization and hemagglutination assays were done essentially as described respectively (Clarke & Casals, 1958; Lopes et al., 1987, 1988).

Preparation of protein extracts, immunoprecipitation and polyacrylamide gel eletrophoresis (PAGE) – These techniques were carried out as described elsewhere (Post et al., 1991).

Nucleic acid extraction and hybridization – Aliquots of each YF vaccine virus 17DD, 213 and 204, usually the equivalent of 10^5 plaque forming units (pfu) were made 1% sodium dodecyl sulphate (SDS) and 5 μg of *Escherichia coli* tRNA, extracted twice with phenol-chloroform (1:1) and ethanol precipitated from 0.2 M sodium acetate. Samples were kept at −70° in ethanol until use. Precipitates were collected by centrifugation (15000 g) and dissolved in water, made 50% formamide-2.2 M formaldehyde in 1X running buffer (20 mM MOPS- 8 mM sodium acetate- 1 mM EDTA; Maniatis, 1989). Gels were run at 100 V for 3 h and blotted onto nitrocellulose filters with 10X SSC (1XSSC is 150 mM sodium chloride-15 mM sodium citrate). Filters were baked for 1h at 80 °C and pre-hybridized for 3 h at 42 °C in 50% formamide- 5X SSC- 20 mM phosphate buffer pH 7 -5X Denhardt solution – 0.2% SDS (Maniatis, 1989). Hybridization was under the same conditions but for 16 h with 1X Denhardt solution and 5 X 10^5 cpm/ml of nick-translated probes (Rigby et al., 1977). Filters were washed in 2X SSC-0.1% SDS at room temperature for 30 min and 0.1% SSC-0.1% SDS at 42 °C for 30 min as the stringent step.

RESULTS

The YF virus 17DD vaccine strain – An approach to understanding the molecular basis of YF virus genetic variability and virulence/attenuation is to determine the genetic changes that occurred during virus propagation in culture. Since the number of passages from the original wild YF strain Asibi to generate the vaccine virus series (17D) is known it should be possible to determine evolution rate and to correlate specific nucleotide/amino acid changes in the viral genome to serial passage either in culture or in embryonated chicken eggs. Therefore, a rather accurate passage history of each virus studied should be available. In Fig. 1 the derivation of YF virus strain 17DD is presented and was compiled from different sources (Monath et al., 1983; J. F. Cunha & A. R. Nicolau, personnal communication). Considering that the 17DD strain history is different from those other strains which have already had their genomic nucleotide sequence determined (Rice et al., 1985; Despres et al., 1987) it should be of interest to analyze the 17DD genome.

Purification of YF 17DD virus from chick embryo (CE) homogenates – Given the large production of YF 17DD virus at FIOCRUZ it would be of utmost importance to obtain genetic information directly on the 17DD virus which is used for half a century for human vaccination against yellow fever. The genomic sequences obtained are more likely to represent a vaccine phenotype than any other presented to date (Rice et al., 1985; Despres et al., 1987).

Initially based on a methology developed for another arbovirus (Cabral, 1986) several experiments were carried out in which a number of parameters were checked. The protocol presented in the Methods section is presently in use in our laboratory. Details on every experiment are available upon request. The Table summarizes the results obtained in fourteen experiments done to optimize the recovery of YF 17DD virus from CE homogenates. The major pitfall was the clarified CE homogenate where the concentration of protamine sulphate was critical. An excess of it would render rubber-like the PEG precipitate virtually impossible to resuspend and therefore several concentrations were tested. According to the Table the recovery of YF 17DD virus, as measured by plaque formation on Vero cells, varied broadly from 0.2% (the 0.02% on experiment 13 is attributed to using a 10% glycerol cushion instead of sucrose 20%) to a maximum of 5.1%. Three other recent preparations were similar in recovery (%) to experiment 14 but intermediate steps were not titered (results not shown).

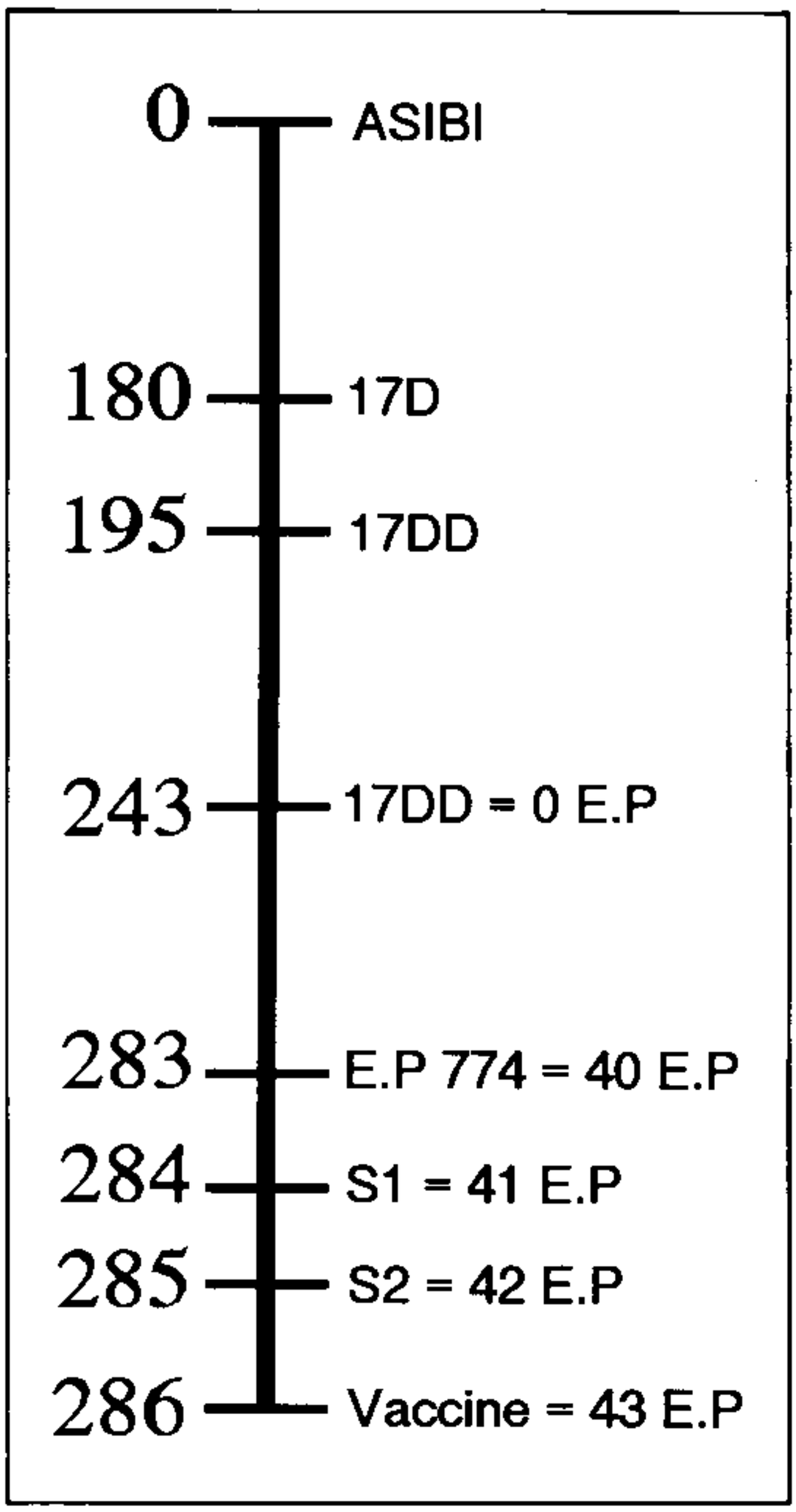

Fig. 1: passage history of YF 17DD. The Asibi strain was subcultured in tissue culture originating the 17D strain at passage 180 which yielded the 17DD strain at passage 195. The 17DD virus was further subcultured until passage 243 when it underwent additional 43 passages in embryonated chicken eggs until the vaccine batch used for 17DD virus purification (passage 286). E. P., egg passage; S1, primary seed lot; S2, secondary seed lot.

In order to confirm the identity of the recovered virus aliquots of preparations 11 and 12 were incubated or not with YF hyperimmune serum and serial dilutions thereof plated on Vero cells. The neutralization pattern of the purified virus was similar to the original 17DD vaccine virus before purification whereas no plaques in any instance were observed in the mock control (results not shown). Moreover, hemagglutination-inhibition using goose red cells and YF hyperimmune serum (results not shown) provided further evidence that the YF virus had been purified from CE homogenates.

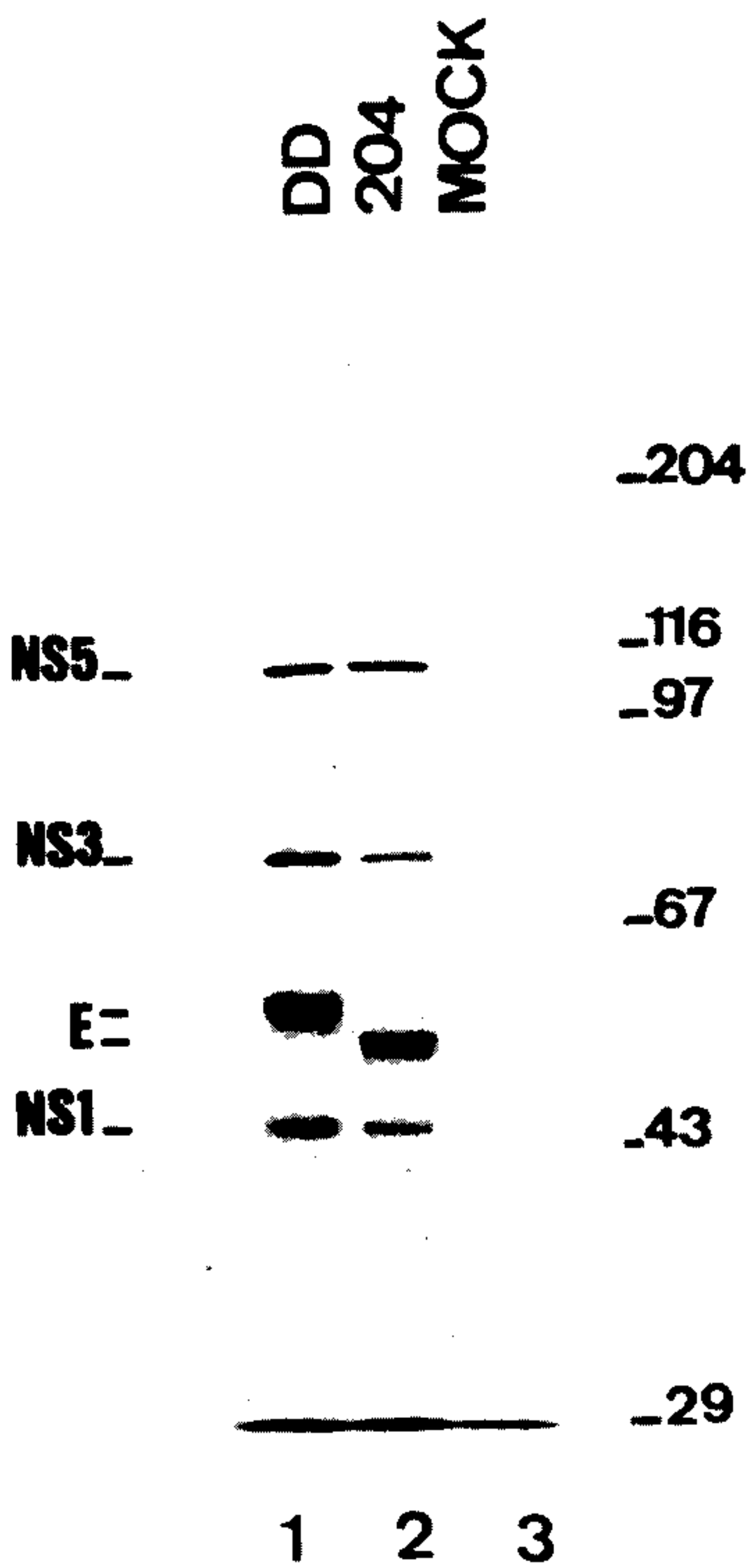

Fig. 2: comparative profile of YF 17D virus proteins. Vero cells were infected at m.o.i. of 1 with each virus and incubated for 42 h, pulsed with radioactive methionine for 1 h. Thereafter, protein extracts were prepared from cell monolayers. Analysis of extracts from cells infected with the 17DD strain (lane 1) and 204 (lane 2) or mock (lane 3) are shown. Aliquots were immunoprecipitated with YF hyperimmune serum and samples analyzed on SDS-PAGE (10%). The gel was treated for fluorography and exposed at −70 °C for 12 h. Molecular weight markers are indicated in kilodaltons on the figure side. YF virus proteins are denotated from top to bottom as NS5, NS3, E and NS1 as originally proposed (Rice et al., 1985). See text for an explanation on E protein migration difference.

Characterization of YF 17DD viral proteins — The first step in characterizing the YF virus 17DD strain was to propagate it in Vero cells to obtain metabolically labeled protein extracts.

TABLE

Purification of 17DD virus from CE homogenates[a]

Exp.	E.H.[b]	DIL[c]	CHE[d]	PEG 1	PEG 2[e]	virus[f]	recov (%)[g]
1	–	–	–	–	–	7.3	–
2	9.3	9.1	–	–	–	–	–
3	9.2	8.9	–	6.9	5.2	–	–
4	8.6	–	–	–	–	–	–
5	8.3	8.3	8.1	–	–	6.2	0.87
6	–	–	8.8	7.8	8.1	7.7	–
7	8.7	–	–	–	–	6.8	1.44
8	8.8	8.6	–	8.0	7.6	7.1	2.02
9	–	8.6	8.2	7.2	7.2	6.7	1.5
10	9.2	9.1	8.5	7.2	7.3	6.5	0.2
11	9.0	8.6	8.5	7.9	7.6	7.3	2.0
12	9.0	8.8	8.5	7.7	7.5	7.6	4.7
13	8.4	8.3	8.2	–	–	5.0	0.04
14	8.5	–	–	7.6	7.7	7.2	5.1

a: total viral titers expressed in $\log_{10}$.
b: embryo homogenate.
c: diluted embryo homogenate.
d: clarified e.h.
e: polyethylene glycol.
f: recovered virus.
g: calculated with values in b and f.

Aliquots thereof were immunoprecipitated, proteins separated by PAGE and visualized after fluorography. These results are shown in Fig. 2. The comigration of proteins identified as NS5, NS3, E and NS1 based on their molecular weights (Chambers et al., 1990) was evident. The YF virus 17D-204 strain is the same viral stock as previously described for cDNA cloning and sequencing (Rice et al., 1985) with four additional passages in SW13 cells. This result further confirms the nature of the purified YF 17DD virus.

The envelope (E) protein of YF 17DD virus migrated slower than its YF 17D-204 counterpart under denaturing and reducing conditions. This observation suggests that additional protein modification occurs in the DD E protein. This modification was recently identified as N-linked glycosylation (Post & Galler, unpublished). The 4 identified proteins constitute altogether about 75% of the encoded viral proteins and therefore we must conclude that the 17DD virus used for human vaccination can be purified from CE homogenates for biochemical and genetic analysis.

Characterization of YF virus RNA – The ultimate experiment to characterize the YF virus 17DD strain was to compare by hybridization its genomic RNA to the RNA of other well known strains, including the 17D-204 reference strain. For this purpose RNA was extracted and separated by size on denaturing agarose gels. Hybridization to filter-bound viral RNAs of cDNA clones (Rice et al., 1985) encompassing the whole YF genome is shown in Fig. 3. The RNA from 2 different 17DD preparations was tested and shown to be similar in lenght to other YF virus strains, namely, 17D-204 and 17D-213/86. Longer exposures of the autoradiograms shown in Fig. 3 did not display subgenomic RNA molecules. This result would suggest that viral particles containing defective viral RNA molecules are not present in human YF vaccine nor in the monkey neurovirulence-tested tissue culture-produced YF virus vaccine-candidate 17D-213/86 strain.

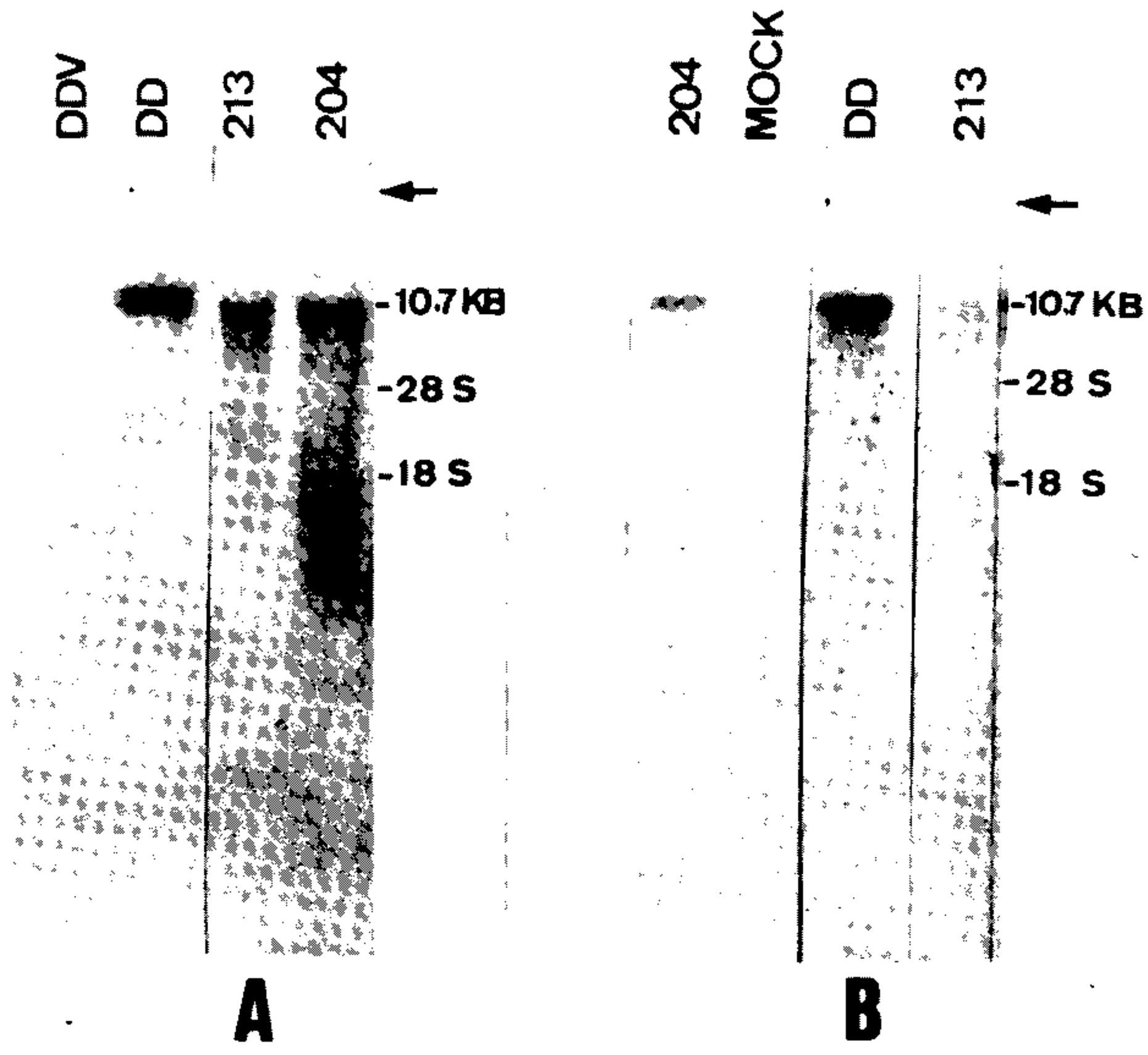

Fig. 3: YF 17D virus RNA analysis. The RNA from each YF virus strain (DD/V; DD; 204 and 213) were extracted and separated by size on denaturing formaldehyde gels. Mock contained *Escherichia coli* tRNA and was treated simultaneously. RNA was transferred to nitrocellulose filters and hybridized to cDNA clones encompassing the whole genome (Rice et al., 1985). Stringent washing was 0.1X SSC at 42 °C for 30 min and filters were exposed for 8 h each at −70 °C without intensifying screens with Kodak X-OMAT films. The arrows point to gel origin and the 10.7 kilobases value for full-length YF viral RNA is deduced from the complete nucleotide sequence (Rice et al., 1985) which produces infectious RNA transcripts (Rice et al., 1989). The values for 28 and 18S correspond to ribosomal RNAs from Vero cells observed after toluidine blue staining of nitrocellulose filters. A and B are experiments in which different 17DD purifications were analysed comparatively to other YF 17D viruses.

DISCUSSION

To study the genetic variability of YF 17D seeds and vaccines and its relevance to viral attenuation a number of such viruses should have their genomic sequences determined. It is also important that the vaccine phenotype of these viruses be well characterized regarding primate infection. The analyses of virus preparations intended for human vaccination and/or monkey neurovirulence test are the most significant as far as genetic variation and the vaccine phenotype are concerned. Therefore, the procedure described here for YF 17DD vaccine virus strain is a step forward to the successful preparation of other vaccine viruses for biochemical and genetic comparisons.

The large production of YF 17DD virus at FIOCRUZ and the availability of viral stocks allowed the establishment of the methodology of virus purification from chick embryo homogenates. It is evident from the Table that the yields are low with the best virus recovery at 5%. This yield has been reproduced in the last 3 experiments with the described protocol. Since several steps were checked for improvements it may not be feasible to recover even more virus from the initial bulk. Nevertheless, the recovered 17DD virus was sufficient in virtually all experiments to be propagated in cultured cells for metabolic labeling of viral proteins and the analysis of viral RNA.

In average about 10^9 pfu (as titered on Vero

cells) were used in each purification experiment which optimally yields about 1-5 x 10^7 pfu final. Since we are primarily interested in determining the genomic sequences it is anticipated that 1-5 x 10^7 pfu will be needed to obtain sequences representative of the whole genomic coding region. This argument is based on experiments in which cDNA synthesis using synthetic oligonucleotide primers and the polymerase chain reaction (PCR; Saiki et al., 1988) was accomplished (Santos, Post & Galler, unpublished). Here the RNA equivalent to as little as 10^6 pfu of each YF 17DD and 213 were used to amplify the whole envelope region (1.8 Kb in length). Since the YF genome is 10.7 Kb long (Rice et al., 1985) 5 x 10^6 should suffice for the coding region but the 5' and 3' ends are considerably more difficult to reverse transcribe (Rice et al., 1988). The initial 300 ml of CE homogenate containing 10^9 pfu corresponds to 50 YF virus-infected chick embryos and this number is much lower than those for maximal weekly YF vaccine production (1000-2000 eggs). Therefore, it should be possible to use this procedure to prepare other 17D vaccine strains than the 17DD currently in use at FIOCRUZ to approach the question of genetic variability of 17D seeds and vaccines.

The analysis of metabolic labeled viral proteins by immunoprecipitation with YF hyperimmune serum and separation by PAGE revealed a difference in migration of 17DD E protein as compared to its YF 17D-204 counterpart. Plotting the respective molecular weights suggested this difference is close to 3 Kd which is the usual value found for N-linked glycans in other glycosylated flavivirus proteins (Smith & Wright., 1985; Post et al., 1990). Indeed, the use of endoglycosydases known to remove N-linked glycans corroborate the hypothesis of YF E protein glycosylation (Post, Santos & Galler, unpublished). Several N-linked glycosylation sites are observed in the nucleotide sequences of several flaviviruses but only one at amino acid position 150 from the E protein amino terminus is conserved. The E protein from many flaviviruses including DEN2 (Smith & Wright, 1985; Ozden & Pourier, 1985), JE (Mason, 1989), TBE, Murray Valley and Rocio (Winckler et al., 1987) and YF (Schlesinger et al., 1983; Deubel et al., 1987) were found to be glycosylated. However, the role of carbohydrate on flavivirus E protein structure and antigenicity remains to be elucidated. It is noteworthy, however, that the detected difference in E glycosylation between the YF 17DD virus used as human vaccine and the plaque-purified YF 17D-204 strain would already support the accumulation of genetic differences among vaccine strains by independent passaging.

The results shown here (Fig. 3) are the first analysis of RNA purified from human vaccine virus (17DD) including the 17D-213/86 strain which was previously monkey neurovirulence tested (R. Marchevsky, personnal communication). Shorter than full-length viral RNA could not be observed but the existence of defective viral RNA with small deletions can not be ruled out. Nevertheless, the growth of purified YF 17DD, 213 and the plaque purified 17D-204 in CEF and Vero cells at high (10) and low (0.1) m.o.i. did not display any multiplication rate differences. This observation together with RNA analysis would suggest that the vaccine phenotype is not dependent on the generation of defective particles, whether interfering or not. Consequently, the accumulation of mutations during serial passage of YF virus is more likely to play a role in changing the viral phenotype. The analysis proposed here of several YF vaccine strains used for human vaccination should contribute to confirm this hypothesis.

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