The In Vivo Differentiation of Strains of Yellow Fever Virus in Mice

By R. FITZGEORGE AND C. J. BRADISH*

formerly of The Microbiological Research Establishment, Porton, Salisbury, England

(Accepted 26 July 1979)

SUMMARY

Strains of yellow fever virus isolated since 1927 in Africa and the Americas, and strains derived from them, have been differentiated by the responses of mice of different ages to intraperitoneal (i.p.) or intracerebral (i.c.) infection. Infection, antibody conversion, protection and death have been presented on age-dose response phase diagrams that serve as in vivo 'fingerprints' for the differentiation of virus strains and their modifications through passage and selection. Correlations between marker characteristics are discussed in terms of the efficiency of infection, regulatory (pre-challenge) and protective (post-challenge) immunity, and the expression of virulence. The requirement in virus strain specification for the resolution of events on pathogenic and immunogenic pathways is discussed.

INTRODUCTION

The identity of a virus strain or variant is established by the pattern of its interactions with a range of responsive hosts. This essential basis for the control of vaccines and therapeutic strategies, or for the specification of infective agents as pathogenic or apathogenic, leads to a number of simplifying correlations. A first correlation is required of the responses of the principal host with those of model or indicator hosts. A second set of correlations may be sought between the phases of the virus–host interaction and the intrinsic characteristics of the infecting virus strain (molecular, serological, in vitro) or of the 'normal' host (genetics, stress, competence) (Bradish et al. 1979).

All such studies require a specification in the virus–host interaction of such qualities as the efficiency of infection, expression of virulence, transmissibility, persistence and immunogenicity. Once the phases of the virus–host interaction have been sufficiently defined, correlations between hosts and with putative in vitro markers may be established.

The present studies with strains of yellow fever virus (YFV) were undertaken in an attempt to define the virus–host interaction and the differentiation of in vivo qualities.

METHODS

Virus strains. The strains of YFV used represent isolations made since 1927 in Africa and the Americas. The YFV strains (Table 1) were obtained as freeze-dried samples through the generosity of Dr J. Casals of the Yale University Arbovirus Research Unit and Dr Y. Robin of the Institut Pasteur de Dakar. These two sources are indicated by Y and D, respectively, in Table 1. All strains were obtained at the lowest passage level available or as

* Address for reprint requests: Sutherlands, Pitton, Salisbury, Wils.
Table 1. Histories of strains of yellow fever virus

<table>
<thead>
<tr>
<th>Strain code</th>
<th>Designation</th>
<th>Origin and passage history*</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1</td>
<td>French viscerotropic</td>
<td>Dakar, 1927: R2, P2, (47), P1, m2</td>
</tr>
<tr>
<td>D2</td>
<td>French neurotropic</td>
<td>Dakar, 1927: R1, m260, (5A), m1</td>
</tr>
<tr>
<td>Y1</td>
<td>Asibi; 5023</td>
<td>Ghana, 1927: human serum, R53, m1</td>
</tr>
<tr>
<td>Y3</td>
<td>Rendu; 82557</td>
<td>Senegal, 1928: human serum, R4, m1</td>
</tr>
<tr>
<td>Y5</td>
<td>Diorbel; SH1276</td>
<td>Senegal, 1953: human serum, m7</td>
</tr>
<tr>
<td>Y6</td>
<td>J.S.S.</td>
<td>Senegal, 1965: human serum, m5</td>
</tr>
<tr>
<td>Y25</td>
<td>BE H69</td>
<td>Brazil, 1935: human serum, R3, m1</td>
</tr>
<tr>
<td>Y26</td>
<td>Suarez; 64846</td>
<td>Brazil, 1955: human serum, m2</td>
</tr>
<tr>
<td>Y27</td>
<td>VI41; 70299</td>
<td>Colombia, 1936: human serum, m3</td>
</tr>
<tr>
<td>Y28</td>
<td>Human vaccine, 1937-38</td>
<td>Colombia, 1959: human serum, m3</td>
</tr>
<tr>
<td>17D</td>
<td>ARILVAX' (RIF free), 1974-78</td>
<td>Human serum, R53 (as in Y1 above), me18, Ce158-218 (Lloyd et al. 1936; Theiler &amp; Smith, 1937)</td>
</tr>
</tbody>
</table>

* Abbreviations: R, Rhesus (Macaca mulatta), passage via mosquito bite, viraemic blood or infected tissue; m, suckling mouse brain; me, minced mouse embryo tissue; C, primary chick embryo cells; Ce, minced chick embryo tissues; P, PS line pig-kidney cell culture; (#), clone picked as plaque from P cell monolayers to generate strains D1 and D2 above.

Fig. 1. A summary of the responses of monkeys (M. mulatta) to i.c., i.s. or i.p. infection by the strains of YFV derived during the passage selection from Y1 Asibi (R53, Table 1) to 17D (1937) in (a) mouse embryos (me), (b) chick embryos (Ce) and (c) mice i.c. The reversion of the 17D vaccine strain to virulence for monkeys i.c. appears to be established within 80 to 130 passages in the brains of mice. Data summarized from Lloyd & Penna, 1933a, b; Lloyd et al. 1936; Theiler & Smith, 1937; Strode, 1951.
Strains of yellow fever virus in mice

long-passaged or selected-adapted material of particular interest as determined by previous studies. The small available quantities of most virus strains necessitated the use of one further suckling mouse brain passage (mi) in this laboratory to produce the working stocks listed. These were stored in minimal amounts of 0.5 ml at -70 °C or in liquid nitrogen. The medium for storage and sample dilution throughout was Parker's medium 199 at pH 7.6 with 10% foetal calf serum. The stability of YFV infectivity during storage will be described elsewhere.

It is evident from Table 1 that some strain passage histories are long and uncertain. The 17D vaccine strain of Theiler and co-workers (Lloyd et al. 1936; Theiler & Smith, 1937; Strode, 1951) is of exceptional interest in view of its production for over 40 years as the standard human yellow fever vaccine. The derivation of the 17D vaccine strain from the 53rd passage of the Asibi strain (Y1 in Table 1) in monkeys is an important example of the changes with passage of some virulence/avirulence marker characteristics. This long course of 'adaptation' is shown in Fig. 1 and could not be reproduced (Strode, 1951).

Assay of 17D YFV infectivity in agar suspension of primary chick embryo cells. Of the strains of YFV considered here only the derivatives of the 17D vaccine strain yielded plaques suitable for the efficient assay of virus infectivity in agar suspensions of primary chick embryo cells. Samples of virus or tissue suspensions to be titrated were diluted serially in medium 199 plus 10% foetal calf serum at pH 7.6. Amounts of 0.5 ml were mixed with 2 x 10⁸ primary chick embryo cells in 2 ml of the same medium at 37 °C. After 15 min at 37 °C, 2.5 ml of 1% agarose (Miles-Seravac Ltd.) in medium 199 at 44 °C were added and the whole rapidly poured into a 5 cm diam. Petri dish. Once set these were kept for 5 days at 37 °C in an atmosphere of air plus 5% CO₂. After flooding with 0.005% neutral red in physiological saline the clear 1 to 2 mm plaques were counted and the virus concentration per ml of initial sample calculated. Replicate estimates of p.f.u./ml correspond very closely with parallel estimates of suckling mouse i.c. LD₅₀ units.

Assay of neutralizing antibody activity. Various samples of human, rabbit and mouse antisera to strains of YFV were assayed for virus neutralizing activity by the plaque reduction procedure. Suspensions of primary chick embryo cells were used as with Semliki Forest virus (Bradish et al. 1971; Fitzgeorge & Bradish, 1973) and adjusted as above for the infectivity assay of 17D YFV. Neutralization indices (SNI) indicating the logarithm of the antibody activity or concentration were calculated as described by Bradish et al. (1962).

For surveys of the neutralizing activities of up to 100 samples a zone inhibition method was used. Here the agarose suspensions of primary chick embryo cells were prepared with a constant confluence of about 1000 YFV (17D) plaques per 5 cm dish. After setting, 6 mm diam. holes were cut to receive 0.03 ml amounts of dilutions of the antisera to be assayed. After incubation for 5 days at 37 °C as above, the plates were stained and the serum neutralization indices (SNI) of the applied samples were indicated as proportional to the diameters of the circles of non-plaque formation that surrounded each sample hole. Standard antisera of known SNI were included in each test series.

Procedures in mice. The mice used in this study were of strains Balb/C (inbred), A2G (limited outbred) and Porton (random outbred). Mice of defined ages (+10%) in groups of five to ten were used for the titration of virus strain samples by the inoculation of 0.025 ml intracerebrally (i.c.) or 0.1 ml intraperitoneally (i.p.) of graded serial tenfold dilutions in buffered physiological saline at pH 7.6. Paralysis was followed by death within 36 h and associated with a high brain infectivity due to YFV. Mice were therefore scored as dead (D) even if killed for tissue and blood sampling at the onset of frank paralysis.

According to virus strain, route and dose, the mice surviving to 14 or 21 days after primary infection were bled for assay of serum neutralizing activity and then lethally challenged by
the inoculation i.c. of $10^4$ p.f.u. in 0.025 ml of the 17D.C1 vaccine strain of YFV. Mice surviving for 14 to 21 days after virulent challenge were scored as protected (P). This category of protection was subdivided further according to the presence (P+) or absence (P-) of detectable neutralizing antibody activity at the time of challenge: thus P+ and P- indicate the incidence of protection in mice showing or lacking antibody conversion. Challenge and other controls were included at equal weight in every test (Bradish et al. 1975).

The infectivities of the virus strain samples used were assayed by direct plaque counting or by titration through the brains of suckling mice. Thus the input dose of potentially infective units was defined as p.f.u. or suckling mouse (SM) i.c. LD$_{50}$ units. This procedure follows that used for unadapted strains of VEE virus (Walder & Bradish, 1975, 1979) which do not regularly form countable plaques.

Since mice of any age were equally sensitive to lethal i.c. infection by the present strains of YFV, the adult mouse i.c. LD$_{50}$ unit was used interchangeably with the suckling mouse i.c. LD$_{50}$ unit as a standard of potential infectivity.

Assessment of responses in mice. The scores in any test showing the incidence of mice dead (D), protected against lethal challenge (P+ or P-) or uninfected and susceptible (S), enable a number of 50% response-doses to be calculated (Reed & Muench, 1938) and related as a ratio to actual inputs of potentially infective units. Thus the number of input infective units required for the LD$_{50}$ was estimated from the incidence of deaths (D) shown through the titration series. Similarly the number of input infective units required for antibody conversion in 50% of the test group was estimated from the incidence of detected neutralizing activity.

The number of input infective units required for infection in 50% of the test group (ID$_{50}$) was estimated from the total incidence of any detectable response to infection (primary death, protection, antibody synthesis and recovery of infective virus). This estimated ID$_{50}$ corresponded with the P-D$_{50}$ if significant protection was observed in the absence of detectable antibody activity.

Efficiency of infection for mice. As in studies with SFV & VEEV (Walder & Bradish, 1975, 1979), the number of infective units required for the ID$_{50}$ in any test indicates the reciprocal of the efficiency of infection. Thus if 1000 p.f.u. are identified with the ID$_{50}$, then the efficiency of infection is only 0.001 under these conditions and 99.9% of input virus is eliminated before progressive replication or immune stimulation can occur. This efficiency of infection is independent of the outcome of infection as death or protection.

RESULTS

Response of mice to intraperitoneal infection

To characterize the response of A2G mice to i.p. infection, groups of seven to ten mice of defined age were inoculated with $10^3$ to $10^4$ infective units of the strains of YFV listed in Table 1. Mice were scored for survival until death (D), protection against lethal challenge (P) or as primarily uninfected and susceptible (S). Except as noted below for the 17D vaccine strains, most mice were infected by the administered doses and showed either paralysis and death, or protection with (P+) or without (P-) detectable antibody conversion.

The results summarized in Fig. 2 show that the % mortalities decline sharply above a certain age at infection so that an age for 50% death:50% protection may be defined in each case. These critical ages at infection for the change from death to protection occur at about 3.5 days old for the 17D(A).C1 vaccine strain at 9 to 12 days old for the majority of
Strains of yellow fever virus in mice

Fig. 2. The responses of groups of 10 or more A2G mice of defined ages to i.p. infection by 10⁹ to 10¹⁰ SM i.c. LD₅₀ units of the various strains of YFV listed in Table 1. ●—●, 17D.C1; □—□, Y₁; ▲—▲, Y₃; ■—■, Y₅; Δ—Δ, Y₆; ○—○, Y₂₇; ▼—▼, D₂. Not shown are strains D₁ (as Y₅) and Y₂₅, Y₂₈ (as Y₂₇). Other abrupt age changes of host response are associated as shown with VEEV and SFV at (b), (c) and (d) (Walder & Bradish, 1975, 1979), herpes simplex virus at (e) (see Discussion and Darlington & Granoff, 1973), lymphocytic choriomeningitis virus at (a) (Lehmann-Grube, 1971) and the graft v. host reaction at (a) (Billingham & Brent, 1957, 1959).

Fig. 3. The average survival times for groups of seven or more A2G mice of 35 to 55 days old to i.c. infection by the various strains of YFV listed in Table 1 and modified as below. (a) Strain D₂; (b) 1st adult mouse brain pass of strain D₂ (D₂.m₁); (c) all Y strains listed in Table 1; (d) strain D₁; (e) strain D₂ neutralized by antiserum from 10⁶ to 10⁷ infective units/ml; (f) strain Y₁ with 3000-fold excess of formalin-inactivated virus.

YFV strains and at 20 to 21 days old for strain D₂. Within the central group of YFV strains [(c) in Fig. 2] showing a protective response at 9 to 12 days old, the African strains (Y₁, Y₃, Y₅, Y₆) tend to show a more sloping characteristic that may indicate an inherent heterogeneity of virus strain population. The South American YFV strains (Y₂₅, Y₂₇, Y₂₈) show the sharpest changes from death to protection for mice 10 to 12 days old at infection.
Fig. 4. The relationship for strains of YFV (Table 1) between survival time in days following i.c. infection and the age in days at infection i.p. for a response of 50% death:50% protection (D/P response) O—O, 10^8 infective units inoculated; ▣—▪, 10^9 ID_{50} inoculated; ▲, strain 17D.C1 at 10^8 infective units; ▼——▼ strain 17D.C1 at 10^3 ID_{50} units; †, the information for monkeys (Macaca mulatta) is extracted from Fig. 1 to show conditions for equivalent D/P response.

Responses of mice to intracerebral infection

Although all of the strains of YFV listed in Table 1 are lethal for adult mice following i.c. infection, there is nevertheless a wide range of survival times before death. This is shown in Fig. 3 for 35- to 55-day-old A2G mice in relation to the number of LD_{50} units administered in 0.025 ml. The strains D2 and D2.m1 (Fig. 3a, b) kill mice most rapidly at 5 to 7 days whereas strain D1 does not kill until 14 to 18 days after infection i.e. The remaining Y strains and 17D vaccine strains all kill mice within 10 to 14 days (Fig. 3c); these results are not shown individually.

When the infectivity of the YFV strain D2 was reduced by antiserum from 10^8 to 10^2 infective units per ml, the survival time in mice was only prolonged from about 7 to 9 days (Fig. 3e). Similarly, when the Y1 strain was inoculated i.c. as 10^{1.5} LD_{50} in the presence of 10^8 initial LD_{50} of formalin-inactivated virus, the survival time was only prolonged from about 11 to 13 days (Fig. 3f).

These results demonstrate that the survival time is a significant in vivo characteristic that is not greatly modified by considerable excess concentrations of inactivated or neutralized virus. Furthermore, the survival times following i.c. infection are only slightly shortened when infective doses increase (Fig. 3). Similar survival times are maintained even when suckling mice are infected i.c. and in adult mice of other strains (Martin, 1969; Darnell et al. 1974).

Relationships between in vivo characteristics

It is important to consider the functional relationships between the in vivo characteristics summarized in Fig. 2 and 3. The age of mouse in days at infection for a response of 50% death:50% protection (from Fig. 2) is shown in Fig. 4 in relation to the survival time for adult mice following i.c. infection (from Fig. 3). The tested strains show a clear reciprocal relationship between these in vivo characteristics for input doses of 10^3 to 10^{3.5} infective units or ID_{50} units. In view of its low efficiency of infection and immunogenicity the 17D(A)
Strains of yellow fever virus in mice

vaccine strain of YFV conforms to the reciprocal relationship (Fig. 4) only when input doses in excess of $10^8$ ID_{50} are considered.

The general relationship between the survival time following i.c. infection (Fig. 3) and the age for immunological maturation to the level required for protection i.p. (Fig. 2) indicates that even the i.c. infection in adult mice is regulated by an effective immunological feed-back. Thus the French 'viscerotropic' strain DI kills adult mice most slowly following i.c. infection and fails to kill even 9-day-old suckling mice following i.p. infection. Recent studies with distinct strains of VEE virus (Walder & Bradish, 1979) have also shown that survival time and age-response characteristics are indicators of the balances between events on pathogenic and immunogenic pathways.

Relationships to tests in monkeys

The wide use of monkeys, particularly Macaca mulatta, for the assessment of the in vivo characteristics of virus strains prompts the comparison of the pathogenic-immunogenic balances in mice and monkeys under different conditions of infection. The early information summarized in Fig. 1 shows the responses of M. mulatta to i.c., i.s. (intraspinal) or i.p. inoculation of YFV strains on the passage-adaptation route between Y1 and the derived 17D vaccine (Table 1). This indication of the strain-route conditions provoking a response of 50% death:50% protection may be compared with those summarized for mice of different ages in Fig. 2. On this basis the 50% death:50% protection responses of monkeys to a YFV strain inoculated i.c., i.s. or i.p. correspond with those of mice between 6 and 12 days old inoculated i.p. Thus, despite detailed differences in tissue tropism and cellular mechanism, the balances between pathogenic and immunogenic events that determine death or protection in monkeys can be paralleled closely in developing mice of the appropriate age at infection. This rationalization of distinct in vivo indicators in monkeys (i.c., i.s., i.p.; Fig. 1) and in mice (i.c., i.p.; Fig. 3) is shown in Fig. 4 and discussed later.

Dose-response relationships for 17D(A) strains of YFV

In view of the features (Fig. 4) shown by the important 17D vaccine strains of YFV, a more detailed study was made of the responses of mice of different ages to the i.p. administration of graded doses. Mice of strain A2G were observed for sickness or paralysis for 21 to 35 days when they were bled for antibody and then challenged i.c. with $10^4$ p.f.u. of the 17D.C1 strain of YFV. Thus host scores were available through graded doses of virus for estimations in mice of different ages of the p.f.u. required for 50% lethality (LD_{50}), 50% protection (PD_{50}, ID_{50}) or for 50% protection with (P+D_{50}) or without (P-D_{50}) detectable antibody activity in serum.

The results (Fig. 5) for these several individual titrations through groups of seven to ten mice indicate that about $10^6$ p.f.u. i.p. are required for the LD_{50} in 3- to 4-day-old mice. This rises rapidly to about $10^6$ p.f.u. i.p. for the LD_{50} in mice of 20 days old or older and defines a steeply rising LD_{50} age characteristic (Fig. 5a).

Estimations of the p.f.u. dose for protection in the absence of detectable antibody synthesis (P-D_{50}) show that 1 p.f.u. i.p. infects and protects mice up to 5 days old. This dose rises rapidly to the requirement for about $10^4$ p.f.u. i.p. for infection and protection at 20 days old (Fig. 5c). For both the 17D mouse passed (17D.m1) and CEC passed (17D.C1) strains the p.f.u. doses i.p. for detectable antibody synthesis (P+D_{50}) were regularly about 30-fold higher than the infecting-protecting dose (P-D_{50}): this is shown by the response lines (b) and (e) in Fig. 5.

Results for actual individual levels of serum antibody activity are not quoted in detail since mice were either strongly positive, with serum neutralization indices of 2·5 to 3·5, or...
Fig. 5. The age–dose response phase diagram for A2G mice infected i.p. Each point on the diagram summarizes a complete titration series in mice of the age shown at the input dose of infective units (p.f.u. or SM i.c. LD₅₀) required for the 50% response. ■, □ and line (a), LD₅₀ for strain 17D.C₁ or 17D.m₁; ▼ and line (b), P⁺D₅₀ or dose for 50% antibody conversion for strain 17D.C₁; ● and line (c), dose for 50% protection and infection (P⁻D₅₀ or ID₅₀) for strain 17D.C₁; broken line (d), LD₅₀ for strain Y₁ from Fig. 6. The phase zones D, P⁺, P⁻ and S of age–dose indicate respectively the outcomes of death, antibody conversion, infection and protection without antibody synthesis, and susceptibility to challenge.

clearly negative (SNI < 1). Intermediate SNI levels were rare. Thus the incidence of positive antibody conversion to SNI 3 ± 0.5 is shown by the P⁺D₅₀ characteristic (Fig. 5b).

A consequence of this sharp dose-dependent switch from undetectable to strong stimulation of antibody synthesis is that these results do not depend upon the actual sensitivity of antibody activity detection. This applies throughout to A2G and Balb/C mice with many strains of YFV.

The multiple dose–response characteristics of the type shown in Fig. 5 form a phase diagram or in vivo fingerprint by which virus strains may be identified by the pattern of interactions of pathogenic and immunogenic events. Thus zone D (Fig. 5) embraces the range of mouse ages and virus doses associated with paralysis and death and, as shown in Fitzgeorge & Bradish (1980), the proliferation of virus in brain to high levels (10⁶ to 10⁸ p.f.u./brain). The intermediate zone P⁺ indicates the range of ages and doses for which protection against virulent challenge and elimination of brain infectivity is also accompanied by high serum antibody activity. The zone P⁻ indicates the range of age–dose conditions for which virus stimulation ensures protection against challenge but not the detectable synthesis of antibody. Virus doses for antibody conversion are about 30-fold higher than those required for infection and protection. The lowest line (c) defines, through the age dependence of the ID₅₀ and P⁻D₅₀, the efficiency of infection and of stimulation. In the zone S the mice are uninfected and normally susceptible to the virulent challenge.
Strains of yellow fever virus in mice

Fig. 6. The age–dose response phase diagrams compared for YFV strains 17D.C1 (broken lines) and Y1 (solid lines); see Fig. 5 for details. L, dose in infective units for i.p. LD50; P+, dose in infective units for 50% antibody conversion i.p.; P-, dose in infective units for 50% infection and protection. Bars on LD50 lines represent age at infection for response of 50% death: protection following administration of 10⁸ infective units i.p. See text for description of zones A and B.

Fig. 7. The age–dose response phase diagrams compared for YFV strains D1 (broken lines) and D2 (solid lines); see Fig. 5 and 6 for other details.
Response phase diagrams and the differentiation of virus strains

When parallel tests like those of Fig. 5 were made in A2G or Balb/C mice of defined ages with the YFV strains Y1, D1 and D2, the phase diagrams shown in Fig. 6 and 7 were obtained. The many distinctions between these patterns of responses to i.p. infection are demonstrated when the lines for the 17D.C1 strain are superimposed upon the phase diagram for the Y1 (Asibi) strain (Fig. 6). For the Y1 strain, mice younger than about 11 days old died with paralysis and CNS involvement, whereas mice older than about 15 days were uniformly protected and showed no infectivity in tissues. In mice from 11 to 15 days old infected i.p. by Y1 (Asibi), the pathogenic (LD50) and immunogenic (ID50, PD50) presentations diverge rapidly due to the development of an efficient immunological regulation.

The differentiation of these typical strains of YFV is shown through several features of these superimposed phase diagrams (Fig. 6). In Zone A the Y1 strain is lethal and the 17D strain protective. In Zone B the Y1 strain is protective but the 17D strains fail to infect. Evidently the efficiency of infection and rate of immune stimulation by the Y1 Asibi strain of YFV are significantly higher than those shown by the 17D vaccine strain. This emphasizes the critical interactions between events on pathogenic and immunogenic pathways that follow infection and stimulation by different strains of YFV.

This differentiation of response phase diagrams has been extended to the strains of virus listed in Table I and is shown for the extreme strains D1 and D2 in Fig. 7. Both of these strains show a higher efficiency of infection so that 10 p.f.u. i.p. is a 50% infective and protective dose even in mice of 30 days old or older. As for all of the strains tested (Fig. 5, 6 and 7), an input dose of about 30 ID50 or P−D50 is required before positive antibody stimulation can be detected in the majority of test mice. These D1 and D2 strains of YFV show LD50 characteristics (Fig. 7) that rise steeply with mouse age; for an i.p. dose of 10^8 infective units the D1 strain protects mice over 8 days old whereas the D2 strain continues to kill mice up to about 21 days old. This information (as for Fig. 2) indicates that these particular strains are most sensitively differentiated in mice of about 15 days old for which D2 is lethal but D1 protective (zone A in Fig. 7).

Appropriately selected tests based on the response phase diagrams (Fig. 5, 6 and 7) distinguish and differentiate each strain of YFV in terms of its age-dependent efficiency of infection, immune stimulation (ID50 and PD50 lines) or lethality (LD50 lines). In most cases two strains of YFV may be differentiated as illustrated above, by more than one of these characteristics in different age-dose zones of the phase diagrams. It is particularly to be noted that any strain of YFV may be lethal or protective and may or may not stimulate vigorous antibody production, according to the age of mouse and virus dose administered.

**DISCUSSION**

The in vivo differentiations outlined in this paper show that efficiency of infection (ID50 per infective units), expression of virulence, regulatory immunity (pre-challenge) and protective immunity (post-challenge) are separate and dose-dependent qualities of the progressive virus-host interaction. These qualities have been resolved more clearly through titrations in mice of different ages and levels of immune competence.

The critical maturation changes that may occur in mice at definite ages of about 3, 7, 11 and 20 days old influence these host responses to some but not all strains of YFV. A similar observation has been made for strains of SFV and VEEV (Walder & Bradish, 1975, 1979). The changes in mice at about 2 to 3 days old indicated by the graft v. host reaction and responses to LCM virus (Billingham & Brent, 1957, 1959; Lehmann-Grube, 1971) may
Strains of yellow fever virus in mice

Table 2. Differentiation of in vivo qualities of YFV strains

<table>
<thead>
<tr>
<th>YFV strain*</th>
<th>Adult mice i.c. survival (days)†</th>
<th>Age for 50% death: protection after i.p. infection (days)‡</th>
<th>Efficiency of infection and protective immunization in adult mice i.p.</th>
</tr>
</thead>
<tbody>
<tr>
<td>D2</td>
<td>5</td>
<td>20–21</td>
<td>0.5–1</td>
</tr>
<tr>
<td>D1</td>
<td>14</td>
<td>8–10</td>
<td>1.5–2</td>
</tr>
<tr>
<td>Y1, Y3, Y5</td>
<td>8–10</td>
<td>10–12</td>
<td>2.5–3</td>
</tr>
<tr>
<td>Y25, Y27, Y28</td>
<td></td>
<td></td>
<td>3–4</td>
</tr>
<tr>
<td>17D.m1</td>
<td>8–10</td>
<td>3–5</td>
<td>4</td>
</tr>
<tr>
<td>17D.C1</td>
<td></td>
<td></td>
<td>5</td>
</tr>
</tbody>
</table>

* For details of strains see Table 1. † Data from Fig. 3. ‡ Data from Fig. 2.

be associated with the maturation of a T-lymphocyte function. This does not exclude other emerging lymphocyte or macrophage interaction functions that may be observed by their effects in mice at about 11 to 13 days old (see Fig. 2 and for herpes simplex virus: Johnson, 1964a, b; Stevens & Cook, 1971; Darlington & Granoff, 1973). A change in the responses of the present strain of A2G mice to HSV-1 has been observed at 12 to 16 days old (Dr E. Boulter, personal communication). It is important to note that these several distinct changes in the susceptibilities of mice up to 30 days old (Fig. 2) are distinct from the single-gene regulation of susceptibility proposed by Darnell et al. (1974) for adult mice over about 50 days old. These phases and compartments of host response to virus infection are considered more fully in Fitzgeorge & Bradish (1980).

Arising from these several in vivo criteria and their presentation as response phase diagrams (Fig. 5, 6 and 7), the strains of YFV virus may be differentiated as summarized in Table 2. The last two columns quantify the efficiency of infection and the events on the immunogenic pathway that may subsequently modulate the expression of virulence signified through the first two columns. According to the strain of virus, there is a very wide range of efficiency of infection in young adult mice from about 3 to over 10⁵ infective units per i.p. ID₅₀; the stimulation of vigorous antibody synthesis in A2G or Balb/C mice uniformly requires about 30-fold higher doses of virus. Thus in all phase diagrams the P–D₅₀ and P+D₅₀ lines are separated by a distance of about 1.5 log dose units.

An examination of Table 2 indicates, as for the phase diagrams, that the in vivo characteristics of LD₅₀–age relationship, survival time following i.c. infection and age for change of response to i.p. infection are mutually related (Fig. 2, 3 and 4) and distinct from the initiating efficiency of infection and stimulation (ID₅₀ and P+D₅₀, i.p.). This emphasizes the early divergence of the pathogenic and immunogenic pathways. The responses of monkeys to i.c., i.p. and i.s. infections by strains of YFV (Fig. 4) appear to parallel those of 6- to 12-day-old mice. This suggests that under appropriate conditions different hosts may show a similar interaction between events on pathogenic and immunogenic pathways. In view of the diversity of antibody and lymphocyte classes and their dynamic involvements with some virus strains and not others (Fig. 2), it is probable that the present operational approach to the resolution of in vivo pathways and interactions may allow correlations to be made between the responses of different hosts before each phase of host response can be interpreted in molecular, cellular or genetic detail.
The strains of YFV of African origin (Y1 to Y18) are marginally and regularly different from those of S. American origin (Y25 to Y28). Thus African strains (Table 2) show a higher efficiency of infection and stimulation and may be more heterogeneous with respect to protection of mice (Fig. 2). A greater antigenic range was noted by Clarke (1960) for African strains of YFV which showed an antibody-combining component not present in S. American strains.

Although the manipulations by which the strains D1, D2 and 17D have been derived (Table 1, Fig. 1) are long and irreproducible, it is clear from the in vivo criteria summarized in Table 2 that these three strains illustrate different routes of variant selection from the parental 'Y' stock. The 17D vaccine strain appears as a selection or variant of greatly impaired efficiency of infection and stimulation yet unchanged capacity to involve the target site once infection is established. The D1 and D2 strains, by contrast, show improved efficiency of infection and protection associated with enhanced (D2) or reduced (D1) involvement of the CNS. These distinct selections emphasize not only that changes in pathogenicity and immunogenicity may be independent but that, as here, separate in vivo characteristics may be required for identification. The recent review by Kantoch (1978) cites several cases in which markers and assessments of vaccine immunogenicity are unrelated to the markers and assessments of vaccine pathogenicity (attenuation). Clearly an improved quantification and resolution of host-response pathways to any series of virus strains must precede their correlation with putative in vitro or molecular markers.

A recent summary of the responses of human volunteers to infection by a stabilized 17D(A) YFV vaccine (Freestone et al. 1977) indicated a subcutaneous dose of about 40 infective units (p.f.u.) for 50% antibody conversion on the 28th day. Positive antibody responses showed a serum neutralization index of about 3 and 2.8 for $10^8$ and $10^9$ infective units, respectively. Natural primary infections by 'wild' strains of YFV usually show specific seroconversion in man in about 3 weeks with levels rising in the following weeks (Theiler & Downs, 1973). During the earlier acute phases of invasion and stimulation human sera are rarely antibody positive. Mason et al. (1973) have studied the responses of Rhesus monkeys to 17D intramuscular vaccination followed by virulent challenge 20 weeks later by 1000 infective units subcutaneously of the Y1 (Asibi) strain of YFV. The 50% protective dose was about 20 infective units (suckling mouse i.c. LD$_{50}$) and only monkeys later shown to be protected had positive serum neutralization indices of about 2.5 from the 4th to the 20th week. The elevation of these serum neutralization indices to about 5 within 2 weeks following challenge demonstrated the relatively poor immunogenicity of the 17D vaccine which failed to stimulate some compartments that then responded rapidly to the 'wild' challenge. These studies generally show that man and monkeys are efficiently infected by less than 20 to 40 infective units of the 17D vaccine strain of YFV but that the stimulation of antibody synthesis or protection may be relatively slow or inefficient even at doses of up to $10^6$ infective units. Although the range of test conditions in such studies precludes closer comparisons, it is probable that the mouse can be used as a much more sensitive indicator of strain differences and of the critical phases of the virus–host interaction that now limit the standardization of vaccine heterogeneity, pathogenicity and immunogenicity.

The authors are grateful to the following colleagues for willing co-operation and debate: Mr D. Titmuss, Mrs I. White, Mr S. Leighton, Dr Lynn Francis, Mr P. Buck, Mrs D. Batter-Hatton and Dr W. Ford. Parts of this study contribute to a thesis by R.F. for the Ph.D. degree of the University of Reading.
Strains of yellow fever virus in mice

REFERENCES


(Received 15 March 1979)