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Replication of Virulent and Attenuated Strains of Yellow Fever Virus in Human Monocytes and Macrophage-Like Cells (U937)

By

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With 5 Figures

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Summary

Virulent and attenuated strains of yellow fever virus were compared for their ability to grow in cultures of unstimulated leucocytes and monocytes derived from human peripheral blood, and of a macrophage-like cell line of human origin, U937. The extent of virus growth in leucocyte cultures varied depending on the strain of virus, multiplicity of infection, presence of diluted antibody in the culture medium but independently of the flavivirus immune status of the donor. The same pattern of differential growth was observed in the three types of cultures used. Although strain related variation in growth occurred within both virulent and attenuated strains, most of the attenuated strains produced higher virus yields than the virulent ones, suggesting that replication in this cell system is not related to the expression of virulence for the host. Replication in human monocytes as an *in vitro* marker of immunogenity for substrains of 17D vaccine virus is discussed.

Introduction

Yellow fever virus (YFV), the prototype of flavivirus, has been shown to replicate in cultures of leucocytes isolated from human peripheral blood (18, 25). In absence of mitotic stimulation, virus replication appears to occur mainly in monocytes, although lymphocytes treated with phyto-hemagglutinin support multiplication of the virus (25).

Interaction between virus and macrophage has been shown to play a key role in natural and experimental infections (12). In the case of YFV, macrophages appear to be an early site for virus multiplication. Invasion of the liver, the main target organ of YFV infection of primates, is preceded by virus multiplication in the Kupffer cells lining the liver sinusoids (24).

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Both virulent (13, 24) and attenuated (22) strains of YFV show *in vivo* a marked tropicity for lymphoreticular tissues, to which replication of the vaccine strain appears to be confined. Resident and circulating macrophages are an important site of replication of dengue virus, another flavivirus (6, 20). A correlation between dengue virus replication in these cells and virulence for the host has been proposed (7), although comparison of strains of different virulence have given contradictory results (2, 7). A similar correlation has been sought for several viruses (12), but with different results suggesting that the importance of the interaction between viruses and macrophages vary for each virus-host situation.

In the present study we compared the growth of several virulent and attenuated strains of YFV in cultures of mononuclear leucocytes and monocytes from human peripheral blood, and of a macrophage-like cell line of human origin (U937). We show that the extent of virus growth in these cells is not related to virulence for the host and that a marked variation in the ability to grow in these cells occurs among both attenuated and virulent strains.

Materials and Methods

Virus Strains

The source and passage history of the YFV strains used are shown in Table 1. Strains An-2336584 and H-2333934 were kindly provided by Dr. F. Pinheiro, strains JSS, French Neurotropic and Suarez were obtained through the courtesy of Dr. R. Shope. Derivation and characterization of variants of the 17D vaccine from Burroughs Wellcome LTD has been previously described (9). Plaque size of the other 17D strains were classified as small, medium and large by comparison with these variants. The dengue-2 virus strain New Guinea C1377, used as a suckling mouse brain passage, had a previous history of 24 passages in suckling mouse brain and 4 passages in LLC-MK_2 cells. Preparation of virus stocks in Vero cells and suckling mouse brain were done as described (9).

Virus Assay

Titration of infectivity of both YFV and dengue 2 were carried out by plaque assay in Vero cells as previously described for YFV (9). The overlay consisted of 1 per cent methylcellulose (Fisher, 4000 centipoise) in medium M199, supplemented with 2,5 per cent fetal calf serum, antibiotics and 20 mM $NaHCO_3$. Titers of stocks of YFV strains, on which multiplicities of infection (MOI) were based, were the geometric mean of at least three determinations. All samples from each experiment were titrated simultaneously. Titration of a fresh vial of 17D-E-SP was included in each plaque assay. In experiments to be compared titre of this standard varied within a range of 0.4 log.

Leucocyte Preparation and Infection

Venous blood from healthy adults was anticoagulated either in citrate-phosphate-dextrose solution, 63 ml for 450 ml of blood (Bolsang CPD, Travenol) or in EDTA at final concentration of 5 mg/ml. Mononuclear leucocytes were separated by isopicnic flotation on Ficoll-Hypaque (1). The leucocyte bands at the interphase of the Ficoll-Hypaque gradients were collected, pooled, diluted and washed three times in Ca^{++} and Mg^{++} free PBS (PBS-A) and resuspended in medium RPMI 1640 supplemented with 20 mM HEPES (N-2-hydroxyethil-piperazine-N-2) ethane sulfonic acid, 10 mM $NaHCO_3$, antibiotics and 10 per cent heath inactivated fetal calf serum (complete medium). At this stage leucocyte suspension (PBL) consisted of <1 per cent polymorphs, 9—24 per cent monocytes and 75—90 per cent lymphocytes as determined

Table 1. *History of yellow fever virus strains*

Strain	Source	Passage history[a]	Plaque size in VK cells	Neuro-virulence for mice[b]
17D-E-SP	Original vaccine,	Plaque purified	Small	V
17D-E-LP	Burroughs Wellcome, LTD,	3× in	Large	V
17D-E-MP	England	VK cell, VK1	Medium	A
17D-C	Original vaccine, lot. 235 I.N.S., Colombia	SMB1	Small	V
17D-B	Original vaccine, lot. 945 F.O.C., Brazil	SMB1	Large	V
17D-U	Original vaccine, lot. 1772 Merrel National Drug. Co. U.S.A.	SMB1	Small (95%) Large (5%)	V
French neurotropic	Dakar, 1927	MB, SMB1		
JSS	Brazil, 1935; Human serum	MB, SMB1	—	—
Suárez	Colombia, 1936; Human serum	SMB2	—	—
PHO-42-H	Venezuela, 1961; Human serum	SMB1	—	—
An-2336584	Brazil, 1973; Monkey organs	SMB2	—	—
H-2333934	Brazil, 1973; Human serum	SMB2	—	—

[a] Strains were used at the last passage indicated. Abbreviation: VK, Vero cell; SMB, suckling mouse brain, MB; unknown number of passages in mouse brain

[b] Strains producing a lethal (V) or inapparent infection (A) in adult mice after intracerebral infection

by morphological criteria. For infection PBL were resuspended at a concentration of 2×10^7 cells/ml and dispensed in 1 ml aliquots in 15 ml centrifuge tubes. Virus preparations, diluted in complete medium were added to achieve the desired MOI in a final volume of 1.5 ml. After two hours of incubation at 36° C cells were washed twice with PBS-A, resuspended at a concentration of 10^6 cells/ml in complete medium, dispensed in 1ml aliquots in glass vials or in 15 mm wells (of a 24 wells plate) and incubated at 36° C in a CO_2 incubator. Replicate vials or plates were frozen at —70° C at selected intervals.

Infection of Adherent Cells

Disposible 35 mm plastic Petri dishes or 35 mm wells (of 6 wells plates) were seeded with a suspension of 2×10^7 PBL. After overnight incubation at 36° C (in a CO_2 incubator) non adherent cells were removed and adherent cells washed three times with PBS-A. At this stage between 62 and 85 per cent of adherent cells phagocitized latex particles. These cultures will be referred to as MØ. The number of adherent cells was determined by direct count on selected fields on a inverted microscope (Photo-Invertoscope IM-35, Zeiss, Oberkochen, Germany) at a magnification of 400×. After counting MØ were infected with 0.1 ml of virus diluted to give the desired MOI. After one hour of adsorption at 36° C cultures were washed three times with PBS-A, replenished with complete medium, counted and incubated at 36° C. Virus yields are referred to the number of cells present at this stage. Sample of the supernatant (0.3 ml), were removed daily and replaced with fresh medium.

Infection of U937 Cells

Cells of a human histiocytic lymphoma line U937 (21), kindly provided by Mr. J. Mc Cown, Walter Reed Army Institute of Research, were grown in roller bottles in complete medium. For virus infection studies cells were washed once in PBS-A and resuspended in complete medium at a concentration of 10^7 cells/ml. One ml aliquots of this suspension were added to 10^6 PFU of virus preparations to give a final volume of 1.5. After a two hours incubation at 36° C with intermittent shaking, cells were washed twice with PBS, resuspended at a concentration of 3×10^5 cells/ml and incubated at 36° C in plastic 15 ml tubes kept in a horizontal position in a roller apparatur. Aliquots of 1 ml of cell suspension were collected daily, and frozed at —70° C until assayed for virus content. Sample portions were replaced with fresh medium.

Serology

Serum or plasma from donors were tested for antibody to YFV or dengue-2 virus by a 50 per cent plaque reduction neutralization test (PRNT), essentially as described (17), using strains 17D-E-SP of YFV, New Guinea C of dengue-2 virus and the plaque assay described, and by a micro-adapted hemagglutination inhibition test (HI) with both YFV and dengue-2 antigens (8). Donors negative with both tests were classified as negative for flavivirus antibody. Donors positive to either of the two viruses by neutralization were usually positive against both antigens by HI and are referred to as positive to either YFV or dengue-2 virus with the understanding that presence of antibody against other flaviviruses cannot be excluded.

The human serum used as a source of YFV antibody in the antibody mediated enhancement experiments, was obtained two months after vaccination with 17-D-YFV from a donor without antibody to flavivirus at the time of vaccination. This serum, heat inactivated in all experiments, had a 50 per cent PRNT end-point of 10^{-3} against YFV and $10^{-1,7}$ against dengue-2 virus.

Results

Virus Replication in Donors with Different Immune Status

In all experiments mononuclear leucocyte cultures infected with various strains of YFV at an MOI of 0.01—1 for MØ and 0.0003—0.1 for PBL supported virus growth as detected by an increase in virus yields in the supernatant or in the whole culture. In both systems peak titers occurred at 2—5 days post-infection and varied depending on the MOI and the virus strain used. Virus yields, based on the same number of total cells, were consistently higher in MØ than in PBL cultures: in one comparison in cells from the same donor, peak titers of 7 different YFV strains were 12-95 (average 47) times higher in MØ cultures than in PBL. Most experiments were performed with blood from donors whose immune status to flavivirus was unknown in advance. Replication of another flavivirus, dengue, in human or monkey leucocytes has been shown to be strongly dependent upon the dengue immune status of the donor (5, 11). Therefore in preliminary experiments we tested the permissiveness to YFV of mononuclear leucocytes from donors either without antibody to flavivirus or with antibody only to YFV or dengue-2 virus as detected by neutralization test. Fig. 1 shows the multiplication of the 17D-E-SP variant in PBL from the three types of donors. Under the experimental conditions used the immune status of the donor did not appear to have major influence on the course of the *in vitro* infection. Comparable results were obtained with other YFV strains (data not shown).

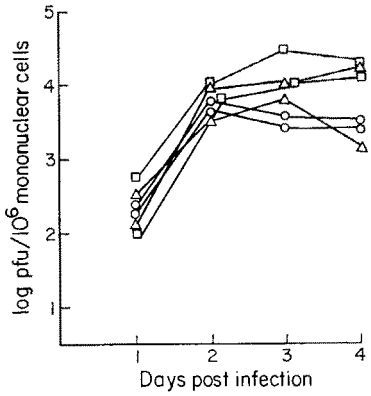

Fig. 1. Replication of 17D-E-SP in peripheral blood mononuclear leucocytes (PBL), from donors negative for flavivirus antibody (○), positive for YFV (△) or Dengue- (□) antibody. Leucocytes were infected at MOI of 0.1. Each point represents the geometric mean of titers from duplicate or triplicate cultures

Replication of Different Strains of γFV in PBL and MØ

YFV strains to be compared were tested simultaneously in cultures of PBL or MØ infected at a MOI of approximately 0.1 (Figs. 2 and 3). The vaccine strains included preparation of the 17D strain form different sources (Brazil, Colombia, U.S.A.) and three variants isolated from the 17D vaccine produced in England (17D-E-SP, MP and LP). In both cell systems 17D substrains showed only slight differences in growth, with the exception of the 17D-E-LP variant which in three

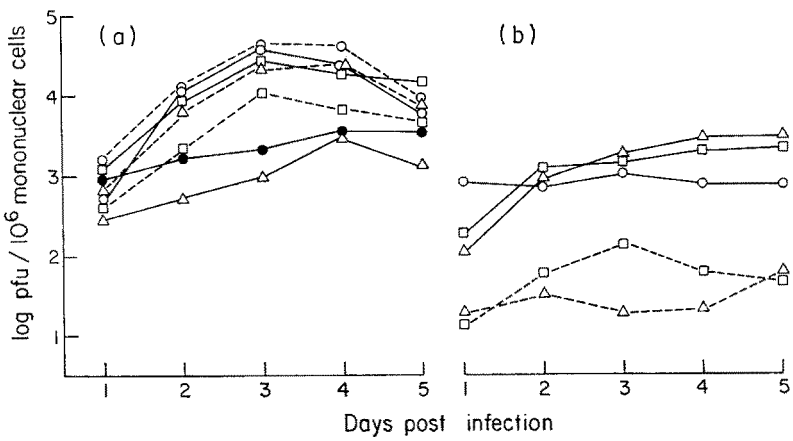

Fig. 2. Comparative replication of attenuated and wild strains of YFV in PBL cultures. Leucocytes from a donor positive for Dengue-2 antibody were infected at MOI of 0.1. Each point represents the geometric mean of titers from duplicate cultures. Symbols of the strains: *a* Attenuated strains: 17D-E-SP (○———○); 17-D-MP (□———□) 17D-E-LP (△———△); 17D-B (○— — —○), 17D-U (△— — —△); 17D-C (□— — —□); French Neurotropic (●———●); *b* Wild type strains: Suárez (□———□); PHO-42-H (△———△); JSS (○———○); An-2336584 (△— — —△); H-233934 (□— — —□)

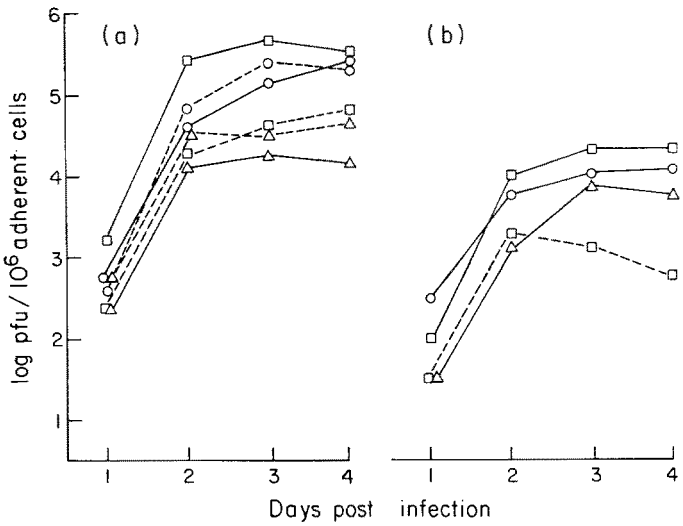

Fig. 3. Comparative replication of attenuated and wild strains of YFV in cultures of adherent mononuclear leucocytes (MØ). MØ, from a donor positive for YFV antibody, were infected at a MOI of 0.1. Each point represents the geometric mean of titers from duplicate cultures. Symbols of the strains (*a* and *b*), are the same as in Fig. 2

replicate experiments in PBL and two in MØ produced peak titers 10—100 fold lower than the other two variants isolated from the same source (17D-E-SP and 17D-E-MP). A representative experiment in each type of culture is shown in Figs. 2a and 3a.

Five wild type YFV strains tested simultaneously showed a marked variation in yields of virus produced, with differences in peak titers up to 100 fold between the strains which grew to the highest and lowest titer (respectively Suárez and An-2336584) (Figs. 2b and 3b). Even with the former wild strain virus yields were lower than those obtained by most of the vaccine strains: in two experiments in PBL and two in MØ cultures strain Suárez produced peak titers 7 to 15 fold lower than strain 17D-B ($p<0.01$ by the t test using the square root of titers from duplicate cultures). In these experiments the various YFV strains tested showed approximately the same pattern of differential virus production as in the ones shown in Figs. 2 and 3.

Although in most comparisons a standard MOI of 0.1 was used, similar differences in growth between a vaccine (17D-U) and a wild strain (PHO-42-H) were observed for MOI of 0.01 and 1 (Fig. 4). Variation between strains in plaquing efficiency in Vero cells did not account for the observed differences in yields, since the same pattern of differential growth was observed titrating leucocyte culture supernatants by intracerebral inoculation of suckling mice. Differences in yields were also not related to variation between strains in the stability to the freezing and thawing steps involved in the sampling procedure, nor to stability at 36° C, as determined by heat inactivation studies on virus stocks diluted 1/50 in complete medium. Although one of the strains (Neurotropic) appeared to be five times more stable than the others, the attenuated and wild

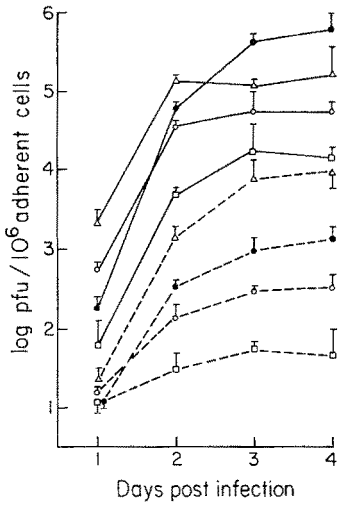

Fig. 4. Multiplication of strains 17D-U (solid line) and PHO-42-H (dotted line) in MØ from a donor positive for YFV antibody. Adherent cells were infected at an MOI of 0.01 (□), 0.1 (○) and 1 (△), and cultured in medium containing a final dilution of $10^{-3.5}$ of either non-immune (empty symbols) or YFV immune (filled symbols) human serum. The range in yield is shown with the geometric mean of duplicate cultures

type strains tested (17D-B, Suarez, PHO-42-H, An-2336584) showed the same rate or decay (2 log/24 hours).

Effect of Diluted Antibody on YFV Growth

Infection of macrophages by different flaviviruses, including YFV, has been shown to be enhanced by subneutralizing concentrations of antibody present in the inoculum or in the culture medium (2, 5, 14, 18, 19). We tested how enhancing antibody would affect the differential replication of YFV strains. Addition to the culture medium of a human antiserum (50 per cent PRNT titre of 10^{-3}), at a final dilution of 10^{-3} to 10^{-5}, resulted in virus yields 3 to 29 fold larger than those of the controls at 3 to 5 days post-infection in either PBL or MØ cultures of 5 out of 7 donors. In four experiments in which different YFV strains were tested simultaneously, enhancement was observed regularly with the vaccine strains, but only in two cases with the wild strain included (PHO-2-H). In both experiments (one of them shown in Fig. 4) approximately the same extent of enhancement occurred for various strains and therefore differences in yield between strains were not substantially affected.

Infection of U937 by YFV Strains

Growth of YFV strains in U937 was comparable with the one observed in PBL and MØ cultures except that virus yields at 24 hours post-infection were much higher in U937 (Fig. 5). At this time differences in yields between strains were maximal.

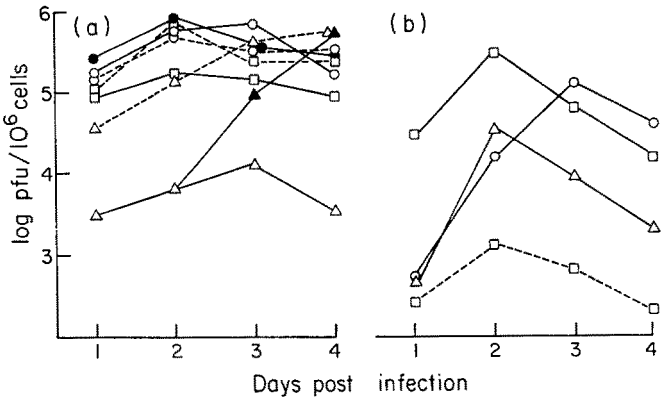

Fig. 5. Comparative replication of attenuated and virulent strains of YFV in U937 cells infected at a MOI of 0.1. Symbols of the strains are the same as in Fig. 2. In *a* solid triangles show the yield of small plaque virus produced by 17D-E-LP

In general the virus recovered after infection of PBL, MØ and U937 with different substrains of 17D had the same plaque size type of the respective virus inoculum. The exception was the growth of the 17D-E-LP variant in U937. The virus produced in these cells during the first 48 hours had the same large plaque characteristic of the parental virus. Thereafter, concomitantly with a marked increase in titer, infectivity consisted predominantly (>90 per cent) of small plaque type virus (Fig. 5a).

Discussion

Wild type and attenuated YFV strains were compared for their ability to grow in cultures of human leucocytes and U937 cells. The five wild type strains used in this study had been isolated from acutely diseased humans or monkeys and therefore were considered virulent in origin. With the exception of one (JSS), these strains had a minimal number of passages in suckling mouse brain, to preserve the composition of the population as close as possible to the original one. The attenuated strains included the Neurotropic strain and several derivatives of the 17D strain. Although all 17D vaccines originate from the strain derived by THEILER and SMITH (23), seeds of different vaccine production centers vary in their history and passage level (26), so that the corresponding vaccine preparations might be expected to differ in some biological properties. In fact, the 17D vaccine preparations used differed in plaque size distribution and, at least, the variants cloned from 17D-E, in neurovirulence for mice (9). When these strains were compared no correlation was found between virulence for primates of the strains and their ability to grow in the three types of cells tested, PBL, MØ and U937. On the contrary, although variation in the efficiency of replication occurred among virulent and attenuated strains, most of the attenuated strains usually produced higher virus yields than the virulent ones. Monocytes were the principal cell type present in MØ cultures and probably the main source of virus replication in PBL, as previously shown by WHEELOCK and EDELMAN (25), and as suggested

in our experiments by the higher yields obtained in MØ compared to PBL cultures. The results presented suggest that for YFV productive infection of monocytes, as detected *in vitro*, is not related to virulence. This conclusion does not necessarily apply to the whole population of mononuclear phagocytic cells that the virus may encounter *in vivo*. It has been shown that macrophages isolated from different anatomical sites or at different stages of maturation may differ in their susceptibility to viruses (3, 15, 16). Even the selection or activation induced by adherence to plastic and *in vitro* cultivation might affect their susceptibility. Virulence for primates of strains of dengue-2 virus correlates with permissiveness of PBL in suspension (7) but not of adherent PBL (2).

Differences in yields between strains were significant, reproducible and unrelated to differences in stability or plaquing efficiency of the various strains. Moreover differences in growth were maintained even when the absolute extent of growth of each virus strain changed depending of factors like multiplicity of infection or presence in the culture medium of diluted antibody.

The reasons for the observed differences remain as a matter of speculation. The virus-cell attachment step appears to be an important factor limiting infection of monocytes by flavivirus. The very occurrence of antibody mediated enhancement of infection suggests that only a fraction of a potentially susceptible cell population becomes infected in the absence of antibody. With different viruses (4), including alphaviruses (10), virulent strains exhibit a lower affinity for cell receptors than avirulent ones. Differences in surface properties among YFV strains might be responsible for differential interactions with human monocytes.

Variation found between 17D substrains is of particular interest. At least one of the substrains tested (17D-E-LP) appeared to replicate less efficiently than the others, as suggested not only by the lower yields obtained but also by the selection against this large plaque variant in favour of a small plaque virus observed in U937 cells. Changes in plaque size composition were observed only upon growth in this cell line. This might be related to the high titers obtained in this system early in the infection, allowing greater chance for mutation and selection to occur, or to a greater selectivity of this cells line compared with primary cultures of leucocytes.

The target cells for the vaccine virus replication in the human host is unknown. Pathogenesis studies in rhesus monkeys infected with 17D strain suggested that virus replication is limited to lymphoreticular tissue (22). Mononuclear phagocytic cells and activated lymphocytes are logical candidates as *in vivo* target cells for the 17D vaccine as suggested by the pathogenesis studies and their *in vitro* susceptibility (25).

Subpopulations of the 17D strain appear to differ in tropism for neural tissue (9) and, as shown in this report, for mononuclear phagocytic cells, both properties relevant to the use of this virus as a vaccine strain. Standardization of the world production of the vaccine has been advocated (26). This should imply a characterization of the various substrains in use and selection of one with optimal attributes. In this regard replication in cultures of human macrophages or macrophage-like cell lines might prove an useful *in vitro* marker eventually related to immunogenicity of different 17 substrains.

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