Infection, Interaction and the Expression of Virulence by Defined Strains of Semliki Forest Virus

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SUMMARY

In these studies in mice, guinea pigs and rabbits infected by intraperitoneal, intracerebral or respiratory routes, the expression of virulence by a virulent/avirulent mixture of known proportion depended on the administered dose and was not a simple marker for the virus population, or for the heterogeneous wild strain which it simulated. This dependence of the virulence of a virus sample upon its dose and heterogeneity is presented quantitatively for each host by a dose–response diagram which is the necessary extension of the simple dose–response curve. The latter may be used to express single response characteristics (death only, protection only) but is inappropriate to the expression of the present dual response–dose characteristics in which protection at low dose gives place to death at high dose, or vice versa. At some proportions of virulent/avirulent sub-populations in the virus inoculum even more complex dual response–dose characteristics may be generated. Thus the specification of virulence requires the presentation of a dose–response diagram for each relevant host and route of administration of virus.

Notwithstanding these seeming complications, basic types of virulent/avirulent interaction have been demonstrated and arranged in sequence according to the susceptibility or responsiveness of the host–route systems investigated. With closer definition of population heterogeneity and dose–response relationships, other virus–host–route systems will probably fit within similar sequences.

These results are interpreted in terms of a dynamic interaction between distinct lethal and protective responses and are relevant to problems involved in the design and testing of live vaccines.

INTRODUCTION

The expression of virulence is the consequence of many factors (Nathanson & Cole, 1970; Bradish, Allner & Maber, 1971; Bradish & Allner, 1972) which may be considered in terms of the genotypic properties of the administered virus population and the responsive potential of the host to virus invasion by the given route. The interaction between these determines the time course and outcome of competition between lethal, intermediate and protective responses. The availability of defined strains of Semliki Forest virus of extreme virulence and avirulence (Bradish et al. 1971) made it possible to investigate these factors, and to probe the role of heterogeneity of the inoculum in the expression of virulence. These mechanisms are important in the design and control of live vaccines which may contain virulent particles at a sufficiently low concentration for their virulence to be overwhelmed by the protective responses triggered by avirulent particles. The dynamic basis of such in vivo interactions has been investigated in the infection of mice, guinea pigs and rabbits by defined strains or mixtures of Semliki Forest viruses.
METHODS

Viruses and infectivity assay. The avirulent (A7.C1, A774) and virulent (V13.C1, L10.C1) strains of Semliki Forest virus (SFV) used in this study were those characterized by Bradish et al. (1971). The assay of virus infectivity by plaque counting in agar suspensions of primary chick embryo cells was also described by these authors.

Infection of mice. Randomly bred Porton white mice were used as weanlings or adults of various ages (± 5 days) up to 200 days old. For each test sample and group, 5 or 10 mice received intraperitoneal (i.p.) or intracerebral (i.c.) inoculation of 0.025 ml of an appropriate virus dilution prepared in Parker's medium 199 with 1 % v/v calf serum or 0.3 % w/v bovine serum albumin. The calf serum was pretested for non-toxicity and efficiency in plaque enhancement. Plaque assays of the same samples were made simultaneously. The sensitivity and pattern of mouse response in relation to age, competence and strain of virus was described by Bradish et al. (1970).

Dual response of mice to infection by SFV. In view of the complex response to primary infection by which some mice in a test group may die and others be immunized (Bradish et al. 1971), the mice were scored as numbers dead (D), protected (P) or uninfected and therefore susceptible (S) to subsequent lethal challenge. The sum D + P + S = N gives the number of mice in the test group. At 14 to 18 days after primary infection all survivors were challenged by i.c. or i.p. inoculation of 100 LD50 (10^3 to 10^4 p.f.u.) of virus of virulent strain L10.C1 or L10.H6.C1; mice which then died specifically within 3 to 14 days were classified as S and those surviving due to primary protection as P. In previous studies the sera of most immunized mice (P) had primary and secondary serum neutralization indices (SNI) of more than 1.5 and 3.5, respectively.

Infection of guinea pigs and rabbits. Random-bred Porton white guinea pigs of 3 to 15 weeks old and Copenhagen white rabbits of 0.5 to 3 kg. body weight were infected, as for mice, with defined doses of virus by i.p., i.v., i.c. or respiratory routes (v.a.) (Bradish et al. 1971). Challenge by 10 to 100 LD50 of a virulent strain of virus (L10.C1, L10.H6.C1) was made by similar procedures at from 6 hr to 30 days later. The responses of test animals following primary infection and later lethal challenge were coded as discussed above for mice and as amplified later.

Presentation of results. Bradish et al. (1971) showed that the infectivity in p.f.u./ml., as assayed in agar suspensions of primary chick embryo cells, corresponded closely with the assay of infectivity in suckling mice and may be taken as the datum unit of potential infectivity against which animal responses may be standardized. In this study the animal responses were expressed as the median infective dose in p.f.u. required for (1), primary infection of 50 % of the test group to give a response of protection or death; p.f.u./ID50. (2) Primary protection of 50 % of the test group; p.f.u./PD50. (3) Primary death of 50 % of the test group; p.f.u./LD50. In each of these test groups 50 % remain uninfected and susceptible to subsequent lethal challenge. At greater doses of virus all animals were infected and it was necessary to define the infective dose required for protection of 50 % and death of 50 % of the test group; p.f.u./'PL'D50. In all of these cases, 50 % doses were calculated by the method of Reed & Muench (1938).
**RESULTS**

*Heterogeneity of virus and progression of virulence markers*

The heterogeneity of virulence of early strains of Semliki Forest virus is illustrated in Fig. 1 by the results for two representative strains (A7.c1, L10.c1) of extreme virulence. Isolated plaques containing from $10^5$ to $10^8$ p.f.u. were cut in 1 cm squares from agar suspensions of primary chick embryo cells and resuspended as described by Bradish *et al.* (1971). Graded or single doses of these resuspended plaque samples were inoculated directly into 25 to 30 day old mice (*i.c.* or *i.p.*) or 3 week old guinea pigs (*i.c.* or *i.p.*) and all animals surviving to 14 days were challenged lethally (*mice *i.p.* or guinea pigs *i.c.*) with 1000 p.f.u. of virulent virus of strain L10.c1 or L10.h6.c1. The incidence of death or protection indicated the virulence of the selected plaque sample. The results for single doses of about 1000 p.f.u. of each plaque sample provided a virulence marker in terms of the incidence of death, whereas the results for graded doses of virus (also Fig. 2) provided the more quantitative virulence markers of p.f.u./LD50 or p.f.u./PD50.

Since there was no overlap of these virulence markers they are presented (Fig. 1) as a progression (*mice *i.c.*, *mice *i.p.*, guinea pigs *i.c.*, guinea pigs *i.p.*) in which the least virulent
Fig. 2. Influence on virulence of passage in hamster brains of virulent (L10.C1) and avirulent (A7.C1) strains of Semliki Forest virus. Weanling hamsters were inoculated intracerebrally with $10^3$ to $10^6$ p.f.u. in clarified brain suspensions from the previous passage. Animals were killed and brain suspensions prepared at 3 or 4 days after infection. A, virulence of strain L10.C1 passages tested in 30-day-old mice, i.p.; B, virulence of strain L10.C1 passages tested in 3-week-old guinea pigs, i.c.; C, virulence of strain A7.C1 passages tested in 30-day-old mice, i.p.; D, virulence of strain A7.C1 passages tested in 3-week-old guinea pigs, i.c.

Clone of virus was benign for mice i.c. (strain A774; $>10^8$ p.f.u./LD$_{50}$) and the most virulent uniformly lethal for all host-routes in the gradient of responsiveness (left to right) up to guinea pigs i.p. A plaque sample in any of the four test blocks of Fig. 1 is uniformly lethal at low dose ($10^3$ p.f.u.) in all tests to the left and uniformly benign in all tests to the right. Appropriate host-route systems were selected for testing each plaque sample according to its likely virulence. Strains A7.C1 and L10.C1 were widely heterogeneous but did not overlap in tests on 40 to 42 plaques from each population (Fig. 1); strain A7.C1 replicated benignly in mice following i.p. inoculation, whereas strain L10.C1 was uniformly lethal. The mean virulence of the parent populations of virus tended to represent the virulence of their more virulent sub-populations. Other early strains of Semliki Forest virus were similarly heterogeneous, and their mean virulences are shown at the top of Fig. 1.

Animal passage as a selection procedure

To test further the possibility that virus of parent strains A7.C1 or L10.C1 contained minority sub-populations of extreme virulence or avirulence, these strains were passed six times sequentially in the brains of weanling hamsters. At each passage the hamster brains were removed and suspended for assay of infectivity and virulence markers before further inoculation into the brains of weanling hamsters.

The virulence markers of p.f.u./LD$_{50}$ (Fig. 2) for strain L10.C1 virus in mice i.p. (line A) or guinea pig i.c. (line B) did not change during the six passages. The virulence markers at each passage for strain A7.C1 did not change in p.f.u./LD$_{50}$ for guinea pigs i.c. (line D) but increased within two passages from $10^5$ to $10^6$ p.f.u./LD$_{50}$ for mice i.p. (line C). Further selection for greater virulence did not occur in passages 3, 4, 5 or 6 and the degree of
Infection, interaction and virulence

Table 1. Response of 100-day-old mice to intraperitoneal infection by Semliki Forest virus in mixed inocula of virulent and avirulent particles

Entries in the body of the table show the numbers of mice dead (D): protected (P): uninfected (S) at the specified inoculum.

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<thead>
<tr>
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<tbody>
<tr>
<td></td>
<td>10³ : 1</td>
<td>10² : 1</td>
</tr>
<tr>
<td>0:2</td>
<td>0:0:10</td>
<td>0:0:10</td>
</tr>
<tr>
<td>2</td>
<td>0:2:8</td>
<td>0:2:8</td>
</tr>
<tr>
<td>20</td>
<td>0:10:0</td>
<td>0:10:0</td>
</tr>
<tr>
<td>200</td>
<td>0:10:0</td>
<td>0:10:0</td>
</tr>
<tr>
<td>2×10⁵</td>
<td>0:10:0</td>
<td>0:10:0</td>
</tr>
<tr>
<td>2×10⁶</td>
<td>0:10:0</td>
<td>0:10:0</td>
</tr>
<tr>
<td>2×10⁷</td>
<td>0:10:0</td>
<td>0:10:0</td>
</tr>
<tr>
<td>p.f.u./PD 50</td>
<td>4:4</td>
<td>4:4</td>
</tr>
<tr>
<td>p.f.u./LD 50</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>p.f.u./PL'D 50</td>
<td>10⁶</td>
<td>10⁶</td>
</tr>
<tr>
<td>% of mice protected in zone between 0 and 100 % lethality</td>
<td>—</td>
<td>85</td>
</tr>
</tbody>
</table>

Virulence shown by L10.C1 was not reached. These experiments support the conclusion that the population represented by strain A7.C1 did not contain a minority of virus particles of the virulence of strain L10.C1. Selection from the parent A7.C1 to a new equilibrium population of higher virulence at the limit of that of the parent (Fig. 1) clearly occurred rapidly within two passages in hamster brains.

Infection of mice: heterogeneity, interaction and expression of virulence

In a series of experiments designed to test the expression of virulence by heterogeneous inocula, groups of 10 to 20 adult mice of 100 + 10 days old, and at maximum susceptibility (Bradish et al. 1971), were inoculated intraperitoneally with 0.025 ml. of virus mixtures prepared to contain from 0 to 10⁴ p.f.u. of the virulent, lethal strain (L10.H6.C1) with from 0 to 10⁶ p.f.u. of avirulent, protective strain (A7.C1). Fourteen days later the survivors were challenged by i.p. inoculation of 100 LD₅₀ of the virulent strains. The responses (Table 1), scored as described in Methods, allowed estimation of the numbers of p.f.u. of virulent and avirulent virus in the mixed inocula which showed the median responses of 50 % dead with 50 % uninfected, 50 % protected with 50 % uninfected, 50 % dead with 50 % protected. The dose–response diagram (Fig. 3) summarizes these results.

Only 4 to 8 p.f.u. were required as the i.p. infective dose for protection (PD₅₀) by the avirulent strain (A7.C1) or for death (LD₅₀) by the virulent strain (L10.H6.C1). When mice were infected by the series of mixtures shown in the middle columns of Table 1 there was a clear interaction between lethal and protective responses and a zone of protection at intermediate doses was followed by death only at the highest doses. Thus the expression of virulence following infection was modified by the dose at which the sample was inoculated.

The dose–response diagram (Fig. 3) for the i.p. infections shows the interaction between lethal and protective responses as the series of mixtures of virulent and avirulent virus
Fig. 3. Dose-response diagram for 100-day-old mice showing proportions of virulent and avirulent infective units (p.f.u.) in mixed inocula giving 1 PLD 50 for intraperitoneal (●) or intracerebral (○) inoculation. Zone S, mice uninfected and susceptible to challenge; zone C, mice protected following i.p. or i.c. infection; zone B, mice protected by i.p. infection but killed by i.c. infection; zone A, mice killed by i.p. or i.c. infection; line M, response to dilutions of mixtures of avirulent and virulent particles in ratio 100 : 1.

which gave the response of 50 % protection with 50 % death. The slope of the i.p. interaction line indicates the approximate relationship that infection was protective only if the number of avirulent infective units (p.f.u.) exceeded the square of the number of virulent infective units (p.f.u.). Thus, as shown by the 45° line M, a virus sample containing subpopulations of avirulent and virulent particles was protective at small doses represented by points below the i.p. interaction line but became lethal at larger doses represented by points above the interaction line. Thus 10³ virulent infective particles (200 i.p. LD 50) failed to kill if in the presence of 10⁶ or more avirulent infective particles. By contrast, the almost horizontal i.c. interaction line shows that no effective avirulent–virulent interaction occurred since the minimal lethal dose of about 2 p.f.u. of the virulent strain ensured death even in the presence of at least 10⁷ avirulent infective units.

Use of the dose–response diagram

The dose–response diagram (Fig. 3) used to express the responses of mice to infection by heterogeneous inocula is an extension of the familiar sigmoid response curve which is restricted to the presentation of a simple response of protection only or death only. The dose–response diagram is an effective phase diagram since inocula of compositions lying in Zone S failed to infect, those in Zone C were protective following i.p. or i.c. inoculation, those in Zone B protective in i.p. inoculation but lethal in i.c. inoculation, and those in Zone A were lethal by both routes.

The line M in the diagram shows the response to dilutions of the virus sample with an avirulent to virulent particle ratio of 100 : 1. This was protective i.p. and i.c. at low doses below 100 p.f.u., protective i.p. and lethal i.c. at doses between 100 and 10⁴ p.f.u., and lethal by both routes above 10⁴ p.f.u. This example illustrates the value of the dose–response diagram in the interpretation of host responses to infection by heterogeneous virus populations.
Interaction of responses in guinea pigs

The responses of 3-week-old guinea pigs to infection through intracerebral or intraperitoneal routes were followed by methods similar to those described in the previous section for mice. Mixed inocula of SFV were prepared to contain known proportions of infective units (p.f.u.) of virulent and avirulent strains. Five guinea pigs were inoculated at each of five tenfold dilutions of six initial mixtures of avirulent/virulent ratio from $10^6$ to $10^8$. Survivors at 14 days after primary infection were challenged by i.c. inoculation of $100$ LD$_{50}$ (1000 p.f.u.) of virulent virus ($10^6$. ci).

The responses observed following intracerebral inoculation are summarized in the dose-response diagram of Fig. 4. Here, as in the dose–response diagram for mice (Fig. 3), it is necessary to distinguish the range of inocula giving responses of 50% uninfected with 50% dead (LD$_{50}$), 50% uninfected with 50% protected (PD$_{50}$) and 50% dead with 50% protected (PLD$_{50}$). The latter response for guinea pigs infected intracerebrally was generated by any of the mixed inocula on the interaction line shown in Fig. 4: thus $10^5$ virulent infective units, equivalent to about $10^4$ LD$_{50}$, failed to cause death if in the presence of about $10^8$ p.f.u. ($10^7$ PD$_{50}$) or more of avirulent infective units.

A feature of the avirulent/virulent interaction following intracerebral infection of the guinea pig is the unit slope of the characteristic (Fig. 4) which indicates that over a wide range of doses greater than $10^4$ p.f.u. a particular virus population having avirulent/virulent particles in the ratio 2000:1 killed about 50% and protected about 50% of the test animals. Test animals were protected by doses of $10$ to $10^4$ p.f.u. at this ratio. There was thus a continuous balance between the lethal response induced by the minority and more virulent subpopulation and the protective response induced by the majority and more avirulent subpopulation.

Intracerebral inocula of avirulent/virulent ratio greater than 2000:1 were protective at all doses above $10$ p.f.u. Similarly, inocula of ratios less than 2000:1 were uniformly lethal at high doses but showed a zone of partial protection at low doses from $10$ to $1000$ p.f.u.
About 80 to 90% of guinea pigs of the present strain were protected by intraperitoneal inoculation of any of the most virulent strains of virus (200 to 300 p.f.u./PD₅₀, i.p.). These apparently anomalous dose–response relationships were noted for the guinea pig in a previous study (Bradish et al. 1971) of infections by several natural strains of SFV. We conclude that, as for the present experiments, these responses reflected the heterogeneity of the virus populations with respect to virulence (Fig. 1) which, after primary replication from low input doses, induced distinct lethal and protective responses which interacted dynamically according to the dose–response diagram of Fig. 4.

Interaction of responses in rabbits

The responses of rabbits (Table 2) to respiratory infection by a range of doses of the natural virulent and avirulent strains of SFV were studied as for mice and guinea pigs above. Procedures for the generation of 0.5 to 2 μm aerosol particles and for the subsequent sampling and animal scoring have been described (Bradish et al. 1971).

The inhalation of about 200 p.f.u. of either strain of virus, equivalent to the retention of about 60 p.f.u., infected about 50% of these rabbits. Infections by 500 to 5000 retained p.f.u. of avirulent virus gave responses of benign protection (25/25) whereas infections by virulent virus or avirulent/virulent mixtures were almost uniformly lethal (51/54).

At doses of retained virus greater than 10⁴ p.f.u. the virulent or mixed infections showed an apparent anomaly of fewer deaths until, at retained doses of over 10⁶ p.f.u., only 3 of 14 rabbits died and all survivors were immune.

By analogy with the interactions already discussed for mice and guinea pigs, the present results for rabbits suggest that the available virulent strains retain a majority proportion (3 : 1 to 10 : 1) of infective units which are avirulent for rabbits in respiratory infection. Alternatively, the massive inhalation of particles which are wholly virulent at lower doses triggers a simultaneous protective response which is sufficiently vigorous to block its lethal potential. In either case, the complex dose–response relationship demonstrates again the competition of protective and lethal responses and indicates a dose of about 10⁶-⁷ retained p.f.u. for 50% protection : 50% death (PLD₅₀).

Time factors in virulent–avirulent interaction

The generally high efficiency and rapidity of infection, even by avirulent particles inoculated intraperitoneally at small doses, and the rapid development of protection in mice (Bradish & Allner, 1972) suggested that the responses to infection by heterogeneous inocula were determined by the rates of replication at primary sites and the consequent rates of development of potential lethal and protective responses which then competed for expression of the terminal response.

To test these rate conditions more fully, the previous experiments with heterogeneous inocula of virulent and avirulent particles were extended so that various doses of the avirulent component were inoculated at a given route from 4 hr to 14 days before inoculation of various doses of the virulent component as the lethal challenge (v.a. in rabbits, i.c. in guinea pigs, i.p. or i.c. in mice). These experiments, otherwise under previous conditions, allowed comparison of the interaction mechanisms in the several host–route combinations. There was a wide range of rates of development of the dose-dependent protective response (Fig. 5). Results on the zero time ordinate correspond with those for the single mixed inoculations described in previous sections. These results thus characterized the early development of the independent protective response and its subsequent competition with the lethal challenge initiated at zero time and developing to the right.
Table 2. Response of rabbits to respiratory infection by virulent (L10.C1), avirulent (A7.C1) or mixed strains of Semliki Forest virus

<table>
<thead>
<tr>
<th>Virus strains inhaled as 0.5 to 2 μm. droplets</th>
<th>Log p.f.u. retained after inhalation</th>
<th>p.f.u. retained for PD50, LD50, PLD50</th>
</tr>
</thead>
<tbody>
<tr>
<td>L10.C1 alone</td>
<td>0:10, 1:0, 3:0, 6:0</td>
<td>10, 10, 10</td>
</tr>
<tr>
<td>A7.C1 + 400 p.f.u. L10.C1</td>
<td>0:18, 0:18, 0:12, 0:11</td>
<td>100, 100, 100</td>
</tr>
<tr>
<td>A7.C1 alone</td>
<td>0:14, 1:0, 3:0, 6:0</td>
<td>10, 10, 10</td>
</tr>
</tbody>
</table>

* Challenge condition.

Table 3. Relationship in 35-day-old mice of route of immunization to route of challenge

<table>
<thead>
<tr>
<th>Challenge by virulent virus (L10.C1)</th>
<th>Dose of avirulent virus (A7.C1) for 50% protection</th>
</tr>
</thead>
<tbody>
<tr>
<td>10⁶-8 p.f.u., i.c. at 6 days after immunization</td>
<td>10⁶-8 p.f.u. = 3 ID 50</td>
</tr>
<tr>
<td>10⁶-8 p.f.u., i.p. at 17 hr after immunization</td>
<td>10⁷-8 p.f.u. = 1 ID 50</td>
</tr>
</tbody>
</table>
Fig. 5. Rate of development of protective response following infection with avirulent virus of strain A7.ui at various doses and by different routes. Animals were infected by various doses of avirulent virus and challenged by virulent virus at various times later. Experimental points show the dose of avirulent virus (± 0.3 log units for mice and ± 0.5 log units for guinea pigs and rabbits) required for 50% protection in each case. A, Guinea pigs, immunization i.p. and challenge i.c. by 10^6 p.f.u. B, Guinea pigs, immunization i.e. and challenge i.c. by 10^5 p.f.u. C, Mice 40 days old, immunization i.e. and challenge i.e. by 10^5 p.f.u. D, Rabbits, immunization i.p. and challenge by inhalation of 10^5 p.f.u. E, Mice 40-day-old, immunization i.p. and challenge i.p. by 10^6 p.f.u. F, Mice 40-day-old, immunization i.p. and challenge i.p. by 10^5 p.f.u.

The administration of 1000 p.f.u. i.p. of avirulent virus showed 50% protection against challenge (i.c. or v.a.) by 1000 p.f.u. of virulent virus at 60 hr in rabbits, at 4½ days in mice and at 9 days in guinea pigs. When, however, mice received the avirulent virus by the i.p. or i.e. route and were challenged by the i.p. route, then 50% protection was established as early as 12 hr.

Further similar experiments (Table 3) in mice confirmed that the time required for development of the 50% protective response was determined by the route of challenge and was almost independent of the route of immunization.

In addition to the near linearity of the lines in Fig. 5 (which showed that the protective response increased approximately exponentially with time) their parallelism showed for guinea pigs and mice that the dose of avirulent virus administered at a given time was proportional to the dose of virulent virus subsequently required to break protection: these features specify further the development of the independent protective response.
Table 4. Summary of virulent–avirulent interactions following infection of mice, guinea pig and rabbits by heterogeneous inocula of SFV

<table>
<thead>
<tr>
<th>Interaction and Fig. reference</th>
<th>Host and route of infection</th>
<th>Nature of response interaction</th>
<th>Development of protective response (Fig. 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Virulent dominant (Fig. 3)</td>
<td>Mice, i.c.</td>
<td>No interaction within available range of doses. Death follows infection by $1 \text{LD}_{50}$ (2 p.f.u.) of virulent virus regardless of simultaneous infection by over $10^7$ avirulent virus particles</td>
<td>Protection if avirulent infection precedes i.c. challenge by 48 hr or more</td>
</tr>
<tr>
<td>(2) Avirulent dominant if above high threshold dose (Table 2)</td>
<td>Rabbits, respiratory</td>
<td>Death follows infection by $1 \text{LD}_{50}$ (60 p.f.u.) of virulent virus unless in presence of simultaneous replication of over $10^6$ avirulent virus particles</td>
<td>Protective response increases approximately 10 fold in 96 hr</td>
</tr>
<tr>
<td>(3) Avirulent dominant if avirulent–virulent ratio above threshold value (Fig. 4)</td>
<td>Guinea pigs, i.c.</td>
<td>Death follows infection by $1 \text{LD}_{50}$ (10 p.f.u.) of virulent virus unless avirulent–virulent particle ratio exceeds 2000:1</td>
<td>Protective response increases approximately 10 fold in 48 hr</td>
</tr>
<tr>
<td>(4) Progressive interaction from low doses, virulent dominant at high doses (Fig. 3)</td>
<td>Mice, i.p.</td>
<td>Progressive interaction of virulent and avirulent responses determined by degree of excess of avirulent particles</td>
<td>Protective response increases approximately 10 fold in 4 hr</td>
</tr>
<tr>
<td>(5) Avirulent dominant (Table 5)</td>
<td>Rabbits, i.v. or i.p.</td>
<td>Infection at any dose by virus of available strains provokes avirulent (protective) response in majority of animals</td>
<td>Dominant</td>
</tr>
</tbody>
</table>

**Comparison of interaction and response effects**

These interaction and response effects for the various host–route combinations (Tables 4 and 5) indicate that the comparative times and rates of development of the competing lethal and protective responses appeared to be the determinants of the terminal response. There were three intermediate levels of response between the overwhelming lethal replication of virulent virus in mice following i.c. infection and the dominant protective replication of any virus in guinea pigs or rabbits following i.p. infection. The protective response was dominant in rabbits at higher doses in respiratory infection and in mice at lower doses in intraperitoneal infection; the intracerebral infection of guinea pigs was intermediate and less influenced by dose. The complex influence of dose-composition and time on interaction and response in each host is well illustrated by the reversal of the order of sensitivity as the dose-composition was modified (Table 5).

**DISCUSSION**

The responses defined here for mice infected by particular strains or mixtures of SFV may be compared with those for West Nile virus and Powassan virus studied by Weiner, Cole & Nathanson (1970) and discussed in relation to the responses of mice to infection by other virulent arboviruses. Three patterns of response were considered: fatal encephalitis preceded...
by early viraemia and invasion of the CNS; inapparent infection without detectable viraemia or CNS involvement; and subclinical encephalitis preceded by trace viraemia and minimal infection of the brain. The range of responses observed in our own studies (Table 4) conform to none of these categories since infections by virulent (lethal) or avirulent (benign-protective) extremes of SFV are all characterized by equally early-high viraemia and involvement of the CNS (Bradish & Allner 1972; Zlotnik, Grant & Batter-Hatton, 1972). Thus, in contrast to the above two patterns of non-fatal encephalitis, the CNS invasion by some strains of SFV, despite its early initiation and efficiency, may be outraced and blocked by host defence mechanisms (Mims, 1964). Similarly vigorous feed-back controls will probably be demonstrated for other virus infections when population heterogeneity (virulence-avirulence) and dose-response relationships are defined more closely.

The regularity of the response characteristics reported in this paper, and their extension over a wide range of doses (1 to $10^8$ p.f.u.) and mixtures of administered virus or times of lethal challenge, indicate a general mechanism of virulent-avirulent interaction by which distinct sub-populations of virus, after preliminary replication at priming sites near that of administration, initiate independent lethal and protective responses. These developing responses then interact, according to the dose, site and time of initiation, to give the terminal responses (death, protection or intermediate) indicated. Our studies on interaction and response have yet to be related to the more detailed non-specific or specific component mechanisms by which, for example, cellular damage may be enhanced or localized in the brain (Zlotnik & Harris, 1970; Zlotnik et al. 1972), or by which virus may be recognized and processed as antigen through the macrophage, T-cell, B-cell system (Mäkelä, Cross & Kosunen, 1971; W.H.O. 1970). An essential and early discrimination of virus as potentially virulent or avirulent is likely to occur in the latter system.

Although the concept of virus heterogeneity within serological identity is well recognized in the many examples of virulent and avirulent strains, it is not always appreciated that even selected virulent or avirulent strains may be widely heterogeneous. We have shown that such heterogeneity of virulence may not be detected in limited tests which reveal only the properties of a subpopulation, often a minority, which is ultimately dominant. In this

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<th>Composition of mixed inoculum</th>
<th>Host and route of administration of virus</th>
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D or P indicate response of death or protection in majority of test group of animals. For detail see Fig. 3 and 4. References to the types of interaction summarized in Table 4 are shown in parentheses.
respect the race for expression between virulent and avirulent subpopulations may be a continuous and dynamic interaction of responses following infection and replication at several sites. Thus, for example, the consideration of host responses to virus infections of the central nervous system should include the influence of the interacting responses triggered by infection and processing at other sites and likely to provoke modifying protective or exacerbating (Webb & Hall, 1972) mechanisms.

In this study unrestricted virulence was expressed by virus populations comprising only one virulent particle per million or more (Fig. 3). Although this situation has been elucidated by dose-response studies in appropriate hosts, in vitro markers are unlikely to detect virulent virus particles in such populations. Thus the search for significant in vitro markers of virulence/avirulence should be preceded in each system by confirmation that the in vivo character is determined by a majority of the population distribution. This concept applies particularly in the search for and selection of candidates for use as live vaccines.

These requirements for the preliminary specification of the population distribution of virulence may be illustrated by the discordant correlations reported for the several distinct isolations of Newcastle Disease virus (Waterson, Pennington & Allan, 1967). Thus, despite an earlier report by Reeve et al. (1971), Moore, Lomniczi & Burke (1972) found no systematic correlation between the relative virulences of 13 strains of NDV and their activities in inhibition of cellular protein or RNA synthesis.

A further consequence of the interaction between responses triggered by virulent and avirulent subpopulations is that direct in vitro markers for avirulence/virulence are required to express in some way the balance between the protective or immunogenic potential of virus and its vigour in invasion of target sites. This information, presumably coded in the virus genome, may be expressed obliquely through a range of structural features and properties in yield, synthesis and stability. Characteristics of the virus particle only indirectly or distantly related to the interaction mechanism by which virulence is expressed are unlikely to offer sensitive markers for virulence.

A live vaccine containing no virulent particles cannot, in the absence of mutation or contamination, show increased virulence during subsequent haphazard passage. However, if the present virulent/avirulent interactions are relevant to other systems, it may be necessary to ensure that a vaccine does not contain a proportion of potentially virulent particles concealed by the interacting protective response provoked in the test animal by the avirulent majority.

An important consequence of the interaction between avirulent and virulent subpopulations is the sensitivity of many properties to the avirulent/virulent ratio in the virus populations. This ratio may change sufficiently within one or two passages to modify the apparent character of a virus in terms of the number of infective units per LD50 or PD50. Similarly, the nature of the response to a particular dose of virus may be changed significantly. Thus, guinea pigs will be either killed or protected if infected intracerebrally (Fig. 4) by 10^6 p.f.u. of SFV samples with avirulent/virulent ratios of 1000 or 5000, respectively. Similarly, the LD50 i.p. for mice (Fig. 3) will be either 3 or 100 p.f.u. for SFV samples with avirulent/virulent ratios of 1 or 10, respectively. Clearly such properties in infection and response are extremely sensitive to the small changes in the avirulent/virulent ratio for the virus population which may be imposed rapidly by selection during passage (Fig. 2) in nature or in the laboratory. It is unnecessary to evoke mutation or contamination in explanation of such cases of rapid strain variation.

It should be emphasized that most of the features of avirulent/virulent interaction discussed here for a model system are noted frequently as anomalies during studies on live
attenuated vaccines. Thus the varied results obtained during the standardization and testing of attenuated poliomyelitis vaccines (Evans, 1967) may be related to their content of varying proportions of virus particles with different degrees of virulence or potentials for initiation of competing protective responses. This extends to the influence of animal species and route of inoculation on the sensitivity of the neurovirulence test for detection of a virulent minority, to the early emergence of strains of modified virulence following passage by different routes in man or monkeys, and to the difficulties of establishing a correlation between neurovirulence (monkeys) and pathogenicity (man) when hosts of distinct responsiveness interact with ill-defined virus populations at various doses. Thus a vaccine to be applied by a given route in man should be tested for virulence in host-route systems of lower responsiveness than man himself. However, host-route systems of very much lower responsiveness would be unsuitable since virus particles protective to man would then be indicated as virulent. The selection of test systems is thus critical and may be guided by the definition of a quantitative gradient of responsiveness of the type discussed (Fig. 1, Table 4 and Bradish et al. 1971).

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