REVIEW ARTICLE

A Multi-Hit Model for the Neutralization of Animal Viruses

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INTRODUCTION

Neutralization of animal viruses by antibodies has been studied in many virus–antiserum–cell systems, and the relevant literature has been extensively reviewed on several occasions (Burnet, Keogh & Lush, 1937; Fazekas de St Groth, 1962; Svehag, 1968; Osterrieth, 1972). There is at present no consensus of opinion on whether a general mechanism of neutralization exists by attachment of a single antibody molecule, or of several antibody molecules, to the surface of a virus particle; both a single-hit mechanism (Dulbecco, Vogt & Strickland, 1956; Fazekas de St Groth, 1962; Mandel, 1960, 1976) and a multi-hit mechanism (Gard, 1957; Westaway, 1965b, 1968a; Hierholzer & Dowdle, 1970; Wallis, 1971) have been proposed.

The discussion to follow deals only with animal viruses; plant and bacterial viruses are excluded because their modes of entry into host cells differ from those of animal viruses and because antibody is not a defence mechanism of their hosts against infection. It should be noted that the data reviewed relates to conditions of antibody excess (rather than to antigen excess). Such experiments are the most useful in the context of neutralization because the percentage law applies, i.e. in the presence of excess antibody, the percentage or fraction measured as surviving virus remains constant with increasing or decreasing virus concentration.

Evidence for a single-hit mechanism of neutralization

The single-hit model is primarily based on apparent first-order kinetics of neutralization. In kinetic neutralization experiments, a large amount of virus is usually incubated at 37 °C with a relatively high concentration of antiserum; at intervals of one or more minutes, samples are taken and immediately diluted 100-fold or more in order to arrest the reaction and also to permit analysis of countable survivors, usually by plaque assay. A typical plot (Fig. 1) of surviving fraction versus time shows an immediate and rapid decrease of survivors; the decrease with time occurs linearly and logarithmically, and is characteristic of a first-order reaction. This is usually interpreted as being the result of binding of a single antibody molecule to a critical site on the surface of each neutralized virus particle (Dulbecco et al. 1956; Mandel, 1960). However, a small 'persistent fraction' of the original virus population survives, indicated by an inflexion or 'levelling off' in the neutralization curve; it is only a true persistent fraction if it resists further addition of (more concentrated)
antibody. The observed persistent fraction may slowly decrease on prolonged incubation, a process of 'immunoinactivation' (Gard, 1957).

The 'single-hit' interpretation of first-order kinetics of neutralization is valid only if the linear logarithmic decrease commences immediately, i.e. at the origin of the plot. If several hits are required to inactivate an infectious particle, then the theoretical curves of Meynell & Meynell (1970) indicate that a 'lag phase' should be observed before the inactivation curve enters the rapid linear phase. We shall refer later to the failure to observe this lag phase or shoulder at the commencement of most kinetic plots of virus neutralization.

Recently, a novel mechanism was proposed by Mandel (1976) who reported that the loss of infectivity of poliovirus after combination with antibody was associated with a change in iso-electric point; this change was interpreted as being caused by a co-operative conformational transition or allosteric change of capsid subunits. Because the observed change occurred even at low multiplicities of antibody (e.g. 50% survival of infectivity), it was assumed that neutralized virions had reacted with only one molecule of antibody. Loss of 'uncoatability' of virus-antibody complexes in an in vitro system was also associated with loss of infectivity. These observations led to a 'critical reaction' hypothesis that neutralization occurs by a one-hit mechanism at any capsid site, providing that the reaction is efficient (that is, producing loss of ability to penetrate or uncoat because of a change in conformation or isoelectric point of surface capsids). The basis of this one-hit model rests on the assumption that a single antibody molecule is required to produce the observed transition, and that this is in accord with apparently first-order kinetics of neutralization. Although no intermediate transitional (or iso-electric) states were observed, the data does not appear to exclude the possibility that a threshold dose of antibody molecules is required before the transition occurs in an all-or-none manner. It seems appropriate to defer further discussion of this model until other evidence relevant to the mechanism of neutralization has been advanced.

Some of the earlier proposals were based on limited data. Gard (1957) warned that 'results obtained with one particular virus-serum system may not possess general applicability... a general theory on the mechanism of neutralization should account for all established facts'. It is our intention to develop a model which will account for facts which tend to be ignored by proponents of the single-hit mechanism of neutralization.

**Evidence compatible with a multi-hit mechanism of neutralization**

The recent review of neutralization by Daniels (1975) is of particular interest because it directs attention to evidence which does not fit with a single-hit model but which is compatible with a multi-hit mechanism of neutralization. The main evidence which fits better with a multi-hit mechanism was summarized as follows (Daniels, 1975), with some additional references included:

1. A lag or shoulder in the kinetic neutralization curve is observed when virus-antibody mixtures are incubated at low temperature (4 °C rather than 37 °C) and at low antibody concentration (Fig. 1; Burnet et al. 1937; Dulbecco et al. 1956; Lafferty, 1963a). This lag is also seen using IgM antibody to poliovirus (Svehag, 1965; Philipson, 1966).

2. The reactions of antibodies with haptens or antigens observed in other systems occur very rapidly, reaching completion in less than 3 seconds (Mayer & Heidelberger, 1942; Schneider & Sehon, 1961; Pecht, Givol & Sela, 1972). Such reaction rates could not possibly be measured in kinetic neutralization tests which measure the surviving fraction in infectious virus-antibody mixtures, sampled at intervals of (normally) 1 min or greater, and then tested in plaque assays for the number of infectious virus particles which survive.
Review: model for animal virus neutralization

Fig. 1. A schematic representation of a typical kinetic neutralization curve showing terms used to describe the kinetic curve. Fraction V/V₀ is a ratio of the amount of virus originally present in the test (V₀) and the amount of virus remaining (V) after a defined reaction time in the assay system. This figure is modified after Fig. 4 of Lafferty (1963) which shows reaction kinetics of neutralization at 0°C with antiserum at a dilution of 1:20 (●) or 1:40 (○) and using influenza A virus. (Figure reproduced with permission.)

(3) The occurrence of a persistent fraction of infectious virus in mixtures of excess antibody and virus (Burnet et al. 1937; Dulbecco et al. 1956; Gard, 1957; Lafferty, 1963b; Wallis & Melnick, 1967; Westaway, 1968a; Wallis, 1971; Majer, 1972; Daniels, 1975), and even using filtered (monodispersed) virus preparations (Ashe, Mage & Notkins, 1969; Majer & Link, 1970; Lewenton-Kriss & Mandel, 1972; Yoshino & Morishima, 1972; Daniels, 1975).


(5) Neutralization of sensitized virus by addition of early acting components of complement, reviewed by Majer (1972), Daniels (1975) and Oldstone (1975). Also discussed by Daniels (1975) is neutralization of some enveloped RNA viruses (after sensitization) by the phospholipase activity of the late-acting components of complement (C5-C9), producing lytic effects demonstrated by electron microscopy (Almeida & Waterson, 1969) or by accessibility of the RNA to ribonuclease digestion (Oroszlan & Gilden, 1970; Radwan & Crawford, 1974; Oldstone, 1975).

(6) Ample evidence exists that the amount of neutralization observed in virus–antibody mixtures is considerably influenced by the host cell system in which the surviving or residual virus infectivity is assayed, using either whole animals or different cell cultures (Kjellén & Schlesinger, 1959; Lafferty, 1963a; Philipson, 1966; Westaway, 1968a).

Items (1) and (2) above cast doubt on the validity of interpretation of observed first-order kinetics of neutralization. If attachment of a single antibody molecule were sufficient to produce neutralization, then the infectious virus–antibody complexes in items (3) to (6) should not be demonstrable. Daniels (1975) concludes from such evidence that 'many
immunoglobulin molecules must bind to the surface to render the virus non-infectious’. Similarly, Westaway (1965b) proposed that ‘neutralization requires specific dispositions or groups of antigen–antibody complexes on the virus surface’ – we shall return to this model later.

Mention should also be made of earlier reports that prolonged incubation of infectious virus–antibody mixtures leads in some instances to additional neutralization, indicating that secondary interactions occur within the already formed virus–antibody complex (Gard, 1957; Kjellén, 1962; Wallis, 1970). These reports inferred that such secondary interaction is a sequel to an earlier attachment of several antibody molecules to a ‘critical site or sites’.

There is some additional and important data relevant to the evidence for multi-hit neutralization which was omitted from Daniels’ (1975) review; specifically we refer to (i) the synergistic effects in neutralization by mixtures of antibody molecules directed at different antigenic determinants on the virus surface, and (ii) some unique features of the reactions of flaviviruses with antibodies.

**Synergistic effects of antibodies**

**Reactions with adenoviruses**

The distribution of antigens (as proteins or structural units) over the adenovirus surface is well defined (reviewed by Norrby, 1971). The capsid is composed of 252 capsomers comprising 12 vertex capsomers (penton bases) and 240 non-vertex capsomers or hexons. Each penton base carries a fibre as a projection, the complex being known as a penton (Valentine & Pereira, 1965; Norrby, 1966). These components are separable and antisera may be prepared against each component. Hence, the reactions of the virus particle with such antisera provide valuable information on the surface distribution of antibodies and on their effects.

The neutralization of adenoviruses is similar to that of other viruses. At 37 °C and in the presence of a high concentration of antiserum, the kinetic neutralization curve is apparently first-order. The initial combination of virus with antibody is complete within 5 min (Kjellén, 1962), after which excess antibody and a persistent, constant fraction of infectious virus remain. There are two main sources of data which indicate the surface distribution of the antibodies on the virus surface: (1) the synergistic effects obtained by mixing antibodies against different virus determinants and (2) the effect of anti-globulin on sensitized virus.

Hexons carry group- and type-specific antigenic determinants at separate sites, whereas penton bases carry predominantly group-specific determinants and antibody to the fibre is more than 70% type-specific (Norrby, 1971; Pettersson, 1971; Wadell, 1972; Willcox & Mautner, 1976a). Kjellén & Pereira (1968) observed that most of the neutralizing activity was produced by anti-hexon serum, a small amount by anti-penton serum, and virtually none by anti-fibre serum. Norrby & Wadell (1972) found that anti-penton serum possessed modest neutralizing activity and anti-fibre serum much less activity; when sheep anti-rabbit serum was added, an 8- or 60-fold (respectively) enhancement of neutralization was observed with the two sera. Anti-penton base serum (obtained by adsorption of anti-penton serum with fibres) had no detectable neutralizing activity (at 1:40), but after the addition of sheep anti-rabbit serum there was significant neutralization at a 1:640 dilution of anti-penton base serum. Wadell (1972) also observed a similar marked enhancement when antisera against hexons (prepared for immunization by anionic exchange chromatography and isoelectric focusing) of several adenoviruses were tested in the absence and presence of sheep anti-rabbit serum.

It may be concluded that attachment of intact antibody to readily available hexons
produced by far the most efficient neutralization, and this can be improved further by addition of anti-globulin. Antibodies to pentons or fibres clearly attach but do not neutralize the virus infectivity, probably because of the low surface concentration of pentons (12 in a total of 252 capsomers). However, neutralization by anti-penton sera is achieved by addition of anti-globulin, hence hexons do not possess unique critical sites denied to pentons.

The importance of the distribution of adenovirus antigens and of antibodies attached to them becomes more apparent when populations of mixed antibodies to specific adenovirus antigens are employed in neutralization tests. In a well-documented experiment with adenovirus type 5, Kjellén & Pereira (1968) observed that IgG fractions of rabbit antisera against heterologous adenovirus types (1, 2 and 12) were able to enhance by 30- to 50-fold the neutralizing ability of homologous serum, although the heterologous serum produced no neutralization alone. The authors suggested that attachment of homotypic antibody causes capsid changes permitting heterologous antibody to enter and attach to subsurface common antigenic determinants thus producing neutralization. However, this hypothesis is not supported by their results which show that virus sensitized by heterologous antibodies was neutralized by anti-globulin. Furthermore, Willcox & Mautner (1976b) found that virions bound type-specific and cross-reacting antibodies impartially, but the manner in which type-specific antibodies were bound to virions produced greater neutralization than that observed with cross-reactive antibodies.

In other studies, Hierholzer & Dowdle (1970) measured low neutralization titres of antisera prepared against hexons or against dodecons (aggregates of 12 pentons) of adenovirus type 8 or of type 9 when the antisera were used separately. An 8- to 16-fold increase in neutralization was observed when both antisera were used together against either virus. Similarly, Norrby & Wadell (1972) noted that antibodies against vertex capsomers (penton bases) or against fibres produced only negligible neutralization but were effective when used together, in an anti-penton serum. Hierholzer & Dowdle (1970) concluded that their data indicate that ‘maximal neutralization titres may involve antigen–antibody reactions at more than one site’.

In summary, adenoviruses are neutralized most readily by antibodies to the most numerically accessible (hexon) antigens. Adenoviruses may also be sensitized by attached antibody which clearly does not neutralize unless augmented by anti-gamma globulin or by additional antibody directed at different antigenic determinants. The augmentation represents a very large synergistic effect. Attachment of a single or several antibody molecules to a defined site(s) on the surface of a virus clearly is not always sufficient to effect neutralization (without augmentation). The distribution of antibodies of different or the same specificity on the virion surface appears to play an essential role in neutralization.

Reactions with phenotypically mixed virus particles

Synergism as noted above in adenovirus neutralization occurs even more dramatically in a situation which is generally ignored in discussions of virus neutralization. Sequential infections of cells with two different enveloped viruses can result in production of phenotypically mixed progeny, that is virus particles with the complete genome of one parent and a coat of mixed antigens. These particles are apparently formed by budding of core particles through cell membranes in which are embedded structural proteins specified by both parental viruses. The reactions of two types of phenotypically mixed virus particles will be described; these virus populations were derived by mixed infection with a pair of serologically related viruses, or by mixed infection with viruses from different virus groups or families which hence have no serological relationships. Such progeny include a proportion of ‘doubly neutralizable’
Table 1. Neutralizing activity of antiserum mixtures with mixed virus populations of immunologically related viruses

| Antiserum Plaque count Surviving fraction Percentage neutralized |
|-----------------------|-------------------|-----------------|-----------------|
| Kunjin* Dengue 2†     | Vo‡               | V§              | (V/Vo)          |
| + -                   | 693 ± 50          | 175 ± 61        | 0.25            | 75              |
| - +                   | 693 ± 50          | 187 ± 30        | 0.27            | 73              |
| + +                   | 3470 ± 250        | 8 ± 5           | 0.002           | 99.8            |

* Hyperimmune rabbit anti-Kunjin virus serum heat inactivated (at 56 °C for 30 min) at a dilution of 1 in 500.
† Hyperimmune rabbit anti-dengue type 2 virus serum heat inactivated (56 °C for 30 min) at a final dilution of 1 in 60.
‡ Total virus present in test (Vo) using control non-immune serum and buffer and assayed (Westaway, 1966) at the appropriate dilution so that about 60 p.f.u. were present on each plate. Mean ± 2 standard errors of the mean is shown. The flavivirus population contained a mixture of 1.0 Kunjin virus particles to 0.9 dengue type 2 particles, as determined by use of antisera in the overlay of the plaque assays (Wecker, 1960; and see Fig. 2).
§ V is the titre (mean ± two standard errors shown) after reaction with antibody for 1 h at 37 °C.

virus particles, that is, neutralized to a relatively small extent by antiserum to either parental virus. When the antisera are used in combination, a strong synergistic effect is observed.

Granoff (1959, 1965) obtained phenotypic mixtures of two strains of Newcastle disease virus, and showed that about 20% of the population was not neutralized by antiserum to either parental strain, but > 99.6% was neutralized when the two antisera were combined. Similar results were reported by Vogt (1967) in neutralization tests with phenotypically mixed progeny from infection with two strains of Rous sarcoma virus; single antisera neutralized a maximum of 67% or 86%, whereas the combined antisera neutralized > 99.9%. The synergistic effects of antibodies in these experiments appear undeniable. However, some caution in interpretation is necessary because similar results were obtained using a mixed population of two related but distinct flaviviruses (not phenotypically mixed) and appropriate antisera (Table 1). A small amount of serological cross-reactivity expressed by each of the two antisera may produce an apparent synergistic effect when the antisera are combined. More definitive results are obtained when the parental viruses are unrelated, as detailed below.

Unassailable evidence of phenotypic mixing and synergistic neutralization is provided in experiments with the progeny of mixed infections by vesicular stomatitis virus (VSV), a rhabdovirus, and any of several unrelated (spherical) enveloped viruses. Choppin & Compans (1970) obtained phenotypic mixtures of the rhabdovirus VSV and the para-influenza virus SV5, a paramyxovirus, and assayed the survivors with VSV genotype after reactions with anti-VSV, with anti-SV5, and with the combined antisera. A greater than 40-fold increase in neutralization was observed when the combined antisera were used compared to their individual effects. Inclusion of SV5 antigens in the VSV genotypes was shown also by a significant reduction in VSV plaque count after reaction only with SV5 antiserum. Unique phenotypically mixed bullet-shaped particles were identified by their ability to adsorb to chicken erythrocytes (confirmed in electron micrographs) and to elute later; both these properties could be conferred only by the spike glycoproteins (hemagglutinin and neuraminidase) specified by SV5 virus. The incorporation of the SV5 proteins in the envelopes of the phenotypically mixed bullet-shaped particles was shown directly in the electron microscope by use of ferritin-labelled antibody, and in a subsequent paper (McSharry, Compans & Choppin, 1971) these particles were purified and shown, by gel electro-
phoresis, to contain the two spike glycoproteins of SV5 virus and all the VSV structural proteins. Some of the above features, including synergistic neutralization by mixed antibodies, were reproduced by Kimura (1973) with a phenotypic mixture obtained as progeny from mixed infection with VSV and Sendai virus (a paramyxovirus like SV5). The results were qualitatively and quantitatively almost identical with those of Choppin & Compans (1970).

In further similar experiments to those of Choppin and colleagues, but using VSV and retroviruses, Zavada & Rosenbergova (1972) added VSV to chick embryo cells pre-infected with avian myeloblastosis virus or to mouse embryo cells pre-infected with murine sarcoma virus. The progeny included VSV genotypes which partially resisted neutralization by anti-VSV serum (compared to neutralization of control VSV); this partial resistance in the presence of anti-VSV serum was overcome (8- to 50-fold reduction in infectivity) by adding the appropriate anti-retrovirus serum. The particles which were partially resistant to anti-VSV serum also showed host range and interference specificity corresponding to the retrovirus involved. Phenotypically mixed infectious particles of VSV genotype were obtained from cells pre-infected with measles virus (a paramyxovirus) or with a defective strain of measles virus. The combined action of anti-VSV serum and anti-measles serum increased neutralization by more than 100-fold compared to the total effect of either antiserum alone (Wild, Cathala & Huppert, 1975/76).

These experiments with phenotypically mixed virus particles carrying unrelated antigens show that while attachment of antibody against one set of (parental) antigens neutralized only a relatively small fraction, a greatly enhanced neutralization was achieved by the addition of antibody to the second (parental) set of antigens. In such situations a single antibody molecule on the surface of the virion is indisputably unable to neutralize infectivity. It seems significant that the surface distribution of antibodies on virus surface antigens plays a key role in the neutralization of enveloped viruses as well as icosahedral adenoviruses.

Reactions of flaviviruses with antibodies

Further data incompatible with a single-hit model is provided by neutralization tests with flaviviruses (formerly the group B arboviruses), a genus of the family Togaviridae. Flaviviruses are particularly suitable for examining mechanisms of virus neutralization. The surface structure is a membrane envelope containing multiple copies of a single glycoprotein V3, mol. wt. in the range 50000 to 60000 (Stollar, 1969; Shapiro et al. 1971) which is separable from the internal core structure of RNA and two smaller proteins (V2 and V1) by treatment with sodium deoxycholate (Westaway & Reedman, 1969; Trent & Qureshi, 1971). The antibody response including the neutralizing activity produced by immunization with separated V3 protein is slightly retarded but otherwise identical to the response to immunization with purified virions (Della-Porta & Westaway, 1977). The genus includes about 40 species which can fairly readily be distinguished by neutralization tests (Westaway, 1966) and in some cases by electrophoretic analyses of the virus-specified proteins (Shapiro et al. 1972; Westaway, 1973; Westaway, McKimm & McLeod, 1977). Extensive cross-reactions occur in haemagglutination-inhibition (HI) tests (Westaway, 1968b) and several subgroups are defined by relationships in neutralization tests (Westaway, 1966; de Madrid & Porterfield, 1974). The serological relationships are interpretable on the basis of the presence of two antigenic determinants on the envelope protein V3, comprising a common or group-specific determinant and a type-specific determinant (Qureshi & Trent, 1973; Westaway, Della-Porta & Reedman, 1974; Trent et al. 1976).

Although parameters of neutralization of flaviviruses have been studied in several
Table 2. Effect of anti-globulin serum and guinea pig complement on the neutralization of the flavivirus Kunjin, sensitized by homologous or heterologous antibodies

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Anti-globulin</th>
<th>Complement</th>
<th>Plaque count[</th>
<th>Surviving fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kunjin*</td>
<td>Dengue 2†</td>
<td></td>
<td>Vo</td>
<td>V</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>-</td>
<td>30750 ± 1500</td>
<td>72 ± 1</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>+</td>
<td>30750 ± 1500</td>
<td>4 ± 2</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>-</td>
<td>179 ± 8</td>
<td>41 ± 10</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>+</td>
<td>179 ± 8</td>
<td>22 ± 7</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td></td>
<td>1564 ± 100</td>
<td>8 ± 1</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>+</td>
<td>1564 ± 1000</td>
<td>5 ± 2</td>
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<tr>
<td>-</td>
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<td></td>
<td>1564 ± 10</td>
<td>36 ± 5</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>+</td>
<td>1564 ± 10</td>
<td>5 ± 1</td>
</tr>
</tbody>
</table>

* As in Table 1 (homologous antibody).
† As in Table 1 (heterologous antibody).
‡ Goat anti-rabbit globulin serum (list no. 71-232, Hyland Laboratories, Los Angeles, Ca.) at a dilution of 1 in 20. The anti-globulin serum was dialysed against normal saline overnight at 4 °C to remove the sodium azide preservative and heat-inactivated (56 °C for 30 min) to destroy any complement. Virus controls in the presence and absence of anti-globulin serum showed no difference in titres.
§ 1 in 4 dilution of fresh guinea pig serum (complement). The complement had no effect on the virus titre in the absence of antiserum.
¶ 0.5 ml of the virus (Vo) was incubated with 0.25 ml of antiserum for 1 h at 37 °C, followed by addition of 0.25 ml of anti-globulin serum, complement or buffer and a further incubation period of 1 h at 37 °C. The surviving virus, V (mean ± 2 standard errors shown), and the original amount of virus in tests, Vo (assayed using non-immune serum and buffer), were assayed by plaque titration. The titre of virus in test (Vo) was determined by assaying at an appropriate dilution so that 30 to 40 p.f.u. were present on each of the replicate plates. (Mean ± 2 standard errors shown.)

A persistent fraction of surviving virus is more evident with early than with late antibody, being as large as 10% in reactions with very early or IgM antibody (Westaway, 1965a, 1968a; Ozaki et al. 1974). Reversibility of the virus–antibody combination occurs more readily with early than with late antiserum (Westaway, 1965a). Virus populations sensitized by antibody are neutralized by added complement or by added anti-globulin. The potentiation by complement is greater with early antiserum than with late antiserum in both homologous-reactions and cross-reactions, and both IgM and IgG antibodies are potentiated (Table 2; Iwasaki & Ogura, 1968a; Westaway, 1965b, 1968a). Virus sensitized by IgM or IgG antibody is neutralized by addition of anti-gamma globulin (Iwasaki & Ogura, 1968b; Westaway, 1968a; Ozaki et al. 1974). Both homologous and heterologous antibodies can sensitize flavivirus and this can be detected by addition of anti-gamma globulin (Table 2).

Enhancement of virus infectivity

Hawkes (1964) reported as much as a 12-fold enhancement of infectivity of Murray Valley encephalitis (MVE) virus which had been reacted with avian anti-MVE serum and assayed by plaque assays in avian cells; only neutralization was observed by assay in mammalian cells. Enhancement developed more rapidly in virus–antibody mixtures at 37 °C than at 0 °C. The maximum enhancement at 37 °C (fourfold) was attained immediately after mixing and was about 75% of that observed after 2 h at 0 °C (5.6-fold) using a 1 in 640 dilution of antiserum; at a 1 in 10 dilution of the same antiserum the enhancement at 0 °C was only threefold. Enhancement decreased if the incubation period (prior to cell infection) was extended further, sometimes falling to zero. Greater enhancement was
observed with hyperimmune than with immune serum, and at lower concentrations. Homologous antisera enhanced to a greater extent and at lower concentrations compared to heterologous antisera. The results showed that both 19S (2-mercaptoethanol sensitive) and 7S antibodies are involved in enhancement which is unstable, decaying with time. Hawkes (1964) suggested that the surface charge on virus particles is altered by the attached antibody to increase the proportion of infectious particles (Ada et al. 1962, reported that only about 1 in 100 MVE virus particles are infectious). Hence virus particles which are non-infectious in normal assay systems (with or without antibody) are thereby rendered infectious.

Antiserum produced in rabbits against Kunjin virus could enhance the infectivity of dengue type 2 virus by 1.7 to 3.4-fold (Table 3) when assayed in mammalian cells. Barrett (1971) reported similar results; early rabbit antisera to Kunjin virus enhanced the plaque counts of MVE virus as much as tenfold. Halstead & O’Rourke (1977) showed that the yield of dengue type 2 virus was enhanced significantly after infection of cells by virus incubated with anti-dengue sera from several species. We were able to conclude that anti-viral antibodies were in fact attached to the ‘enhanced’ dengue virus particles by the observation that they were neutralized following addition of complement or of anti-globulin antibodies (Table 3). Dose–response curves (Fig. 2) of the neutralization of Kunjin virus using decreasing concentrations of rabbit anti-Kunjin serum and assayed in mammalian cells, indicated that enhancement of plaquing efficiency occurred at low concentrations (10^{-4.5} to 10^{-5.0}) of hyperimmune serum, similar to the results in the avian system (Hawkes, 1964).

The enhancement reaction appears to be inversely related to the number of antibody molecules attached to the virus surface (Hawkes & Lafferty, 1967; Fig. 2). Hawkes & Lafferty (1967) showed that the maximum enhancement was dependent on the period of incubation, on the antiserum concentration and on the temperature. The more prolonged the incubation period the lower the concentration of antiserum needed to cause maximum enhancement. With MVE virus (and also with rabbit pox virus) the enhancement rapidly reached a maximum which declined to barely significant levels. In some cases neutralization occurred after prolonged incubation. Enhancement apparently represents an early stage in the neutralization process in which one or a small number of antibody molecules are attached to the virus particle, but these are insufficient to neutralize. As the incubation is continued more antibodies can attach to the virus, and eventually the virus–antibody complex loses its infectivity.

If, under the conditions of the above experiments, enhancement is the result of attachment of a single antibody molecule to the surface of each ‘enhanced’ virus particle, the loss of this increased infectivity with time suggests that the attachment of additional antibody
molecules produces neutralization. Because of the surface structure of flaviviruses (multiple copies of a single protein) all sites should be 'critical' in terms of a 'single-hit' hypothesis, and hence the enhancement phenomenon is incompatible with the single-hit hypothesis of neutralization.

Retardation of virus penetration into cells

Further evidence is available of resistance to neutralization in the presence of demonstrable effects of antibody. The rate of penetration of West Nile virus into cells was retarded in the presence of homologous antiserum; this was shown by removal of the virus–serum mixture from cell monolayers after the adsorption period and replacement with further antiserum (at the same final concentration) or with diluent only. The plaque count was significantly reduced by removal and replacement with further antiserum (but not with buffer) after 1 h, whereas the plaque counts of controls (no antiserum during the first hour adsorption period) were unaffected (Westaway, 1965a).

Another example of the effect of antibodies on the rate of cell penetration can be seen in the Wecker (1960) neutralization test. If homologous antiserum is incorporated in the overlay media in a plaque assay of a flavivirus, plaque formation is inhibited. However, if heterologous antiserum is used then only the rate of development of the plaques and not the count is affected. For example, anti-dengue type 2 hyperimmune serum at a dilution of 1 in 100 completely inhibited formation of dengue type 2 plaques but did not affect the number of Kunjin virus plaques. However, a significant difference in the rate of development of these
Fig. 3. Effect of a 1 in 100 dilution of hyperimmune anti-dengue type 2 virus serum in the overlay on the development of Kunjin virus plaques in mammalian cells. The heterologous antiserum did not affect the plaque count, but did reduce the rate of development of plaques. Homologous antisera totally inhibited plaque formation (not shown). Assay method after Wecker (1960).
Table 4. Comparison of neutralization titres (NT) and of haemagglutination inhibition (HI) titres in IgG antibody fractions against homologous flaviviruses*

<table>
<thead>
<tr>
<th>Antibody to Kunjin virus† (electrophoretically slow or fast fractions)</th>
<th>Test virus</th>
<th>Antibody titres</th>
</tr>
</thead>
<tbody>
<tr>
<td>Late slow IgG</td>
<td>Kunjin</td>
<td>145‡ 160§</td>
</tr>
<tr>
<td>Late fast IgG</td>
<td>Kunjin</td>
<td>188‡ 320</td>
</tr>
<tr>
<td>Early fast IgG</td>
<td>Kunjin</td>
<td>224‡ 160</td>
</tr>
<tr>
<td>Early fast IgG</td>
<td>WN</td>
<td></td>
</tr>
<tr>
<td>Early fast IgG</td>
<td>MVE</td>
<td></td>
</tr>
</tbody>
</table>

* Data derived from Westaway (1968a, data used with permission).
† Sera from rabbit 18 were collected either 7 days after intravenous inoculation (early) or after four weekly inoculations, followed by one 2 months later and then by bleeding 7 days after this final inoculation (late).
‡ Fig. 4.
§ Table 7.
|| Flaviviruses West Nile (WN) and Murray Valley encephalitis (MVE).
¶ Table 9.

plaques was observed (Fig. 3). The reverse situation using Kunjin antiserum and the same viruses was also observed. Similarly, results were originally reported by Wecker (1960) with poliovirus types. It appears that virus particles complexed with heterologous antibodies are able to infect cells but the presence of such antibody limits the rate of spread of infection.

Haemagglutination inhibition and neutralization

The inability of attached antibody molecules to neutralize virus particles becomes obvious when cross-reacting antibody titres against flaviviruses are compared by neutralization tests and by haemagglutination-inhibition (HI) tests. Purified infectious virus particles function as haemagglutinin (HA) in HI tests and the antibody titres to such HA are identical with those obtained using crude HA preparations containing subviral particles (Westaway, Shew & Della-Porta, 1975). The titres in neutralization tests (50% plaque reduction) and in HI tests, representing reactions of antibody at the minimum detectable dose of antibody in both tests, are sometimes coincident, especially early in the immune response. Later in the immune response, neutralization titres are often greater than HI titres, probably due to increases in antibody avidity of IgG. For example, the titres of antibodies to Kunjin virus in three separate fractions of IgG from rabbit antiserum differed in the two tests by less than twofold in homologous reactions (Table 4). However, in cross-reactions, the titres in the two tests showed a large difference (HI much greater than neutralization). Clearly, attachment of cross-reacting antibody at low concentrations to virus particles is sufficient to inhibit HA activity but produces no neutralization. Such virus particles are sensitized, however, because addition of complement produces neutralization (Table 9 in Westaway, 1968a). This different reactivity of the same antibody preparation in the two tests is well recognized among arbovirus epidemiologists (Casals, 1973), but tends to be ignored in discussions of mechanisms of virus neutralization. Recent data from Trent et al. (1976) show the same phenomenon with several flaviviruses in a most striking manner (Table 5).

Multi-hit model of mechanism of neutralization

The assertion that most, if not all, of the above evidence is in conflict with the single-hit model requires that an adequate alternative model be presented which is compatible with all
Table 5. Comparison of neutralization titres (NT) and of haemagglutination-inhibition (HI) titres in hyperimmune sera against flaviviruses

<table>
<thead>
<tr>
<th>Antiserum against (virus)†</th>
<th>Test</th>
<th>Normalized antibody titres to (virus antigen)†</th>
<th>Ratio of homologous titres to geometric mean titres of cross reactions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>SLE</td>
<td>JE</td>
</tr>
<tr>
<td>SLE</td>
<td>NT</td>
<td>100</td>
<td>0·5</td>
</tr>
<tr>
<td></td>
<td>HI</td>
<td>100</td>
<td>25</td>
</tr>
<tr>
<td>JE</td>
<td>NT</td>
<td>2·7</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>HI</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>WN</td>
<td>NT</td>
<td>1</td>
<td>0·3</td>
</tr>
<tr>
<td></td>
<td>HI</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>D2</td>
<td>NT</td>
<td>0·25</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>HI</td>
<td>12</td>
<td>12</td>
</tr>
</tbody>
</table>

* Based on Table 2 of Trent et al. (1976, data used with permission).
† Flaviviruses St Louis encephalitis (SLE), Japanese encephalitis (JE), West Nile (WN) and dengue 2 (D2).
‡ All values in italics normalized with respect to homologous titres. Homologous titre shown in brackets.

of the data. The model that follows attempts to achieve this aim. The essence of the model is based on the statement of Westaway (1965b): 'Neutralization requires specific dispositions or groups of antigen–antibody complexes on the virus surface. The form of these groups is purely conjectural but for the present purpose they may be envisaged as forming variable "critical areas" each with a certain statistical probability of rendering the virus non-infectious for a specific host cell; criticality refers only to the prevention of infection without any implication of damage to the virus particle'. This conclusion arose from consideration of the role of the host cell in neutralization and from analyses of plaque neutralization by early and late antisera in homologous reactions and in cross-reactions using several flaviviruses (Westaway, 1965a, b). The observed combining power or avidity of antibodies in specific reactions (expressed as the slope in linear dose–response curves) was related by an appropriate equation to the ratio of neutralized virus (with the critical site(s) occupied) to surviving virus [critical site(s) not occupied or not completed; Westaway, 1965b]. The opportunity to complete critical areas is obviously greater in homologous reactions (more antigenic sites are available) than in heterologous reactions.

The model is presented schematically in Fig. 4 and 5. The number and distribution of antigenic sites are hypothetical but the antigenic specificities are assumed to be similar to those of flaviviruses (a group-specific and a type-specific determinant with the latter functioning as a cross-reactive subgroup determinant in heterologous reactions). The model thus also permits an interpretation of the large differences observed between neutralization titres and HI titres in heterologous reactions of flaviviruses (see previous section). Virus particles complexed with homologous antibodies (Fig. 4) are represented in case I and case II. Case I depicts a virus particle which has sufficient antibody molecules bound to form a ‘critical area(s)’ which results in neutralization of infectivity for host A or B. In case II there are insufficient antibody molecules attached to form the ‘critical area(s)’ which would render the particle non-infectious for host B. In this case, the virus particle is sensitized and could be neutralized by the addition of anti-gamma globulin. In both cases I and II, the ability of virus particles to agglutinate red blood cells is inhibited.

Reactions of heterologous antibodies with the virus particle (Fig. 5) are represented by cases III and IV. In case III, antibody molecules are attached to enough group- and subgroup-determinants to form a ‘critical area(s)’ recognized by some cell populations but not
Neutralization

Host A: +
Host B: +

HA-inhibition

Fig. 4. Model for multi-hit mechanism for neutralization of virus infectivity by antibody. Homologous antibody system: ○ – represents antibody molecule attached to type-specific determinant, ♂ – represents antibody attached to a group-specific determinant. The shaded area represents a critical area of attached antibody, sufficient to produce neutralization (Westaway, 1965b). The requirements for neutralization are more stringent for cells of host B. Although in case II the virus–antibody complex is still infectious for host B, it is sensitized and could be neutralized by addition of anti-gamma globulin.

by others. Some of these particles may be sensitized to neutralization by anti-gamma globulin, but less so compared to case II. In case IV the distribution of antigen–antibody complexes on the virus particle is insufficient to form ‘critical area(s)’ in relation to any host and hence neutralization cannot occur. Furthermore, the complexes may be too widely spaced to sensitize the virus for neutralization by anti-gamma globulin. However, the particles may be sensitized to the lytic effect of complement as this is not dependent on the formation of ‘critical areas(s)’. In cases III and IV, there is still sufficient antibody attached to (sterically) inhibit haemagglutination.

The synergistic effect of combined antibodies and other phenomena discussed earlier is also readily interpreted in terms of the multi-hit model. When specific antibodies against only one (repeating) antigen are unable to attain a sufficient concentration on the surface of virions to complete critical areas, these are readily completed when additional antibodies of different specificity attach to adjacent (different) antigenic sites. Conversely, reversibility of neutralization occurs after dilution of virus–antibody mixtures because of loss of attachment of one or more antibody molecules from a formerly completed critical area. Addition of anti-globulin to sensitized virus particles stabilizes antigen–antibody complexes and hence facilitates completion of critical areas. In this model the persistent fraction in neutralization mixtures represents a small proportion of virus–antibody complexes in which critical areas cannot be completed because previously attached antibody interferes sterically with attachment of (say) one additional antibody molecule required to complete a critical area. In accordance with this concept of steric interference, the persistent fraction tends to be larger when the specific antibody population includes larger IgM molecules (Westaway, 1968a).
Review: model for animal virus neutralization

Fig. 5. Model for multi-hit mechanism for neutralization of virus infectivity by antibody. Heterologous antibody system: @ — represents antibody molecule attached to group-specific determinant. ® — represents (cross-reacting) antibody attached to a subgroup determinant, which functions as a type-specific determinant in homologous reactions. The shaded area represents a 'critical area' of attached antibody which in case III produces neutralization in host A but is inadequate to produce neutralization in host B. The probability of attachment of cross-reacting antibody to subgroup determinants is lower than in the homologous reaction, and hence critical areas are formed less readily. For example, in case IV the attached antibody molecules are too dispersed on the surface of the virion to complete a 'critical area' for either host, but sufficient to (sterically) inhibit haemagglutination (HA).

CONCLUSION

The manner in which a cell responds to a virus particle complexed with antibody molecules so that the infectivity is neutralized in the specific cell remains undefined. It may involve some allosteric transition in the virus capsid which occurs when antibodies are attached (Lewenton-Kriss & Mandel, 1972; Mandel, 1976), or the antibody-coated virus particle may activate some cellular nuclease (Dales & Kajioka, 1964; Joklik, 1964; Mandel, 1967).

The allosteric transition postulated by Mandel (1976) may be unique to poliovirus because not all viruses show spontaneous conformational changes. Clearly the requirement of only one antibody molecule in his critical reaction hypothesis is not compatible with the previously discussed evidence, especially of the dependence of neutralization on host cell, enhancement of infectivity by antibody, neutralization of sensitized virus, and neutralization by synergistic action of two antibody populations; some of these phenomena were observed with either or both enveloped and icosahedral viruses, including poliovirus. In contrast, all this and other evidence is readily interpreted in the context of the multi-hit model.

Studies of neutralization of the infectivity of animal viruses by antibody, studied using in vitro tests, provide only a limited understanding of the defence mechanisms that make animals resistant to virus infections. The interaction of virus plus antibody in the whole
animal is much more complex than in cultured cells, because of the presence of undiluted serum, complement and various cell types involved in immunity (Allison, 1974) as well as susceptible cells. However, in vitro antibody tests do indicate whether an animal has been previously infected with a particular virus, assist in differentiating virus strains, and sometimes provide an index for assessing an animal’s resistance to infection. An understanding of the basic mechanisms involved in the neutralization of virus infectivity by antibodies contributes to the interpretation of neutralization tests, supplies information on the interaction of viruses, antibodies and animal cells, and should provide a rational basis for the selection of antigens for use in virus vaccines. These interests would be better served by focusing attention on a multi-hit mechanism of neutralization rather than on the restrictive single-hit hypothesis. While it may be possible to employ ad hoc assumptions in the single-hit hypothesis and thence interpret some of the challenging observations presented, only the multi-hit hypothesis offers a rational explanation of all the data discussed in this review.

Note added in Proof. In a recent study (D. W. Trent, 1977, Journal of Virology 32, 608–618) done using a competitive solid phase radioimmunoassay, it has been confirmed that the envelope glycoproteins of the flaviviruses St Louis encephalitis, Japanese encephalitis and dengue viruses contain type-specific, complex-reactive and group reactive antigenic determinants. This confirms the presence of these three classes of determinants, as proposed in our model.

REFERENCES


 REVIEW: MODEL FOR ANIMAL VIRUS NEUTRALIZATION


Review: model for animal virus neutralization


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