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Neutralization of Animal Viruses

With 10 Figures

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Abbreviations

FMDV, foot-and-mouth disease virus
HA, Haemagglutinin
HCMV, human cytomegalovirus
HIV, HIV-1, human immunodeficiency virus type 1
HSV-1, herpes simplex virus type 1
influenza virus, influenza virus type A
LCMV, lymphocytic choriomeningitis virus
LDV, lactate dehydrogenase-elevating virus
mab, monoclonal antibody
MHV-4, mouse hepatitis virus type 4
NA, neuraminidase
NANA, N-acetyl neuraminic acid
NDV, Newcastle disease virus
PIV-3, parainfluenza virus type 3
poliovirus, poliovirus type 1
RSV, respiratory syncytial virus
SLEV, Saint Louis encephalitis virus
TBEV, tick-borne encephalitis virus
TGEV, transmissible gastroenteritis virus
TMEV, Theiler's murine encephalomyelitis virus
VEEV, Venezuelan equine encephalomyelitis virus
VSV, vesicular stomatitis virus

1 Introduction

Understanding neutralization is particularly relevant to an appreciation of the interaction between a virus and its antibody-synthesizing host since it is likely that viruses and the antibody system have evolved in response to reciprocally imposed selective pressures. Neutralization of viruses which only infect non-antibody-synthesizing hosts, while of considerable interest from a number of points of view is de facto without any such evolutionary significance. In this second category are viruses of plants, invertebrates, vertebrates below fish in the evolutionary scale which do not synthesize antibody and most bacteria. Viruses of organisms parasitic on or commensal with antibody-synthesizing vertebrates, such as enteric bacteria, protozoa or metazoan parasites, will be in contact with antibody at some stage of their existence, and arthropod-borne viruses which have a higher vertebrate as second host are obviously bona fide members of the first category.

There is an urgent need to understand the principles by which antibodies inactivate virus infectivity since, at present, we are unable to rationally construct effective vaccines against new agents like the human immunodeficiency viruses or to improve existing vaccines. The intention of this volume is to comprehensively review neutralization and where possible to construct a unifying theory which can be tested by experimentation. The major conclusion is that it is not possible to say that a virus is neutralized by any one mechanism, but rather that there is a mechanism peculiar to the particular permutation of conditions prevailing; these include properties of the virus itself other than the relevant neutralization epitope, the neutralization epitope, the isotype of immunoglobin, the cell receptor and the virus: immunoglobulin ratio. Thus a virus may be classified as being neutralized according to several different mechanisms, and the appearance of a virus under only one category of neutralization mechanism probably means that other mechanisms operating under different circumstances have not yet been uncovered.

Some early work suffered from the (false) assumption that there was only one mechanism of neutralization, and interpretation of much of the early work was ambiguous because of the uncertainty of the composition of polyclonal antisera even when monospecific reagents were available. Nonetheless there is a wealth of important data in early reviews (FAZEKAS DE ST GROTH 1962; SVEHAG 1968; DANIELS 1975; DELLA-PORTA and WESTAWAY 1978; MANDEL 1979) and in some more recent articles (DIMMOCK 1984, 1987; MANDEL 1984, 1985; MCCULLOUGH 1986; COOPER 1987; IORIO 1988). Other reviews concentrate on specific groups of viruses: Arenaviridae (HOWARD 1986),

rotaviruses (MATSUI et al. 1989), foot-and-mouth disease virus (FMDV; McCULLOUGH et al. 1992), human immunodeficiency virus (HIV; NARA et al. 1991; McKEATING 1992; PUTNEY 1992), polioviruses and rhinoviruses (MOSSER et al. 1989), Paramyxoviridae (NORRBY 1990), type A influenza viruses (OUTLAW and DIMMOCK 1991) and lentiviruses (PEDERSEN 1989) More general treatments of antibody–antigen interactions are GETZOFF et al. (1987, 1989), and of antibody structure BURTON (1990).

2 Immunoglobulin G Neutralization by Inhibition of Attachment of Virus to the Cell

There are surprizingly few documented examples of immunoglobulin G (IgG) inhibiting attachment of virus to its cell receptor (Table 1). For this to occur it would seem that the IgG has to bind directly to or close by the viral attachment site or to change its conformation so that it no longer functions. Table 1 includes only data which show some indication of direct proportionality between neutralization and inhibition of attachment. For this reason some data where authors have ascribed neutralization to inhibition of attachment have been excluded, for example Venezuelan equine encephalomyelitis virus (VEEV) where there was a 90% reduction in attachment for 99.998% neutralization (ROEHRIG et al. 1988), poliovirus where monoclonal antibody (mab) ICJ27 caused an 80% reduction in attachment but 99% neutralization (EMINI et al. 1983a) and transmissible gastroenteritis virus (TGEV) where mabs to site A of the major surface protein inhibited attachment by 78%–96% but neutralized by up to 99.999999% (SUÑÉ et al. 1990). Another problem is that experimenters sometimes use only one concentration of IgG (e.g. LEE et al. 1981), which renders the conclusion that the observed inhibition of attachment is the primary cause of neutralization open to doubt. This is aptly illustrated by the interaction of TGEV with anti-site A mabs, where there is inhibition of attachment of already neutralized virus by a vast excess of antibody (SUÑÉ et al. 1990). Thus much of the work cited in Table 1 should be appraised with caution. However, the study by IORIO et al. (1989) on Newcastle disease virus (NDV) pays very careful regard to the correspondance between inhibition of attachment and neutralization and shows clearly that some neutralizing mabs directed against the haemagglutinin-neuraminidase (HN) protein block attachment while others do not; the latter inhibit neither haemagglutination nor neuraminidase (NA) activity. Another group of mabs gives a degree of inhibition of attachment which is less than the observed neutralization. It is also noteworthy that attachment of rhinovirus 14 was diminished even by Fab fragments of IgG (COLONNO et al. 1989) and, furthermore, this occurred with Fabs reactive with any of the four major antigenic sites.

Neutralizing IgG to reovirus is directed against the $\sigma1$ protein, which is the attachment protein (WEINER and FIELDS 1977; LEE et al. 1981), but the relationship between the neutralization site and the attachment site(s) on $\sigma1$ is not yet clear. Again there is the unresolved problem of quantitation mentioned above where a mab (G-5) with a neutralization titre of 1/12500 (BURSTIN et al. 1982) inhibited attachment at a dilution of 1/10 by only 60% (MARATOS-

Table 1. IgG neutralization by inhibition of attachment of virus to the cell

Family	Virus	Comment	References
Corona	TGEV	Anti-B; anti-C, anti-D	SUNE et al. 1990
Bunya	La Crosse virus	Site A	KINGSFORD et al. 1991
Herpes	Epstein–Barr virus	Anti-gp350 mabs[a]	NEMEROW et al. 1987
		Anti-gp350, anti-gp220	MILLER and HUTT-FLETCHER 1988
	HSV-1	Anti-gC[a], anti-gD[a]	FULLER and SPEAR 1985[a]; HIGHLANDER et al. 1987
		Anti-gB[a]; anti-gD[a]	KUHN et al. 1990
	Bovine herpes virus 4		DUBUISSON et al. 1990[c,d]
Myxo	Influenza virus		EISENLOHR et al. 1987; OUTLAW et al. 1990e; OUTLAW and DIMMOCK 1993[e]
Paramyxo	NDV	Anti-HN	IORIO et al. 1989[a], 1991
Picorna	Poliovirus types 2 and 3[b]		KELLER 1966; see Fig. 7[a]
	FMDV	Anti-VP1, 141–160	BAXT et al. 1984[a]; FOX et al. 1989
	Rhinovirus		COLONNO et al. 1989
Reo	Reovirus		LEE et al. 1981
	Rotavirus	Anti-VP7 (38K)[a], anti-VP4 (VP8*)[a]	SABARA et al. 1985; KUKUHARA et al. 1988 RUGGERI and GREENBERG 1991
Retro	Maedi-visna virus	Fibroblasts only	KENNEDY-STOSKOPF and NARAYAN 1986
	Mouse mammary tumour virus		MASSEY and SCHOCHETMAN 1981a[c]
	HIV-1	Anti-gp120[a]	FIELDS et al. 1988; HO et al. 1988[a]; SUN et al. 1989[f], POSNER et al. 1991
		Anti-gp120 carbohydrate	HANSEN et al. 1991[c]

[a] Only some antibodies; others do not inhibit attachment.
[b] No mabs to type 1 inhibit attachment.
[c] Inferred as mabs fail to neutralize when added after attachment.
[d] Neutralization with pairs of mabs; maximum 25%–30% neutralization.
[e] Contribute to but do not fully account for neutralization.
[f] Some mabs neutralize poorly.

FLIER et al. 1983). Nevertheless, despite this and the lack of quantitative data the best interpretation is that IgG prevents attachment. The situation is complicated by evidence that σ1 may have two receptor domains, a low-affinity carbohydrate-specific and a high-affinity protein-specific site (DERMODY et al. 1990), which may be used in succession by the virus during infection of a cell. The first site also mediates haemagglutination and it has been demonstrated through the use of antibody escape mutants that some mabs can inhibit haemagglutination without causing neutralization (BURSTIN et al. 1982). Neutralization escape mutants have substitutions at amino acids 340 and 419 while the carbohydrate receptor spans 198–204. Unfortunately this story may not be as straightforward as it appears, since reovirus neutralized with mab A-12 failed to attach to L cells but attached to the macrophage-like cell P388D1, presumably via Fc receptors, but was not infectious (BURSTIN et al. 1983). Clearly, the substantial (95%) loss of infectivity was not due to inhibition of attachment but to some defect in a post-attachment stage, and the data of LEE et al. (1981) therefore require re-evaluation. However, unlike some attachment-inhibiting antibodies (see below), those to σ1 of reovirus and to VP7 of the related rotaviruses neutralize efficiently and, for the latter, there is reasonable quantitative correspondence between the two parameters, suggesting that they may be biologically significant (SABARA et al. 1985; KUKUHARA et al. 1988). However, RUGGERI and GREENBERG (1991) find that only neutralizing antibody to VP4 of rotavirus blocks attachment albeit inefficiently; neutralizing antibody to VP7 does not. The situation is even more complicated since VP4 is cleaved by trypsin to form VP8* and VP5*, and only mabs to VP8* inhibit attachment.

Some attachment-inhibiting mabs neutralize very inefficiently: mabs specific for herpes simplex virus (HSV) type 1 gC and gD glycoproteins block attachment, but only at high concentrations (FULLER and SPEAR 1985; HIGHLANDER et al. 1987), whereas other anti-gC and anti-gD mabs neutralize efficiently without blocking attachment (Table 3). Glycoproteins D (and also B) are not in fact required for attachment in vitro although they are needed for penetration (FULLER and SPEAR 1987; HIGHLANDER et al. 1987, 1988). In a similar way, mabs to sites B, C and D of the major protein of TGEV give only 50% neutralization and inhibit attachment to the same extent (SUÑÉ et al. 1990) (Table 1).

For FMDV there is good evidence that the major neutralization site and the attachment site are both on VP1 between residues 141 and 160, and it seems likely that neutralizing antibody acts by sterically preventing attachment. However, there is at least one other neutralization site and antibody to this does not interfere with attachment (Table 3; BAXT et al. 1984). These aspects are discussed with reference to the three-dimensional structure of the particle (ACHARYA et al. 1989). Poliovirus and rhinovirus, also members of the Picornaviridae, have four or five neutralization sites and a topographically separate attachment site which is not immunogenic: there are mabs to poliovirus types 2 and 3 which inhibit attachment but none to type 1. The latter obviously have a different mechanism of neutralization from FMDV (see

MINOR 1990 and Sect. 23). However, there are early reports of antisera and even Fabs to type 1 virus which inhibit attachment (KELLER 1966). In a study of rhinovirus neutralization, COLONNO et al. (1989) showed that monoclonal IgG to each of the four major antigenic sites and Fabs derived from them inhibit attachment. They also all cause a shift in pI of the virus particle from 6.7 to 1.8–3.2 and it is not clear whether inhibition of attachment is due to steric inhibition by Fab or whether the changes in conformation give rise to the pI shift (see Sect. 23.6).

The major neutralization protein of type A influenza virus is the haemagglutinin (HA, Fig. 1). Neutralization is complex. Of the systems so far studied, where saturating amounts of IgG are used, antibody does *not* affect attachment of neutralized virus to cells (see Table 3). However, neutralizing IgG did prevent attachment of virus to cells of a B-lymphoma line (EISENLOHR et al. 1987). Attachement to red blood cells (rbc's) is also abrogated where

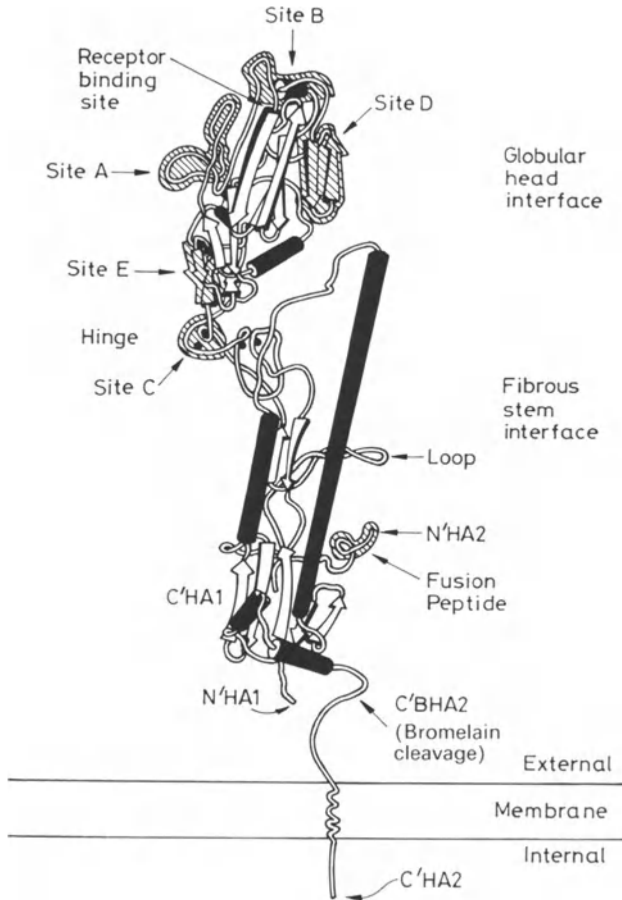


Fig. 1. Diagrammatic representation of the structure of a monomer of the H3 haemagglutinin of influenza virus A. The *shaded areas* show the positions of antigenic sites A–E. (From MURPHY and WEBSTER 1990, after WILEY et al. 1981)

virus is neutralized by IgG (POSSEE et al. 1982; OUTLAW et al. 1990). The situation differs when amounts of IgG just sufficient to cause neutralization (a 'low' IgG:virus ratio) are used. Here there is some (40%–60%) inhibition of attachment (to baby hamster kidney, BHK, and epithelial cells of tracheal organ cultures), although not enough to account for neutralization. Neutralization increases with the IgG concentration but attachment returns to the level seen with infectious virus (OUTLAW et al. 1990). Also, different mabs can affect attachment to a different degree, e.g. for the same amount of neutralization, attachment of X31 (H3N2) to BHK cells is inhibited by HC19 by 50% and by HC100 by 95%; however, in neither case did this account for the amount of neutralization observed (OUTLAW and DIMMOCK 1993). One explanation of the influence of IgG concentration on inhibition of attachment is that at low concentration IgG has freedom to undergo axial rotation and flexion (BURTON 1985; FEINSTEIN et al. 1986; PUMPHREY 1986) and is thus more effective at preventing the juxtaposition of the virus attachment site and cell receptor unit. At high IgG:virus ratios antibody is packed more tightly in an ordered array which is less mobile and interferes less with attachment. Thus the cell receptor unit, if long enough, may be able to interdigitate with the IgG and make contact with the viral attachment site as suggested by DIMMOCK (1987).

Some neutralizing mabs block attachment of HIV-1 to cells bearing its CD4 receptor (SUN et al. 1989). The virus attachment site is composed of a number of interacting parts of gp120 (OLSHEVSKY et al. 1990) to which amino acid residues of the C3 region (423–437 of strain IIIB) contribute (LASKY et al. 1987; Fig. 2A) and against which these mabs were prepared. An odd feature of the data of SUN et al. (1989) is that the mabs all inhibit attachment to the same extent while differing in ability to neutralize the infectivity of virions. MCKEATING and WILLEY (1989) refer to other neutralizing mabs which recognize the CD4-binding sequence. SUN et al. (1989) find attachment-inhibiting antibodies in only 2 of 65 sera from both asymptomatic and symptomatic HIV^+ individuals, although attachment-inhibiting antibodies have also been reported by others (HO et al. 1988) and would appear to be immunorecessive. Others are described by FIELDS et al. (1988), who show by quantitative electron microscopy that 'antibodies to gp120', presumably a polyclonal antiserum, reduce by 10-fold attachment of virus particles to $CD4^+$ lymphocytes; there were no data on neutralization but the antiserum can hardly be otherwise. Goat antiserum to gp120 also blocked attachment of virus particles (HO et al. 1988), as did the human mab F105 which recognizes a conformational determinant (POSNER et al. 1991).

Several reports comment that antibodies which inhibit virus attachment are inefficient at neutralization in terms of the concentration of IgG required (HSV-1, FULLER and SPEAR 1985; TEGV, SUÑÉ et al. 1990). Another category of antibody inhibits attachment of soluble envelope protein but nevertheless does not neutralize (Table 2). LASKY et al. (1987) and DOWBENKO et al. (1988) describe three mabs, of the same competition group, which block attachment of soluble gp120 to CD4; mab 5C2 maps to the C3 region (amino

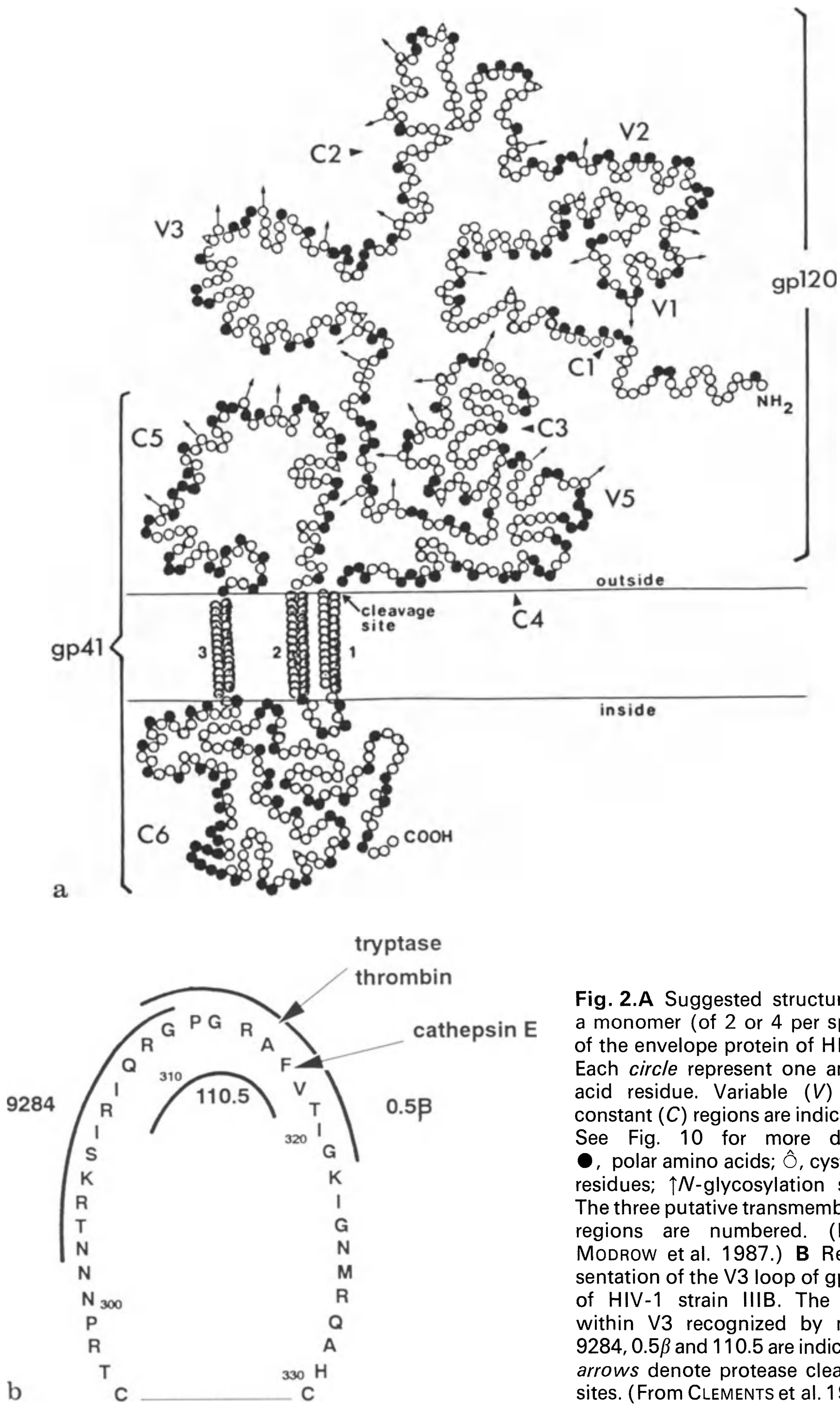


Fig. 2.A Suggested structure of a monomer (of 2 or 4 per spike) of the envelope protein of HIV-1. Each *circle* represent one amino acid residue. Variable (*V*) and constant (*C*) regions are indicated. See Fig. 10 for more detail. ●, polar amino acids; ○̂, cysteine residues; ↑*N*-glycosylation sites. The three putative transmembrane regions are numbered. (From MODROW et al. 1987.) **B** Representation of the V3 loop of gp120 of HIV-1 strain IIIB. The sites within V3 recognized by mabs 9284, 0.5*β* and 110.5 are indicated; *arrows* denote protease cleavage sites. (From CLEMENTS et al. 1991)

Table 2. IgG which inhibits attachment of soluble envelope proteins to cell receptors but does not neutralize

Family	Virus	Comment	References
Retro	HIV-1	Anti-gp 120	LASKY et al. 1987; DOWBENKO et al. 1988; SCHNITTMAN et al. 1988
Rhabdo	Rabies virus	Anti-G	BRACCI et al. 1988; CUSI et al. 1991

acid residues 423–437 of the IIIB strain) of gp120 (Fig. 1) and the others are close by, indicating along with other data that this sequence is relevant to the CD4-binding site (but see OLSHEVSKY et al. 1990 below). These mabs are confirmed as non-neutralizing (L.A. Lasky, personal communication). SCHNITTMAN et al. (1988) also find no correlation between neutralization and inhibition of binding of soluble gp120 to CD4. Similarly, attachment of the rabies virus G protein to the nicotinic acetylcholine receptor is inhibited by a mab which does not neutralize virus infectivity (BRACCI et al. 1988; CUSI et al. 1991). One explanation is that inhibition of attachment results from steric inhibition of the interaction of the virus binding site to the cell receptor and, as these viruses have from around one hundred to several hundred attachment sites, most will need to be engaged by antibody to achieve neutralization; secondly, if the interaction between attachment site and receptor is of high affinity, antibody may compete unfavourably with the receptor. The HIV-1 gp120–CD4 binding affinity is in the order of 10^9 M^{-1} (LASKY et al. 1987), while that of mabs ranges from 10^6 to $10^{9.5}$ M^{-1} (GERHARD et al. 1980; BROWN et al. 1990). Another factor relevant to Lasky's data is that the CD4-binding site is conformational and composed of elements from the constant region domains C3 and C4; mutation of residues within 423–437 had only a marginal effect on binding (OLSHEVSKY et al. 1990).

A general point which emerges from this discussion of neutralization of viruses with multiple attachment sites is that inhibition of attachment cannot be achieved by a single antibody molecule and is not predicted to follow the apparent single-hit kinetics that all neutralization reactions are so far reported to do (see Sect. 20).

An unusual phenomenon is the dissociation of about 80% of rotavirus attached to cells by the subsequent addition of mab to VP4 or its trypsin cleavage product VP8*. Mabs to the other major surface protein, VP7, do not release bound virus (RUGGERI and GREENBERG 1991). Prebound mabs to VP4 and VP8* prevent attachment. This may be the result of reversible interaction between a virus attachment site and cell receptor and binding of a mab which prevents reattachment, or to a more complex interaction where binding of antibody to attached virus induces dissociation.

Conclusion. Inhibition of attachment is a relatively uncommon cause of neutralization and is often an inefficient process; data require interpreting with care to ensure that inhibition of attachment and neutralization correspond quantitatively since it is possible that inhibition of attachment of already neutralized virus can occur.

3 Immunoglobulin G Neutralization Which Does Not Inhibit Attachment of Virus to the Cell

A list of IgG-neutralized viruses which attach to cells is listed in Table 3; what is known of the mechanisms of neutralization will be discussed in the appropriate sections below. As far as is known Table 3 excludes attachment which is peculiar to virus-IgG complexes, although evidence that non-neutralized and neutralized virus use the same receptor is rarely available. This has been demonstrated by competition for attachment between neutralized and non-neutralized virus (with TGEV: SUÑÉ et al. 1990) or by enzymic destruction of the sialic acid receptor of influenza virus (OUTLAW et al. 1990). There is, however, the example of neutralized vesicular stomatitis virus (VSV) using a different receptor (SCHLEGEL and WADE 1983): excess infectious virus failed to block its attachment and an excess of soluble cell receptor—the lipid phosphatidylserine—prevented attachment of infectious but not neutralized virus to cells.

Recent understanding about functional domains of proteins suggests why attachment of so many viruses should be unaffected by neutralizing IgG. We now know that neutralization sites and virus attachment sites on some viruses, such as type A influenza (Fig. 1), are distinct, so that binding of IgG to the former need not necessarily block attachment. In addition, some virus attachment sites are non-immunogenic (see Sect. 22.2.1).

The fact that viruses can bind IgG and yet not be neutralized has long been known from data on non-neutralizing antibody, and in some infections infectious virus–antibody complexes are found in the circulation. A more precise demonstration that a virus particle can bind IgG without affecting its ability to attach to cells is provided by some mab-escape mutants which, although no longer neutralized, still bind the selecting antibody (Table 4A), although in the case of HIV-1 this is seen as a decrease in efficiency of neutralization rather than an absolute abrogation of neutralizing activity (NARA et al. 1990). One explanation for this phenomenon is that neutralization of wild-type virus is caused by a conformational change which is triggered by the binding of antibody to the epitope and that the above escape mutants carry a mutation in that pathway rather than the epitope itself. Most escape mutants, however, do not bind the selecting mab. An analogous situation occurs where apparently similar epitopes exist in different naturally occurring strains; all strains bind the same monoclonal IgG but only one of them is neutralized (Table 4B).

I shall consider two scenarios in which (1) subsaturating and (2) saturating amounts of IgG do not block attachment. Animal viruses have multiple

Table 3. IgG neutralization which does not inhibit attachment of virus to the cell

Family	Virus	Comment	References
Adeno	Adenovirus	Anti-hexon, anti-penton base, anti-penton fibre[b]	WOHLFART et al. 1985.
		Anti-hexon	WOHLFART 1988
Bunya	La Crosse virus	Site G	KINGSFORD et al. 1991[a]
	Dugbe virus		GREEN et al. 1992
Corona	TGEV	Site A	NGUYEN et al. 1986; SUÑÉ et al. 1990
Herpes	HSV-1	Anti-gB, anti-gC[a], anti-gD[a], anti-gH	HIGHLANDER et al. 1987, 1988; FULLER and SPEAR 1985, 1987; FULLER et al. 1989; KÜHN et al. 1990; MATIS et al. 1992
	Epstein–Barr virus	Anti-gp85	MILLER and HUTT-FLETCHER 1988; HADDAD and HUTT-FLETCHER 1989
	Monkey CMV		FARRELL and SHELLAM 1990
	Pseudorabies virus	1A, 1B	ELOIT et al. 1990
Myxo	Influenza virus	Anti-HA	HULTIN and MCKEE 1952; DOURMASHKIN and TYRRELL 1974; POSSEE and DIMMOCK 1981; POSSEE et al. 1982; DIMMOCK et al. 1984; TAYLOR and DIMMOCK 1985a; OUTLAW et al. 1990
Paramyxo	NDV		RUBIN and FRANKLIN 1957; GRANOFF 1965; RUSSELL 1984; IORIO et al. 1989[a]
	Simian virus 5		MERZ et al. 1981
Picorna	Poliovirus 1, 3		HOLLAND and HOYER 1962; MANDEL 1967a; EMINI et al. 1983a; see Fig. 7[a].
	FMDV		BAXT et al. 1984[a]
Pox	Rabbit pox virus		JOKLIK 1964; DALES and KAJIOKA 1964
	Vaccinia virus	Anti-14 K	RODRIGUES et al. 1985
Reo	Reovirus	With mab A-12	BURSTIN et al. 1983
	Rotavirus	Anti-VP3 (now VP4)	KUKUHARA et al. 1988; RUGGERI and GREENBERG 1991[a]
Retro	HIV-1	Anti-gp120	HO et al. 1988[a]; SKINNER et al. 1988[a,c]; DOWBENKO et al. 1988[a]; LINSLEY et al. 1988[c]; JAVAHERIAN et al. 1989; NARA 1989

(Continued)

Table 3. (*Continued*)

Family	Virus	Comment	References
Rhabdo	Rabies virus		DIETZSCHOLD et al. 1987
	VSV		SCHLEGEL and WADE 1983
Toga	VEEV		ROEHRIG et al. 1988

[a] Only some antibodies; others inhibit attachment.
[b] Aggregates virions and increases attachment 3- to 5-fold.
[c] Tested with soluble gp120 protein, not virus.

Table 4. Viruses which bind neutralizing monoclonal antibody without loss of infectivity

Family	Virus	References
A. Neutralization-escape mutants which still bind the selecting antibody		
Corona	TGEV	SUÑÉ et al. 1990
Herpes	HSV-1	MUGGERIDGE et al. 1988
Myxo	Influenza A virus	GITELMAN et al. 1986
Paramyxo	Sendai virus	PORTNER et al. 1987
Picorna	Poliovirus	DIAMOND et al. 1985; THOMAS et al. 1986; BLONDEL et al. 1986
	FMDV	McCAHON et al. 1989
	Coxsackie virus	PRABHAKAR et al. 1987
Reo	Bluetongue virus	WHITE and EATON 1990
Retro	Feline leukaemia virus	NICOLAISEN-STROUSS et al. 1987
	HIV-1	McKEATING et al. 1989; WILLEY et al. 1989; NARA et al. 1990; WILSON et al. 1990
B. Naturally occurring viruses binding a monoclonal antibody which neutralizes only other strains of virus		
Flavi	Yellow fever virus	BUCKLEY and GOULD 1985
Paramyxo	PIV-3	COELINGH and TIERNEY 1989a
Picorna	Poliovirus	CRAINIC et al. 1983
Reo	Bluetongue virus	WHITE and EATON 1990
Retro	HIV-1, strain RF	LAKE et al. 1992
Rhabdo	Rabies virus	FLAMAND et al. 1980
Toga	Sindbis virus	STANLEY et al. 1985

attachment sites, so at subsaturating concentrations there is no reason a priori why IgG should inhibit attachment since there will be attachment sites unencumbered by antibody. How many IgG molecules are in fact required to bind to virus in order to neutralize it? There are data from studies with adenovirus, poliovirus and influenza virus: when adenovirus was neutralized

by 63% there were 1.4 molecules of anti-hexon antibody, presumably IgG, bound per virion (WOHLFART 1988). However when poliovirus was similarly neutralized there were found to be four molecules of IgG per virion (ICENOGLE et al. 1983) even though a figure of one molecule per virion is expected if neutralization follows single-hit kinetics—the reasons for this will be addressed later (see Sect. 20). Even so, four molecules of IgG per virion should not prevent attachment of poliovirus to its host cell since the particle is composed of 60 subunits and even with bivalent binding there are 52 attachment sites free to make contact with cells. (However with some other mabs $\geqslant 15$ molecules IgG per virion are required for the same amount of neutralization: see Sect. 23.5.) When influenza virus was neutralized to 63% there were about 70 IgG molecules per virion (TAYLOR et al. 1987) and since each virion has 1000 (HA spikes, each of which is a trimer with three attachment sites, there will be at least 2580 attachment sites unencumbered with antibody (that is assuming the worst possible scenario with each IgG molecule bivalently attached to two separate HA trimers and blocking attachment to the cell by each HA trimer to which IgG is bound, i.e. $(1000-140) \times 3$).

At high ratios of IgG:virus there is again no reason a priori why attachment should be inhibited providing that IgG does not directly bind to the virus attachment site or alter its conformation and that the cell receptor is long enough to penetrate through the fringe of IgG surrounding the virus to reach the virion attachment site (see Sect. 7). Indeed, this is borne out experimentally with type A influenza virus even at saturating concentrations of IgG (TAYLOR et al. 1987; OUTLAW et al. 1990). The maximum length of most IgG molecules in extended conformation is about 16 nm (about 4 nm per domain; SILVERTON et al. 1977) but flexing at the hinge to a T-shaped structure reduces this to around 10 nm. Some, such as human IgG3, are considerably longer due to an extended region between the C_H1 and C_H2 domains. Data on the extent to which proteins project above the plasma membrane are now emerging; many exceed 10 nm and range in length upto 30 nm (CD43; SPRINGER 1990).

Conclusion. Many combinations of virus and antibody result in neutralized virus which attaches to cells. Loss of infectivity must therefore be due to antibody-induced defects in virus function(s) which have effect after attachment; these are discussed in the following sections.

4 Immunoglobulin G Neutralization by Aggregation of Virions

IgG, Ig A and Ig M molecules all have the flexibility to form aggregates by crosslinking virus particles. Early work on aggregation is well reviewed by SVEHAG (1968), and there are later discussions by STEENSGAARD and FRICH (1979), JACOBSEN and STEENSGAARD (1979), THOMPSON and JACKSON (1984) and DAVIES and PADLAN (1990). A priori, the number of infectious units is reduced when virus is aggregated, and this can be achieved by any antibody which recognizes a virion epitope, be it intrinsically neutralizing or not (Fig. 9). There are more recent data on aggregation of polyomavirus (OLDSTONE et al. 1974), adenovirus (anti-fibre) (WOHLFART et al. 1985), FMDV (BAXT et al. 1984), poliovirus (BRIOEN et al. 1983, 1985a; ICENOGLE 1983; THOMAS et al. 1985, 1986; TANIGUCHI and UROSAWA 1987; MOSSER et al. 1989), rhinovirus (COLONNO et al. 1989), type A influenza virus (ARMSTRONG and DIMMOCK 1992; ARMSTRONG et al. 1990; OUTLAW et al. 1990; OUTLAW and DIMMOCK 1990) and La Crosse virus (KINGSFORD et al. 1991). Some of this discussed below and some in Sect. 23, which deals specifically with neutralization of polioviruses and rhinoviruses.

In an electron-microscope study, ALMEIDA et al. (1963) and others (see SVEHAG 1968) clearly show that aggregation by what is probably IgG depends on the ratio of antibody:virus. There is an optimum ratio for aggregate formation, for when antibody is in excess there are no free epitopes and hence no aggregation (Fig. 3A,B). This has been demonstrated for influenza virus with IgG (LAFFERTY and OERTELIS 1963; TAYLOR et al. 1987; OUTLAW and DIMMOCK 1990), IgM (ARMSTRONG et al. 1990) and IgA (ARMSTRONG and DIMMOCK 1992). These virus–antibody complexes are not particularly stable in vitro as normal mixing procedures prior to titration abolish any loss of infectivity, even with the highly avid IgM (ARMSTRONG et al. 1990). However, there are intrinsic uncertainties about the extent to which preparation of specimens for electron microscopy contributes to aggregation and clearly a better approach is to analyze complexes formed in solution, as with polyoma virus (OLDSTONE et al. 1974) and poliovirus (ICENOGLE et al. 1983; THOMAS et al. 1986). The latter reports show very nicely that aggregation of poliovirus in solution was minimal at high concentrations of antibody. Only with a minority of mabs was infectivity restored on disaggregation at high antibody concentration (MOSSER et al. 1989). Electron microscope data for polyoma virus and data obtained from virus in solution agree that although one or two molecules of IgG per virion caused aggregation, about ten molecules per virion were required before there was significant loss of infectivity (OLDSTONE

et al. 1974), suggesting that, like influenza virus, aggregates are unstable and infectivity is restored during titration. Thus although in principle aggregation reduces infectivity in proportion to the number of virions contained in each aggregate, it is uncertain to what extent this contributes to neutralization in vivo. On the other hand, it is clear that virus–antibody complexes occur in vivo and the deposition of such complexes in blood vessels, joints, kidneys, etc., can be a major contributary factor to the pathology of the associated disease.

The state of aggregation of viruses, whose attachment to cells is not inhibited by IgG, does not appear to affect the ability of aggregated virus to attach to cells (type A influenza virus and monoclonal IgG2a, OUTLAW et al. 1990; poliovirus type 3 with mabs 25-5-5 or 134, Fig. 6, P.D. Minor, unpublished data). Further, reaction of FMDV with mabs that were unusually efficient at aggregating, only inhibited attachment by 50%–70% (BAXT et al. 1984).

IgG–influenza virus complexes were only found when IgG was present at low, often sub-neutralizing, concentrations and the ability to aggregate varied between mabs directed to different antigenic sites (OUTLAW et al. 1990). Since all the mabs were of the same isotype, variation in formation of aggregates may relate to the relative position of the site on the HA (WILEY et al. 1981; WRIGLEY et al. 1983; POUMBOURIOS et al. 1990). TANIGUCHI and UROSAWA (1987) and MOSSER et al. (1989) pursue the same argument. The latter distinguish between mabs which bind to site 3A of poliovirus type 1 and rhinovirus type 14 and neutralize at low concentration without aggregation and those binding to site 2, where neutralization requires a high concentration of antibody and virus is aggregated. They suggest that the difference lies in whether or not repeated epitopes on a single virion are the appropriate distance apart—not too close and not too distant and in the correct orientation—to be crosslinked by IgG. If an IgG is unable to bind bivalently to a virion it is then able to bind to a second virion and to form aggregates. Presumably the mabs to FMDV referred to above, which caused more aggregation than did others to different epitopes, fall into this category (BAXT et al. 1984), as do epitopes on the adenovirus fibre which cause aggregation more readily than those of the penton base or hexon (WOHLFART et al. 1985), although study of different antibody:virus ratios would be required to establish this. OUTLAW et al. (1990) conclude that aggregation may contribute to neutralization of influenza virus if those aggregates are sufficiently stable but at no time is aggregation sufficient to be completely responsible for the loss of infectivity observed. However, the combined loss of infectivity due to aggregation and inhibition of attachment fit very closely to the initial part of the observed neutralization curve although, it should be noted, this was true only for one of three mabs studied.

As already mentioned there is no aggregation or inhibition of attachment at higher IgG:virus ratios and another mechanism of neutralization must be operating. The fact that three mechanisms of neutralization may be operating

simultaneously at low IgG:virus ratios (aggregation, inhibition of attachment, post-attachment neutralization) may explain the observation that attachment of the first 200 molecules of IgG to influenza virus causes a 30-fold reduction in infectivity, whereas attachment of the next 200 molecules of IgG causes a mere three fold additional loss of infectivity (see figure 6 of TAYLOR et al. 1987).

Lastly, of some practical importance are the observations that infectious particles are maintained within an aggregate of intrinsically neutralized poliovirus (WALLIS and MELNICK 1967) and that the infectivity of influenza virus is restored by aggregation with non-infectious virus (DALES and PONS 1976), probably by the transfer of antibody to the added virus.

Conclusion. Two points to emerge from this section are, first, that the mechanism of neutralization may be affected qualitatively by the immunoglobulin:virus ratio and, secondly, that antibody may effect several different properties of the virus. Thus care must be taken to distinguish between the mechanism of neutralization and epiphenomena, by studying a range of immunoglobulin: virus ratios.

5 Immunoglobulin G Neutralization Mechanisms Which Operate After Attachment of Virus–Antibody Complex to a Cell Receptor Unit

Potential targets of neutralization include inhibition of fusion at the plasma membrane, inhibition of endocytosis, inhibition of uncoating by fusion or other events which occur after endocytosis, and inhibition of events necessary for infection which occur after uncoating.

5.1 Inhibition of Fusion at the Plasma Membrane

See Sect. 5.3 below.

5.2 Inhibition of Endocytosis

Inhibition of endocytosis would probably operate by antibody interfering with the binding of a sufficient number of cell receptor units to initiate endocytosis. No examples are known with IgG, although this appears to be one of the way in which influenza virus is neutralized by IgM (see Sect. 10 and TAYLOR and DIMMOCK 1985b; OUTLAW and DIMMOCK 1990). It would seem that IgG is too small to interfere with endocytosis. However, IgG might interfere with endocytosis if the relative molecular dimensions of IgG and cell receptor were the same as IgM and the cell receptor in the example cited.

5.3 Inhibition of Fusion of Viral and Cellular Membranes

Most enveloped viruses are uncoated by fusion with the wall of the endocytotic or endosomal vesicle in which they have been internalized when the internal milieu of the vesicle drops to a pH of 5–6. Others (paramyxoviruses, herpesviruses, HIV-1) fuse with the plasma membrane at neutral pH (WHITE 1990) and these provide most of the currently known examples of neutralization acting by inhibition of the fusion process.

Paramyxoviruses have two major surface glycoprotein neutralization antigens: an attachment protein called the haemagglutinin-neuraminidase (HN), or in those viruses which lack neuraminidase activity simply G, and a fusion (F) protein. The relative efficiency of HN/G and F as neutralization

antigens varies from virus to virus; for example, F is the major neutralization antigen of respiratory syncytical virus, whereas in NDV neutralization operates through both HN and F (NORRBY 1990). HN/G and F have a number of functional domains but the structure–function relationship is not known. Not all HN/G- and F-specific mabs neutralize: for instance, of the eight antigenic sites of the human parainfluenza virus 3 (PIV-3) F protein, only four are neutralizing (COELINGH and TIERNEY 1989a), and of the six sites on the HN protein only three are neutralizing (COELINGH 1986). Otherwise there is at present little insight into the mode of action of these neutralizing antibodies. F-specific antibodies could be expected to neutralize by inhibiting the penetration of virus by fusion at the plasma membrane, but this has not yet been studied; they do, however, inhibit penetration (RUSSELL 1984). While mabs to sites AB, B and C both neutralized and inhibited fusion of PIV-3-infected cells when incorporated into the overlay medium, half of those to site A did not inhibit fusion at all, suggesting that they neutralize by a different mechanism (COELINGH and TIERNEY 1989a). Intriguingly, the activity of some neutralizing anti-HN mabs in inhibiting haemolysis of rbc infers that HN has fusogenic activity in its own right, but the relative contribution of HN and F to viral fusion is unknown (NORRBY 1990). However HN and F proteins interact in a specific and interdependent manner to effect fusion (Hu et al. 1992). All mabs neutralizing rabies virus inhibit low-pH-mediated fusion of cells (DIETZSCHOLD et al. 1987). Some neutralizing mabs also inhibited fusion (syncytium formation) by the corona-virus murine hepatitis virus type 4 (MHV-4; LUYTJES et al. 1989; ROUTLEDGE et al. 1991) and bovine leukaemia virus (BRUCK et al. 1982b).

Fusion can also be studied by labelling membranes of viruses with a lipid-soluble fluorescent probe such as octadecyl rhodamine B chloride (R_{18}); at high R_{18} concentration fluorescence is quenched, but when diluted by lateral diffusion following fusion with another membrane fluorescence increases. This dequenching technique has been used to show that neutralizing IgG directed against gp85 of Epstein–Barr virus does not prevent attachment but inhibits the fusion induced by virus particles (MILLER and HUTT-FLETCHER 1988) or virosomes (HADDAD and HUTT-FLETCHER 1989).

There is evidence too that HSV enters cells by fusion and IgGs against certain epitopes of envelope glycoproteins gB, gD and gH, which permit attachment but block penetration, can be considered as strong candidates for the fusion-inhibition mode of neutralization (FULLER and SPEAR 1985, 1987; FULLER et al. 1989; HIGHLANDER et al. 1987, 1988; KÜHN et al. 1990). FULLER and SPEAR (1987) and FULLER et al. (1989) show very neatly that the infectivity of anti-gD or anti-gH neutralized virus which has attached to cells can be substantially (by about two or more orders of magnitude) restored by treatment with the fusogenic agent polyethylene glycol. This impressive result suggests that at least some of this neutralized virus is defective in fusion activity only; what is not explained is why 99% of virus is still neutralized.

The diversity of HSV neutralization proteins indicates the complexity of the virus–cell interaction: gC is also a neutralization protein but is not essential for infectivity in vitro. gB, gC and gD are all involved in attachment to cells and act non-cooperatively (KÜHN et al. 1990). Penetration, however, appears to require the three proteins working in concert as cells expressing gB or gD alone do not fuse (JOHNSON et al. 1984). Electron microscope studies show, too, that gB and gD form morphologically distinct spikes (see FULLER et al. 1989).

There is a body of evidence which suggests that antibodies of the V3 loop of the gp120 of HIV-1 neutralize by inhibiting fusion of the viral envelope with that of the cell, but much of this is circumstantial. [However, see FREED et al. (1991) and GRIMAILA et al. (1992), who show that mutations in this region affect fusion and neutralization, as well as other aspects of multiplication.] Ideally, for an unequivocal demonstration mabs should be used and neutralization, attachment of virus to cells and fusion of virus to cells should be measured. However, antisera have often been employed, making it much more difficult to be sure that the antibody of one specificity is responsible for inhibiting different biological properties (e.g. neutralization and fusion-inhibition: LIFSON et al. 1986). Furthermore, all data obtained so far on fusion relate to fusion between cells; the extrapolation to fusion between virions and cells may not be valid as a mutation in CD4 abrogates syncytium formation by HIV-1 without affecting the ability of cells bearing the mutated CD4 to be infected (CAMERINI and SEED 1990). The clearest evidence relating neutralization and fusion comes from SKINNER et al. (1988), who show that neutralizing mabs (0.5β and 9284, Fig. 2B) do not inhibit attachment of free gp120 but do inhibit syncytium formation in infected cell cultures. LINSLEY et al. (1988) demonstrate that it takes about 1000-fold more of mab 110.4 to inhibit attachment of isolated gp120 than it does to initiate neutralization. Mabs of the 110.3–110.6 series are IgG1 and neutralize HIV-1 infectivity and inhibit syncytium formation. These mabs react with amino acids 279–472 of gp120. Mab 110.5 selects mutants with a change in the V3 loop at position 308 (Arg to Lys; KINNEY-THOMAS et al. 1988). There are supporting data from sera to gp160 or to peptides encompassing the V3 region of gp120 (see Figs. 2.10) which have both neutralizing and fusion-inhibiting activity (PALKER et al. 1988; RUSCHE et al. 1988; JAVAHERIAN et al. 1989; LAMAN et al. 1992). Evidence that these are virus strain specific or can be blocked by peptides from the V3 region futher indicates their specificity. BROLIDEN et al. (1990) report independently that five neutralizing IgGs map to residues 304–323 of the V3 region. The activity of anti-peptide IgGs which inhibit fusion centres on amino acids 312–319; sera obtained from 6 of 14 HIV-infected men reacted with this region and were mainly IgG1 (GOUDSMIT et al. 1988a). This antibody is also neutralizing (GOUDSMIT et al. 1988b).

The mechanism by which fusion is inhibited is thought by CLEMENTS et al. (1991) to result from neutralizing IgG preventing proteolytic cleavage of the V3 loop at a site which is common to HIV-1, HIV-2 and simian

immunodeficiency virus (SIV). They demonstrate that three mabs (including the neutralizing, fusion-inhibiting mabs 110.5 and 9284 mentioned above) prevent cleavage by thrombin and cathepsia E (see Fig. 2B). Furthermore, some human sera inhibit cleavage and this is proportional to their neutralizing activity. They hypothesize that neutralizing antibodies directed to the V3 loop do not inhibit attachment but inhibit the cleavage event which is necessary for fusion of viral and cellular membranes, and thus prevent entry of the viral genome into the cell. In contrast, antibodies to the viral attachment site prevent attachment (Table 1); both specificities are present in human post-infection sera.

It is remarkable that there are so few examples of IgG which neutralizes by inhibiting the low-pH-requiring fusion of viral and cellular membranes. Attachment and endocytosis of West Nile virus are unaffected by neutralizing IgG but the viral genome is not released into the cytoplasm, indicating that it is the fusion of viral and cellular membranes which has been inhibited (GOLLINS and PORTERFIELD 1985). Monoclonal IgG to sites D, E and G of La Crosse virus cause substantial inhibition of fusion of virus to BHK cells, while antibody to site B has little effect and evidently neutralizes by a post-fusion event (L. Kingsford, personal communication), Fusion of VSV to the plasma membrane at low pH as shown by fluorescence dequenching of R_{18}-labelled viral lipid is inhibited by antibody to G protein added post-attachment (BLUMENTHAL et al. 1987). The fusogenic activity of type A influenza virus was examined in a model system using neutralizing mabs to the HA which inhibit pH-5.9-induced haemolysis of rbcs (KIDA et al. 1983). Fusion and haemolysis are related but not identical events (PURI et al., 1990). This is interesting as mabs to sites II and IV do not normally inhibit haemagglutination and hence do not act by inhibiting attachment. Virus incubated at pH 5.9 before being added to rbcs still causes haemolysis, its HA having presumably undergone the conformational change necessary for this process; mabs to site III and IV bind to pH 5.9-virus, and these virus–mab complexes bind to rbcs and cause haemagglutination, but haemolysis is inhibited. KIDA et al. (1983) conclude that mab inhibits a stage in haemolysis which occurs after the conformational change to the HA. Additionally, KIDA et al. (1983) find that if virus is first incubated with mab at pH 7, and then dropped to pH 5.9 and mixed with rbcs, virus attaches to rbcs but there is no haemagglutination and no haemolysis. Here, the authors conclude that mabs prevent the initial conformational change happening to the HA. (It is not explained how the pH-5.9 form of the HA permits the mabs to inhibit haemagglutination; also, these data must be interpreted with caution since it is far from certain how similar is the behaviour of rbcs and dividing cells in culture.) In another set of experiments YODEN et al. (1986) use electron spin resonance to show that neutralizing mabs to different antigenic sites inhibit the low-pH-induced conformational change of isolated HA by two different modes. WHARTON et al. (1986) examined another model system, the fusion of influenza virus with liposomes. Again, its relevance to infection of living

cells is uncertain, especially as the interaction of virus and these liposomes did not require sialic acid-bearing cell receptor molecules. Recently, the endocytic fusion activity of three strains of human and avian type A influenza viruses was examined using virions labelled in their lipid envelopes with the fluorescent probe R_{18}. The results were complex, as at relatively low concentrations of neutralizing monoclonal IgGs to the HA there was some inhibition of virus attachment. However, fusion of the virus which does attach and is internalized is inhibited by at least 80% (OUTLAW and DIMMOCK 1993). The combined inhibition of attachment and inhibition of fusion can account for the extent of neutralization at the 63% level but at higher mab concentrations neutralization is too great to be explained in this way and it is presumed that at least one other mechanism of neutralization is then operating. Neutralization by relatively low ratios of IgG:virus (OUTLAW et al. 1990; OUTLAW and DIMMOCK 1993) differs from earlier work using high ratios of IgG:virus where attachment was not inhibited and viral RNA entered the nucleus apparently normally (see Sect. 3, 23.3 and review by OUTLAW and DIMMOCK 1991). Fusion experiments at high IgG:virus ratios have not been done. The fusion together of cells at low pH by high concentrations of viruses parallels intraendosomal fusion and this technique has been used to show that neutralizing IgG does not inhibit the fusogenic properties of the bunyavirus Dugbe (GREEN et al. 1992).

Conclusion. Inhibition of fusion of enveloped viruses with cell membranes is one of the ways in which IgG can cause neutralization

5.4 Inhibition of Non-fusion Uncoating

Neutralized vaccinia virus is internalized in vacuoles in L cells and is gradually degraded (DALES and KAJIOKA 1964). Similarly, adenovirus neutralized with antibody to hexon is internalized and remains sequestered in vesicles (EVERITT et al. 1986). Glutaraldehyde-fixed adenovirus behaves in the same way, leading to the conclusion that antibody causes a failure in uncoating.

5.5 Inhibition of Events Which Occur After Primary Uncoating

Even after an enveloped virus particle has lost its membrane and entered the cytoplasm (primary uncoating) the resulting viral genome–protein complex probably has to undergo other processes, such as transport to the appropriate intracellular site of replication or secondary uncoating, before the genome can be expressed. Many viruses also contain enzymes, such as the RNA-dependent RNA polymerase of negative-strand viruses and reverse transcriptase of retroviruses, which are essential for replication. In theory the

activity of these could be modulated as the result of antibody binding to an external epitope of a coat protein and transducing a signal in the presumed (and poorly understood) manner by which cell membrane protein receptors indicate the presence of their bound ligand to the interior of the cell. There is little direct evidence for this at present in regard to neutralization but a close analogy is provided by the modulation of expression of measles virus gene products in the cell by antibody bound to viral antigens embedded in the outside surface of the plasma membrane both in vitro (OLDSTONE et al. 1980; FUJINAMI and OLDSTONE 1979, 1980, 1984; BARRETT et al. 1985; ZINNHEIMER-DREIKORN and KOSCHEL 1990) and in vivo (LIEBERT et al., 1990).

IgG neutralization of influenza virus at a high antibody:virus ratio does not inhibit attachment, internalization, fusion or transport of the genome to the cell nucleus (POSSEE and DIMMOCK 1981; POSSEE et al. 1982; TAYLOR and DIMMOCK 1985a; OUTLAW et al. 1990). However, there is no primary or secondary transcription of the virus RNA (RIGG et al. 1989; POSSEE et al. 1982). Initial suspicion that the virion polymerase from neutralized virus was directly affected was not substantiated and no defect was detected in its ability to recognize and use capped messenger (m) RNA to prime the synthesis of its own mRNA in the normal way (RIGG et al. 1989). Explanation of this paradox seems to lie in the failure of the inner core of the virus to undergo secondary uncoating: virion RNA from infectious virus becomes RNase sensitive whereas that from neutralized virus remains resistant, and RIGG et al. (1989) postulated that virion RNA and transcriptase of neutralized virus is potentially fully functional but is not released from the virion core structure. What now appears more likely in the light of more recent work with IgA is that secondary uncoating takes place but that the viral RNP does not undergo an 'activation' step (possibly a relaxation of the RNP structure) which is necessary for transcription to proceed (ARMSTRONG and DIMMOCK 1992).

Earlier work on HSV also suggested that its genome was not released from neutralized virus which has been internalized by the cell (YOSHINO and TANIGUCHI 1967), although it is not clear at what stage of uncoating this operated. A similar explanation may apply to the IgG-neutralization of the lentivirus visna-maedi in macrophage-like cells (although neutralizing IgG inhibits attachment to fibroblasts). Neutralized virus particles are uncoated but no DNA is synthesized and there is no transcription (KENNEDY-STOSKOPF and NARAYAN 1986).

Inhibition of an event occurring after primary uncoating is presumed to operate in the neutralization of HSV-1 (MATIS et al. 1992) and La Crosse virus by IgG to site B (L. Kingsford, personal communication) where attachment, internalization and fusion properties of these viruses are still functional.

Antibodies do not usually enter cells, strictly speaking, although they are transported through them in vesicles (e.g. transcytosis of IgG across the placenta, IgA to mucosal surfaces). In a similar way, IgGs that do not inhibit the attachment to those viruses which enter cells by endocytosis will be

endocytosed as part of the virus-antibody complex, and it would be interesting to know if they had any other destiny than to enter secondary lysosomes. MAZIER et al. (1986) suggest that antibodies can enter the cytoplasm and act intracellularly on *Plasmodium* sporozoites. Antibodies can be introduced into cells artificially by, for example, fusion with antibody-loaded rbcs (DOXSEY et al. 1985) or by spontaneous translocation after fatty acylation (KABANOV et al. 1989a). With the latter, substantial intracellular neutralization of influenza virus and respiratory syncytial virus (RSV) was achieved but it remains to be seen if there are any circumstances in which antibodies act in this way in vivo.

Conclusion. There is circumstantial evidence that neutralizing antibodies can render enveloped viruses non-infectious by transducing an inhibitory signal across the viral membrane which prevents the functioning of an internal virion component.

6 Neutralization Which Occurs by Virus Binding Antibody After It Has Attached to a Cell

After attaching to a cell some viruses can then be neutralized by addition of antibodies of certain specificities. It is assumed that viruses whose attachment to cells is inhibited by IgG (Table 1) are not neutralized in this way, and indeed this is a criterion often used to define post-attachment neutralization. However, although mab to VP8*, the trypsin cleavage product of VP4 of rhesus monkey rotavirus, blocks attachment it also has the capacity to cause the release of virus already attached to MA104 cells (RUGGERI and GREENBERG 1991). Neutralizing mabs to VP5*, the other cleavage product of VP4 and VP7, do not cause release of virus. With this proviso the addition of antibody after attachment can be and has been used to distinguish between 'inhibition of attachment' and 'inhibition of post-attachment' mechanisms of neutralization. Viruses listed in Table 3 whose attachment to cells is not inhibited by neutralizing IgG are all candidates for post-attachment neutralization. It is an important concept as it indicates that IgG can act at a relatively late stage of virus–cell interaction. However, there is selectivity of antibody action as post-attachment neutralization of adenovirus was achieved with antibody to hexon but not to penton or penton base proteins (WOHLFART 1988). One obvious factor in the process of post-attachment neutralization is the rate at which attached virus is internalized, as antibody can only neutralize when it is outside the cell (but see above and KABANOV et al. 1989b). For example, NDV which has attached to cells at 4°C can be neutralized by either anti-F or anti-HN mabs, but if infected cells are raised to 37°C susceptibility to neutralization ceases after 90 s (RUSSELL 1984). Possibly this is also the reason why IgG added to TGEV after attachment to cells is 1000-fold less effective than when used conventionally (SUÑÉ et al. 1990). Antibody could conceivably act by sterically blocking the nucleation of cell receptors necessary for endocytosis or fusion, stabilizing the structure of a viral protein so that a required conformational change or proteolytic cleavage does not take place or by imposing an unfavourable charge. Alternatively, the relevant antibody may recognize a viral epitope which is revealed only after virus has attached to the cell or an epitope consisting of residues of the viral coat protein and the cell receptor formed de novo, as suggested by KJELLÉN (1985). Into the former category comes neutralization of adenovirus by IgG specific to the penton base. At pH 7 there is only 50% neutralization, but when virus was held at pH 5 this increased to 94% (WOHLFART 1988): it is inferred that free penton base antibody either binds and is unable to neutralize until the complex is in a pH-5 environment, or is endocytosed together with virus and

does not bind or neutralize until the internal milieu of the endosomal vesicle reaches pH5 and causes new epitopes to be revealed.

On binding to cells, novel 'transitional epitopes' become exposed on the surface of Sindbis virus particles. These are temperature dependent and do not appear until virus bound at 4° C is raised to 37°C; their formation does not require low pH or internalization by the cell. They are short lived. 'Transitional epitopes' are recognized by mabs which bind poorly or not at all to free virions. Such mabs do not neutralize per se, but retard internalization and may allow other conventional neutralizing mabs, which recognise non-transitional epitopes, a longer period in which to act (FLYNN et al. 1990).

Post-attachment neutralization was demonstrated for West Nile virus (GOLLINS and PORTERFIELD 1985). The fact that virus was internalized by the cell and that inhibition of fusion and neutralization required similar concentration suggested that these parameters were causally linked. However, since a polyclonal serum was used it is not known if the relevant antibodies recognized native or cell receptor-bound virus.

HSV-1 was neutralized by mabs to gB or gD added after virus attached to cells at 4°C (HIGHLANDER et al. 1987, 1988). These and other data discussed above (FULLER and SPEAR 1985, 1987; FULLER et al. 1989) suggest that inhibition of fusion with the plasma membrane is responsible.

Certain epitopes mediate post-attachment neutralization better than others and mabs to the $E2^c$ site of VEEV proved to be the most effective (ROEHRIG et al. 1988).

Conclusion. Some antibodies can neutralize after virus has attached to a cell. An interesting suggestion is that new epitopes, which are relevant to neutralization, are exposed or formed de novo after a virus has bound to its receptor.

7 Role of the Cell in Neutralization

Viruses have evolved attachment sites which bind specifically to a particular component of the exterior surface of the cell, the cell receptor unit, and allow them to get close enough to the cell to gain entry. Viruses are known to attach to the lipid (phosphotidylserine, phosphotidylinositol or GM ganglioside: VSV) or carbohydrate (sialyoligosaccharides attached to unknown proteins or glycolipids: influenza type A, B and C viruses, paramyxoviruses, polyomavirus, encephalomyocarditis virus, reoviruses), but most common is attachment to a protein moiety (see LENTZ 1990). Information has been slow in coming but transfection of cloned genes for putative attachment proteins into cells to which viruses do not normally attach is providing unambiguous data. Attachment proteins are often those concerned with intercellular communication, particularly between cells of the immune system: intercellular adhesion molecule 1 (ICAM-1) is the attachment protein for rhinovirus, a member of the immunoglobulin superfamily for poliovirus, class I major histocompatibility complex (MHC) for Semliki forest virus, adenovirus and human cytomegalovirus (HCMV), class II MHC for lactic dehydrogenase-elevating virus (LDV), complement receptor (CR)2 the receptor for C3d, a degradation product of complement component 3 for Epstein–Barr virus, IgA receptor for hepatitis B virus, integrins for FMDV, CD4 for HIV-1, HIV-2, SIV, and membrane-bound IgM for murine leukaemia virus. Other cell receptor units are molecules concerned with homeostasis such as the β-adrenergic receptor for reovirus or the acetylcholine receptor for rabies virus (LENTZ 1990). Some caution is needed in this area as the identification of cell receptors for viruses has in some cases yet to be confirmed; in addition a virus may use more than one receptor, as described below.

Also emerging is evidence that some viruses have receptors which act in succession, virus attaching initially to a low-affinity receptor and then to a high-affinity receptor. It is thought that sialic acid (DERMODY et al. 1990) and the β-adrenergic receptor, respectively, subserve these functions for reovirus type 3. Attachment sites for both are located on the σ1 protein; the low-affinity interaction is synonymous with haemagglutination and is identified by mabs which inhibit the interaction with red cells but do not neutralize (BURSTIN et al. 1982). However as reovirus infects cells which do not express the β-adrenergic receptor, it must be able to use one or more additional receptors (EL-GHORR et al. 1992). The situation is parallelled by HSV-1. Envelope glycoproteins gB, gC, gD and gH are neutralization antigens; gB, gC and gD are involved in attachment although gC is not necessary for

infectivity in vitro; gB, gD and gH are essential for penetration of virus into the cell. Some mabs and some monospecific polyclonal antibodies to gB, gC and gD neutralize by inhibiting attachment; this is inefficient and requires large amounts of antibody (FULLER and SPEAR 1985; KÜHN et al. 1990; and see Sect. 2) and it seems likely that this is interfering with recognition of a low-affinity receptor, probably heparan sulphate (WUDUNN and SPEAR 1989). Other efficient neutralizing antibodies do not inhibit attachment and interfere with some later event such as fusion (see above and Tables 1, 3). The fibroblast growth factor receptor is a high-affinity receptor for HSV-1 (KANER et al. 1990).

The importance of the cell receptor to the mechanism of neutralization is whether or not antibody affects the virus–receptor interaction. Some attachment sites are not immunogenic, possibly due to them being hidden in a cavity (e.g. rhinovirus, ROSSMANN et al. 1987; influenza virus, WILEY et al. 1981). As already noted, combination of antibody with other sites on the surface of the virion does not necessarily affect interaction with the cell receptor unit (Table 3). Of course antibody will inhibit this interaction if it binds directly with or closely enough to the virus attachment site, and this is more likely with the large polymeric immunoglobulins. It may also depend on the size, charge and flexibility of the cell receptor unit; if this is shorter than the neutralizing antibody then it is likely that neutralized virus will not attach to the cell (DIMMOCK 1987). The linear dimension of each immunoglobulin domain is about 4 nm (SILVERTON et al. 1977), meaning that the shortest type of IgG molecule varies in length from somewhere between 10 and 16 nm, depending on the angle of flexion. Using the dimensions of cell surface molecules cited by SPRINGER (1990), it can thus be surmised that IgG (see Table 5) does not necessarily inhibit attachment of HIV-1 to its CD4 receptor (about 16 nm), and Table 3 shows that this theory is borne out by observation. The topography of the epitope is also relevant since the further

Table 5. The maximum and minimum distance (nm) that an immunoglobulin would project diametrically from the surface of a virion

	IgG	IgA dimer	IgM
Maximum	16	32	30
Minimum	10 (T-conformation)	10 (bridge conformation)	10 (staple/crab conformation)

Maximum and minimum refer to the dimensions of the immunoglobulin and ignore the fact that its epitope might be below the highest point on the surface and/or that the immunoglobulin could lie at an acute angle to the surface.
Approximate sizes in nanometers. IgG based on SILVERTON et al. (1977) for human IgG1: about 4 nm/domain; IgA and IgM based on FEINSTEIN et al. (1971, 1986).

from the distal end of the neutralization protein that an epitope is situated, the less far will a bound immunoglobulin molecule protrude.

Evidence that the cell can affect the extent of neutralization quantitatively was foreshadowed by TYRRELL and HORSFALL (1953), who discovered that the residual infectivity of neutralized influenza virus depended on the tissue used for titration (mouse brain or lung; chick allantois, chorion or extracted chorio-allantoic membrane), although the possibility that complement or phagocytosis affected the outcome was not ruled out. Less equivocal data came later when it was found that the degree of neutralization (of vesicular stomatitis virus, influenza virus, ECHO virus 4 or 72) depended by an order of magnitude on what type of cell was used for titrating residual infectivity (KJELLÉN and SCHLESINGER 1959; LAFFERTY 1963a; KJELLÉN and VON ZEIPEL 1984; KJELLÉN 1985). A more extreme example is La Crosse virus which was neutralized by one mab when assayed on BHK cells but not on mosquito cells and by another mab only when assayed on the mosquito cells (GRADY and KINCH 1985). These differences probably reflect use of different cell receptors on vertebrate and insect cells. The same conclusion is likely from data showing that a mab specific for the HA of a type A influenza virus (H5) neutralizes on Madin–Darby canine kidney (MDCK) but not in chicken embryos (PHILPOTT et al. 1989). An interesting difference in neutralization efficiency of NDV in CV1 and Madin–Darby bovine kidney (MDBK) cells emerged only when Fab fragments of polyclonal anti-F and anti-HN were used. Fabs specific for both F and HN neutralized less well in CV1, although IgG and Fabs neutralized to almost the same extent in MDBK cells (MERZ et al. 1981). A different type of assay also suggested that the cell influenced the efficacy of antibody when it was found that the spread of rabies virus was inhibited better in cultures of epithelial cells than neuroblastoma cells (LODMELL and EWALT 1987). Finally, CHESEBRO and WEHRLY (1988) show that while mabs and human sera neutralize HIV-1 when $CD4^+$ lymphocytes are used as the assay system, they are inactive on CD4-expressing HeLa cells, suggesting that the environment of the receptor is also important.

Such data make it important to determine whether or not neutralization studies in vitro reflect the situation in vivo. Outlaw and and co-workers took type A influenza virus neutralized by IgG, IgM and IgA and compared its interaction with BHK cells to ciliated epithelial cells on primary organ cultures of mouse trachea. Care was taken to use only mouse components: mouse antibody, mouse cells and a virus strain which had been adapted to multiply productively in ciliated tracheal epithelial cells to avoid artefacts arising from using a mixture of reagents from different species. No major difference between the mechanism of neutralization in cell monolayers and that in ciliated tracheal cells was found suggesting that at least in this instance standard laboratory cell culture work can illuminate in vivo neutralization (OUTLAW et al. 1990; OUTLAW and DIMMOCK 1990). Another cell used extensively to study virus–receptor interactions and the effects of antibodies is the rbc. Like other cells, rbc's interact with type A influenza virus via surface

N-acetyl neuraminic acid (NANA). In a three-way comparison with BHK and tracheal epithelial cells, the authors above show that neutralizing IgG, IgA and IgM all inhibit attachment of virus to erythocytes to a greater extent than to the other cells. Attachment of IgG-neutralized virus to a B-lymphoma cell line is also inhibited (EISENLOHR et al. 1987). In the rbc, most NANA is carried by glycophorin A, a molecule which is 5 nm is length (VIITALA and JÄRNEFELT 1985). It is not surprising therefore that IgG (about 10–16 nm long) interferes with the rbc–virus interaction.

Mabs distinguish between the interaction of NDV with rbcs and chick embryo cells: mabs to one of the two neutralization sites also inhibit haemagglutination while other mabs have only neutralizing activity (UMINO et al. 1990a). Such differences in the interaction of viruses with rbcs and dividing cells is not uncommon and indicate that the red blood cell is an unsuitable model for the study of neutralization (see also LU et al. 1982; YODEN et al. 1982; BURSTIN et al. 1982; KIDA et al. 1982; NISHIKAWA et al. 1983; RUSSELL et al. 1983; GITELMAN et al. 1986; HEINZ et al. 1983a; PHILPOTT et al. 1989; DERMODY et al. 1990).

Conclusion. The cell, or more accurately the cell receptor, is an integral part of the neutralization reaction. A cell system which reflects the situation in vivo should preferably be used in neutralization studies.

8 Antibody-Dependent Enhancement of Infectivity by Neutralizing Antibody: Fc and Complement Receptors

The binding of neutralizing antibody does not automatically mean that a neutralizable virus will lose infectivity. A virus can bind a certain number of molecules of antibody—a subneutralizing amount—which can either have no detectable effect on its infectivity, or permit it to infect cells by attaching to receptors for the antibody (or receptors for molecules of the complement system which have attached to the bound antibody) instead of the usual virus-specific receptor. The latter phenomenon, called antibody-dependent enhancement (ADE) is a well known property of certain antisera but it is only recently, through the use of mabs, that it has it been possible to demonstrate unequivocally that the same immunoglobulin molecule can, depending on the circumstances, be both neutralizing and enhancing (PORTERFIELD and CARDOSA 1984).

There are Fc receptors for IgG, IgA, IgE and IgM (LYNCH and SANDOR 1990). Fc receptors for IgG include high-affinity receptors (FcγRI) which can bind monomeric mouse IgG2a or human IgG1 and two types of low-affinity receptor (FcγRII and FcγRIII) which bind complexes of antigen and antibody (UNKELESS et al. 1988). The Fc receptor acts as a cell receptor unit for virus–antibody complexes as described above. Bound antibody (IgM, murine IgGs 1, 2a, 2b and human IgGs 1, 2, 3) can also result in the activation and binding of complement components. There are receptors of C1q itself, for the products of split C3 (CR1, CR2, CR3 and CR4) and for C4 and C5 (ROITT et al. 1989) which could bind adventitiously to virions. Cells bearing CR3 receptors enhanced the infectivity of West Nile virus only after it had bound IgM and activated complement (CORDOSA et al. 1983). Fc and complement receptors are presumably of greatest significance to viruses which are neutralized by inhibition of attachment to their normal cell receptor. They would permit infection of the normal target cell, if it also possessed Fc or complement receptors, or of cells which normally lack the relevant cell receptor unit. There is a wide range of such cell types including neutrophils, monocytes, macrophages, follicular dendritic cells, NK cells, B cells and epithelial cells of the glomerulus, cervix and nasopharynx (ROITT et al. 1989).

Analysis of the enhancement of infectivity by neutralizing antibodies is only possible where monoclonal antibodies are used; consequently, early work with polyclonal sera will not be discussed here. Subneutralizing amounts of neutralizing mabs are reported to enhance the infectivity in vitro of reovirus, Sindbis virus, West Nile virus, HIV-1, feline infectious peritonitis virus, TGEV, dengue virus and type A influenza virus (BURSTIN et al. 1983; CHANAS et al.

1982; PEIRIS et al. 1982; TAKEDA et al. 1988; OLSEN et al. 1992; DELMAS et al. 1986; KURANE et al. 1992; MORENS and HALSTEAD 1990; TAMURA et al. 1991) and in vivo of Japanese encephalitis virus (GOULD and BUCKLEY 1989). Some herpesviruses encode their own Fc receptors and inoculation of HCMV-infected fibroblasts with infectious HIV-1–IgG complexes resulted in HIV being able to infect cells which are normally resistant (MCKEATING et al.1990). There is concern that HIV or SIV infections may be exacerbated by antibody-mediated enhancement (TAKEDA et al. 1988, 1990; ROBINSON et al. 1989, 1990a, b, 1991; MONTEFIORE et al. 1990; HOMSY et al. 1989, 1990) but the issue is still undecided (MORENS and HALSTEAD 1990). One complication is that there is antibody-enhanced infection of human cell lines, but not of primary blood monocytes or peritoneal macrophages (SHADDUCK et al. 1991); another is that enhancement can be mediated by various permutations of the Fc receptor, the CR2 complement receptor for virion-bound C3d and the CD4 virus receptor (ROBINSON et al. 1989; 1990a; TAKEDA et al. 1990; GRAS and DORMONT 1991). Exactly what is necessary for enhancement to take place is uncertain since of 132 combinations of yellow fever virus strains (12) and mabs (11) only six combinations gave enhanced virulence in vivo, and neither of the two enhancing mabs was neutralizing (BARRETT and GOULD 1986). Although it seems unlikely that ADE would be relevant to viruses like influenza, since all cells already express its sialic acid receptors, there was a 25-fold increase in infectivity with both anti-HA and anti-NA sera (TAMURA et al. 1991), but the authors suggest that there may be distinct neutralizing and enhancing epitopes.

An entirely different form of enhancement, not mediated through Fc receptors, was proposed for HSV-2 for an IgG mab to gD (which neutralizes HSV-1 but not HSV-2). MINSON et al. (1986) suggest that it does so by binding to, and confering infectivity on, non-infectious particles.

Conclusion. Subneutralizing amounts of neutralizing antibody molecules can actually enhance infectivity. The in vivo significance is not understood. Quantitative implications will be discussed later.

9 Neutralization by Polymeric Immunoglobulin A

Polymeric IgA is synthesized by plasma cells of the mucosal immune system which comprises linked elements of the gut and respiratory systems and protects mucosal surfaces (BRANDTZAEG 1989; MCGHEE and MESTECKY 1990). It gets there by binding to a poly-Ig receptor on the basal surface of an epithelial cell and being internalized in an endocytic vesicle which is transported to the apical surface membrane with which it fuses. It is then released by proteolytic cleavage of the poly-Ig receptor, part of which becomes the 'secretory component' of the IgA molecule (UNDERDOWN 1990). IgA is a dimer in man but this and higher multiples are found in mice. Monomers and polymers are also found in serum, and these lack the secretory component. More IgA is synthesized than any other immunoglobulin isotype but as it is turned over more quickly there is less IgA than IgG. The structure of IgA has been described by FEINSTEIN et al. (1971). Dimensions are listed in Table 5.

Neutralization of type A influenza viruses has been studied with secretory polyclonal polymeric IgA purified from bile (TAYLOR and DIMMOCK 1985a) and monoclonal polymeric IgA lacking the secretory component (OUTLAW and DIMMOCK 1990; ARMSTRONG and DIMMOCK 1992). The mechanism of neutralization of type A influenza virus by polyclonal and monoclonal IgA appears identical, suggesting that the secretory component plays no part in neutralization. This is in contrast with the profound differences in the mechanism of neutralization between polymeric IgA and monomeric IgA derived from it by reduction with sulphydryl reagents (see below and TAYLOR and DIMMOCK 1985a).

Low levels of IgA can be detected on influenza virus by gold labelling without there being any neutralization (ARMSTRONG and DIMMOCK 1992). Thus, as with IgG, a virion binds more than one molecule of IgA before neutralization ensues. IgA neutralizes efficiently to $>99.9\%$. Neutralized virus attaches to normal cellular receptors (NANA) since this is prevented by pretreatment of cells with neuraminidase. Loss of infectivity is primarily due to inhibition of the virion's low-pH fusion activity: virus attaches and is internalized, but then the internal virion NP antigen remains cytoplasmic rather than being transported to the cell nucleus like the NP from non-neutralized virus. It seems that virus neutralized in this way is internalized but, lacking fusion activity, it remains in vesicles until it is degraded (ARMSTRONG and DIMMOCK 1992). The neutralization mechanism changes with increasing amounts of applied IgA as attachment is then reduced; furthermore, some of the virus which does attach is not internalized and can be removed by post-

attachment digestion with neuraminidase (OUTLAW and DIMMOCK 1990). Thus IgA can neutralize influenza virus by inhibition of fusion, inhibition of internalization and inhibition of attachment as the ratio of IgA:virus increases. Presumably, inhibition of fusion appears to be the primary cause of neutralization and inhibition of internalization and attachment are phenomena affecting already neutralized virus. However, inhibition of fusion is not the only inhibitory effect of IgA, as neutralized virus did not regain infectivity when artifically fused with the plasma membrane, despite the normal localization of M1 antigen in the cytoplasm and NP antigen in the nucleus. Artificial fusion gives full infectivity with non-neutralized virus (ARMSTRONG and DIMMOCK 1992). How IgA acts under these circumstances is discussed later (Sect. 24.3). Virus is also aggregated at low virus: IgA ratios, but this appears to have little or any affect on virus infectivity in vitro, presumably because these aggregates are unstable (OUTLAW and DIMMOCK 1990). IgA has the structure of a flexible rod and can combine with virions in a number of permutations. In contrast to polymeric IgA, monomeric immunoglobulin molecules derived from it by differential reduction have no effect on virus attachment, internalization or uncoating when used at high concentration (TAYLOR and DIMMOCK 1985a), evidence that polymerization of immunoglobulins affects the mechanism of neutralization. The larger polymeric structure may inhibit endocytosis of attached neutralized virus by preventing the formation of a critical number of cell receptor unit–virus attachment site interactions needed for internalization, but further work is needed to test this hypothesis.

Few other neutralizing IgAs have been described: WANG (1986) obtained a high frequency of IgA-secreting hybridomas to the HA and NA of influenza virus and anti-HA mabs were used to selected escape mutants: only half (15/31) could be assigned to groups defined by IgG mabs. PAGE et al. (1988) describe a neutralizing monoclonal IgA to type 1 poliovirus which maps with some IgG2a mabs to site 3B. It neutralized with the same characteristics as the majority of IgGs: relatively inefficiently, by presumptive aggregation and without recovery of infectivity at high antibody concentrations (see Sect. 23; MOSSER et al. 1989). Secretory IgA neutralized TGEV without inhibiting attachment (NGUYEN et al. 1986). HUSSAIN et al. (1988) report a neutralizing monoclonal IgA to a continuous epitope of gp90 of equine infectious anaemia virus. A neutralizing monoclonal IgA protected mice against Sendai virus (MAZANEC et al. 1987).

Conclusion. IgA is neutralizing; the mechanism of neutralization of its polymeric form may differ from that of the monomer, possibly because of the greater bulk and increased span of the former.

10 Neutralization by Immunoglobulin M

The pentameric nature of IgM ensures high avidity (about 10^7-fold greater than Fab) for structures bearing repeated identical antigenic determinants, but in general IgM is of low specificity since its variable region genes are not subject to hypermutation modification that occurs in cells synthesizing IgG or IgA and increases the affinity of these immunoglobulins. Even if this were not so, there are no memory cells in which mutations favouring increased affinity could be preserved. IgM has long been known to be neutralizing either alone or in the presence of complement (Sect. 15). Complexes of IgM bound to picornaviruses have been visualized by electron microscopy (SVEHAG and BLOTH 1967; BROWN and SMALE 1970; ALMEIDA and WATERSON 1969). When attached by a majority of its binding sites IgM adopts the 'staple' or 'crab' conformation but when only a minority of binding sites are engaged it has a more upright stance (FEINSTEIN et al. 1971, 1986; ARMSTRONG et al. 1990). The upright conformation of IgM does not activate complement.

Despite its large size (see below) IgM bound to virus is not necessarily neutralizing (HSV-1: DANIELS et al. 1970) and quantitative electron microscope studies with immune gold labelling show that up to eight molecules of IgM can bind per influenza virion without causing any concomitant loss of infectivity (ARMSTRONG et al. 1990). Both influenza and the nairovirus Dugbe (GREEN et al. 1992) are aggregated by IgM but only within a narrow window of IgM:virus. For both viruses this is a low amount of IgM, calculated for influenza virus at about five molecules per virion. Aggregates are easily dispersed by physical agitation and infectivity restored. Aggregation only occurred at a concentration of IgM which is intrinsically non-neutralizing. This may also have been the reason why a human IgM mab to influenza virus which had haemagglutination-inhibiting activity failed to neutralize (CASCINO et al. 1986).

With higher concentrations of IgM neutralization occurs. In the few systems so far studied this resulted partly from inhibition of attachment and partly from the failure of attached virus to be internalized (poliovirus, MANDEL 1967A; influenza virus, TAYLOR and DIMMOCK 1985b; OUTLAW and DIMMOCK 1990; murine cytomegalovirus (FARRELL and SHELLAM 1990); Dugbe virus, GREEN et al. 1992). IgM to the mucin-type carbohydrate of HIV-1 is presumed to inhibit attachment as it did not neutralize when added after attachment of virus to the host cell (HANSEN et al. 1991). It may seem surprising that the bulky IgM molecule, 30 nm in diameter in planar conformation, does not interfere more with attachment. However, the crab conformation rises only

10 nm above the surface of the virus, which is less than the maximum length of an IgG molecule (about 15 nm), and also there are NANA-bearing proteins extending up to 30 nm from the surface of cells (SPRINGER 1990) which could interdigitate with bound immunoglobulin and reach the virus attachment site. Inhibition of attachment of influenza virus by IgM was much more effective at 4°C than 37°C (TAYLOR and DIMMOCK 1985b), suggesting that increased thermal agitation favours the necessary interdigitation of viral and receptor molecules.

Inhibition of internalization of virus by IgM is thought to result from IgM blocking recruitment of sufficient cell receptor units to trigger endocytosis (DIMMOCK 1987). Surprisingly, the ability of Dugbe virus or influenza virus to fuse BSC-1 cells at acid pH was unaffected by IgM neutralization (GREEN et al. 1992; S.J. Armstrong, unpublished data), suggesting that fewer virus–receptor contacts are needed for this purpose than for endocytosis. The situation with IgM-neutralized vaccinia virus appears different as it attached to and entered cells but was not uncoated (RODRIGUEZ 1985), but this IgM neutralized 200 plaque-forming units (pfu) by only 30%.

IgM neutralization of influenza virus is initiated by the binding of more than eight molecules of IgM per virion (ARMSTRONG et al. 1990). The process appears relatively inefficient as even when half the surface area of the particle is covered by IgM crabs—a total of around 35 molecules—there is only 50% loss of infectivity. This inefficiency is underlined by the fact that maximum neutralization with this mouse polyclonal IgM was 87%. However, immune rabbit IgM preparations neutralized influenza (TAYLOR and DIMMOCK 1985b) and Dugbe viruses (GREEN et al. 1992) by > 99%.

It can be calculated from their relative areas that a maximum of about 70 IgM molecules in the crab conformation can theoretically be accommodated on an influenza virion. However, quantitative studies show that, at high IgM concentrations, influenza virus binds about twice that number of IgM molecules, suggesting that under these conditions IgM is packed in a planar conformation at a right angle or more acutely to the surface of the virus. This was confirmed by electron microscope measurements that show a fringe of antibody of average length 19 nm, far exceeding the dimensions expected of a crab (about 10 nm high) but less than the maximum planar diameter of 30 nm (ARMSTRONG et al. 1990; Table 5). The fringe was thought to consist of planar molecules which had 'fallen over', partly overlapping each other like plates in a plate rack. It is surprising, in view of the densely packed IgM, that neutralization reached a maximum of only 87%. The seems inconsistent with the notion of neutralization 'critical sites' where only one in 72 HA spikes mediate neutralization by IgG (TAYLOR et al. 1987), since saturating amounts of IgM—about 148 molecules per virion—would bind to a minimum of 148 HA spikes. However, the number of HA spikes bound is likely to be even greater than this; for example, if it is arbitrarily assumed that an IgM molecule in semivertical planar conformation fas four of its ten binding sites engaged with antigen, then, out of the grand total of 1000 HA spikes on a virion, there

will be up to 148 × 4 (592) HA spikes per virion engaged with IgM if each IgM binding site binds to a separate HA spike.

Neutralization of both influenza and Dugbe viruses is enhanced about eight fold by complement, with the result that infectious complexes formed between virus and subneutralizing amounts of IgM are inactivated (GREEN et al. 1992; ARMSTRONG et al. 1990). However, lower amounts of IgM, which were detectable on virus by gold labelling, did not lead to neutralization when complement was added, suggesting that a critical amount of IgM was necessary to achieve this (S.J. Armstrong, unpublished data). Non-neutralizing IgM also inactivated PIV in the presence of complement (VASANTHA et al. 1988). Influenza and Dugbe virus neutralized with the highest concentrations of IgM fixed complement poorly. However, such molecules are in the planar conformation (see above) and lack flexion at the junction of the C_H2–C_H3 domains which exposes the binding site for C1q. This was confirmed when the amount of free complement was found (GREEN et al. 1992) to be inversely proportional to the concentration of applied IgM.

The picornaviruses FMDV and poliovirus are both neutralized by IgM. Early electron microscope studies show that IgM binds to the trypsin-sensitive site on the vertex of FMDV, which is only one of three sites recognized by IgM (BROWN and SMALE 1970). Its multivalent nature is clearly seen in a similar study of anti-poliovirus IgM (SVEHAG and BLOTH 1967; SVEHAG et al. 1967). THOMAS et al. (1986) describe a neutralizing monoclonal IgM to type 1 poliovirus. At low antibody:virus ratios it aggregated virus, but these aggregates disappeared abruptly as the antibody concentration was increased. Digestion with papain disaggregated virus and restored infectivity but the authors do not say if the resulting Fabs remain attached. IgM also shifted poliovirus pI to an acid value.

REIGEL et al. (1984) report on the spectrum of immunoglobulin isotypes produced to ECHO virus 11: IgM was synthesized to VP1, VP2 and VP3 but IgG 'almost exclusively' to only VP1. The mechanism by which such a selective response operates and the significance of the isotype distribution in vivo are not known, although in general expression of immunoglobulin isotypes is under interleukin (IL) control (KLAUS 1990a, b).

IgM to the gp41 polypeptide of HIV-1 neutralized most effectively when added to virus before inoculation but was also effective after virus had attached to cells at 4°C (DALGLEISH et al. 1988). Whereas VSV pseudotyped by HIV-1 is neutralized by IgG1 to the gp120 component of the envelope protein, neither IgG1 nor the above IgM to gp41 was able to do so (KINNEY-THOMAS et al. 1988). No explanation was offered.

Conclusion. IgM is neutralizing. Its stance on the surface of a virion depends on the ratio of IgM:virus, at low ratios it adopts the 'staple' or 'crab' conformation but at high ratios molecules are packed together in a planar arrangement approximately perpendicular to the surface of the virion. Only the former fixes complement and this can lead to inactivation of infectivity by subneutralizing amounts of IgM. IgM 'crabs' do not necessarily block attachment to cell receptors, not so surprising when it is remembered that IgM crabs and IgG molecules are about the same height.

11 The Relevance of Immunoglobulin Isotype to Neutralization

The immunoglobulin isotypes G1, G2, G3 and G4 (in man) and G1, G2a, G2b and G3 (in mouse), A and M differ variously in size (Table 5) and other properties relating mainly to the sequence and structure of the constant region of their heavy chains (ROITT et al. 1989; BURTON 1990). The latter has implications for protection in vivo in regard to interaction with cells bearing the appropriate Fc receptor, activation of complement and aggregation (NISSONOFF and PRESSMAN 1958; MØLLER 1979; RODWELL et al. 1980) (see Sect. 8, 15 and 4, respectively). Properties of IgA and IgM are discussed in Sect. 9 and 10, respectively. However, as regards the relevance of immunoglobulin isotype to neutralization itself, no rigorously controlled comparative study has yet been carried out, since past studies have compared immunoglobulins which vary in both isotype and paratope. The means to remedy this situation and to create familes of isotypes all with identical paratopes are available now through the selection of heavy chain gene switch mutants which occur spontaneously in hybridomas secreting mabs (HALE et al. 1987) or through recombinant DNA technology. A complete set of immunoglobulin isotypes consisting of chimaeras of human immunoglobulin heavy chains and a mouse paratope from a mab to the NP hapten (4-hydroxy-3-nitrophenacetyl) was created by BRÜGGEMANN et al. (1987), and these have been used, for example, to make a true comparison of their relative complement-fixing abilities (VALIM and LACHMANN 1991). Similar families of neutralizing antibodies would be of great interest in both in vitro and in vivo studies, particularly since there is evidence of asymmetric production of different IgG isotypes during infection and immunization with viruses or virus proteins. For example, it appears that IgG2a is produced in response to acute infections in mice and IgG1 during persistent infections (lymphocytic choriomeningitis virus, LCMV, THOMSEN et al. 1985; TISHON et al. 1991), and it may be relevant to this that of adoptively transfered neutralizing mabs those of IgG2a isotype protected while those of IgG1 isotype mapping to the same site did not (BALDRIDGE and BUCHMEIER 1992). Furthermore, it appears that virus infection can influence which virus-specific isotypes are produced by affecting helper T cells and the production of cytokines that contribute to the control/switch of immunoglobulin isotype synthesis: interferon-γ affected the switch to IgG2a synthesis and IL-4 to IgG1 (adenovirus, LDV in mice: COUTELIER et al. 1990). In human sera the main neutralizing isotypes to HSV-1 was IgG1 (MATHIESEN et al. 1988a) but after HSV infection of mice it was IgG2a and IgG2b, although the overall antiviral response was IgG1 (MCKENDALL and

WOO 1988). A similar asymmetric production of IgG isotypes is seen which different presentations of the same immunogen: infection of mice with many different viruses gave predominantly an IgG2a response, while immunization with viral protein stimulated mainly IgG1 (COUTELIER et al. 1987). The immunoglobulin isotypes produced may also be affected by the dose and route of immunogen (FMDV, PIATTI et al. 1991; poliovirus VP1 epitope C3, LECLERC et al. 1990) and by the adjuvant employed (non-viral immunogen, KARAJOUNI and HADJIPETRON-KOUROUNAKIS 1990; or HIV-1, HAIGWOOD et al. 1991). Carbohydrate immunogens yield IgG3 (PERLMUTTER et al. 1978). An asymmetry in IgG isotype response to gp120 and gp41 also occurs in human HIV-1 infections (GOUDSMIT et al. 1988a; MATHIESEN et al. 1988b, 1989a, 1989b; CHIODI et al. 1989).

Conclusion. We need to know which is the most effective isotype of neutralizing antibody in vitro and in vivo, and how to stimulate it.

12 Viral Carbohydrates, Proteins and Neutralization

12.1 Carbohydrates and Neutralization

Most neutralization antigens are proteins but viruses can also be neutralized by antibody to host antigens, probably carbohydrate. This is present primarily in the form of glycoprotein but enveloped viruses also have cellular glycolipids. Enveloped viruses have an unknown mechanism for excluding the bulk of host protein present in the membranes from which they bud, but it cannot be excluded that small amounts of host protein, usually designated as contaminants, are an integral part of the virus particle. Examples of neutralizing antibodies specific for host antigens are found in influenza virus (HAUKENES 1977), infectious bronchitis virus (ALMEIDA and WATERSON 1969), the lentivirus equine infectious anaemia virus (MONTELARO et al. 1990) and HIV-1 (HANSEN et al. 1991). However, as with anti-virus protein antibodies, binding is not necessarily synonymous with neutralization (influenza virus: BRESCHKIN et al. 1981). Mabs in particular give precise evidence of specificity and 4 to 20 were found to have neutralizing activity to HIV-1 (HANSEN et al. 1990), particularly those to mucin-type carbohydrate (HANSEN et al. 1991). However, viral carbohydrate is usually not immunogenic as it is provided by the cell and antibodies would only be generated during infection if immunological tolerance were broken. It is conceivable, though, that antibodies to bacterial carbohydrate crossreact with viral carbohydrate. The same strictures apply to the lipid component of viruses. Early data on the inhibition of influenza virus with antibody to host carbohydrate have to be interpreted with care since the antibody acted not on the virus particle but on the *cell* (LAVER and WEBSTER 1966), possibly by inhibiting virus attachment. More recent chemical data demonstrate that six of the oligosaccharide side chains covalently bound to the HA are antigenically related to the host (WARD et al. 1981).

Carbohydrate moieties may also alter the antigenicity of glycoproteins as revealed by deglycosylation (Rauscher leukaemia and influenza A viruses, ALEXANDER and ELDER 1984; tick-borne encephalitis virus, TBEV, GUIRAKHOO et al. 1989; bovine herpesvirus, VAN DRUNEN LITTEL-VAN DEN HURK et al. 1990) but the antigenicity of murine leukaemia virus was not affected when its carbohydrate was removed (PIEROTTI et al. 1981). However, deglycosylation of Rauscher leukaemia virus (ELDER et al. 1986), HIV-1 gp120 (PUTNEY et al. 1986) or flaviviruses (WINKLER et al. 1987) had no discernible effect on their antigenicity or immunogenicity. Some HIV-1-neutralizing mabs recognize non-glycosylated gp120 expressed in *Escherichia coli* (KINNEY-

THOMAS et al. 1988). Carbohydrate may form part of an epitope or be necessary for a protein achieving its correct conformation (murine leukaemia virus, PIEROTTI et al. 1981; bovine leukaemia virus, Bruck et al. 1984; NDV, LONG et al. 1986; influenza C virus, SUGAWARA et al. 1988; LCMV, WRIGHT et al. 1989; Sendai virus, VIDAL et al. 1989; HIV-1, MOORE et al. 1990; HO et al. 1991a). Virions containing carbohydrate-free influenza virus HA were efficiently precipitated by five different neutralizing mabs (BASAK and COMPANS 1983). VAN DRUNEN LITTEL-VAN DEN HURK et al. (1990) think that carbohydrate is an integral part of three epitopes of gI, the main neutralization protein of bovine herpes virus. An antigenic site may be hidden or revealed by the acquisition or loss of a carbohydrate moiety (influenza virus, SKEHEL et al. 1984; SMITH et al. 1991; rotavirus, MACKOW et al. 1988; rabies virus WUNNER et al. 1985; mumps virus, GOTOH et al. 1988; KÖVAMEES et al. 1990; human PIV-3 COELINGH and TIERNEY 1989b; HIV-1, DAVIS et al. 1990; HSV-1: MUGGERIDGE et al. 1990). Such changes may be an evolutionary ploy which ensures survival in the host species or in individuals (Sect. 22.2.1). RASMUSSEN et al. (1988) believe that carbohydrate has another function and acts to protect the neutralization protein, p86, of HCMV from protease degradation.

Currently there is considerable interest in the development of vaccines to HIV, and much is being made of the SIV–primate model (DESROSIERS et al. 1989; MURPHEY-CORB et al. 1989, 1991; STOTT et al. 1990). However, there is suspicion that protection depends, at least in part, upon immunity generated to host components present in the challenge virus (STOTT 1991; LE GRAND et al. 1992, and see GROOPMAN 1991) since (a) protection does not correlate with antibody to SIV (DESROSIERS et al. 1989; MURPHEY-CORB et al. 1991; CARLSON et al. 1990), (b) there are high levels of antibody against cellular components, (c) animals can be protected by immunization with non-infected cells of the same species as those in which the challenge virus was grown, and (d) protected animals have about 10-fold more anti-cell antibody than those which were not protected (STOTT et al. 1990). Further, monkeys were also protected by immunization with a heterologous virus grown in the same cells as the challenge SIV (CRANAGE et al. 1992).

At the time of writing only preliminary data are available and it is not certain if antibody or cellular immunity is primarily responsible for protection, or if protein or carbohydrate epitopes are involved. Crossreaction between anti-cell antibodies and SIV particles is another possibility.

Conclusion. Carbohydrate moieties may have a variety of functions—or no function—in neutralization.

12.2 Proteins and Neutralization

Most viruses of vertebrate animals have two or more proteins in their outer structure. Exceptions are the Rhabdoviridae, Retroviridae and Arenaviridae, members of which have a single glycosylated envelope protein which mediates

neutralization. At least one protein mediates attachment, but other proteins (e.g. the NA of influenza virus) may be needed for release of progeny virions from the cell. Evolution of multiple outer proteins may also provide a necessary flexibility of response to the pressures exerted by the immune response. Unlike phages, where attachment is mediated through a minority of the total surface protein and in some instances by one or a few molecules, animal viruses have a substantial proportion of their surface proteins able to perform this function.

Of the two or more proteins which most animal viruses present on their outer surface, one or more mediates neutralization. This in part depends on the division of function between viral proteins; for example, the business of infection may be the property of one protein (HA of influenza virus: WILEY and SKEHEL 1987) or of at least four proteins (gB, gC, gD, gH of HSV-1: Sects. 2, 3). A single protein may have several distinct neutralization sites (for example, up to eight in the envelope protein, gp51, of bovine leukaemia virus BRUCK et al. 1982a) which may be linear in sequence, with or without a requirement for a specific conformation, or discontinuous (Sect. 13). There are many examples of viruses with two or more neutralization proteins and those given below are intended only to illustrate the principles involved.

Influenza virus has two surface proteins, the haemagglutinin (HA) and the neuraminidase (NA), which act independently in neutralization. The HA of subtype H3 has five potential neutralization sites (A–E, Fig. 1), all of which are conformational (WILEY et al. 1981; DANIELS et al. 1983). It is remarkable that of the many HA-specific mabs isolated, so few are non-neutralizing (BRESCHKIN et al. 1981; PHILPOTT et al. 1989); this is not an artefact of selection in the sense that neutralization is rarely used as criterion. BRESCHKIN et al. (1981) has competition mapped a non-neutralizing mab to one of the neutralization sites, but this site was not assigned to the physical structure; unfortunately the mab no longer exists. GERHARD et al. (1980) report mabs which bind the HA but do not inhibit haemagglutination except at high concentration (>10–130 μg/ml); no neutralization data were recorded. The HA is a homotrimer (WILEY et al. 1977) and some epitopes of sites B and D depend on its quaternary structure (BROWN et al. 1990). Neutralization of virions by antibody to the other major glycoprotein, the NA, is weak (SETO and ROTT 1966; WEBSTER and LAVER 1967; WEBSTER et al. 1968) and in appropriate proportions is mediated by aggregation (COMPANS et al. 1969; KENDAL and MADELEY 1970). Anti-NA also prevents release of virus from the vicinity of the cell (Sect. 16). In paramyxoviruses, attachment and entry (by fusion at the plasma membrane) are largely, but not entirely, separate functions of the HN and F proteins and both can mediate neutralization (see Sect. 5.3 and NORRBY 1990).

Herpesviruses have several neutralization proteins. Four of the surface glycoproteins (gB, gC, gD and gH), which are believed to be separate entities, can mediate neutralization. Anti-gB, anti-gC and anti-gD each block attachment but do so very inefficiently (see Sect. 2). Anti-gB, anti-gD and anti-gH can each block entry. It is not known precisely what function(s)

are inhibited but the fact that attachment is blocked by antibodies to three different proteins may imply that gB, gC and gD each contributes to the same process, or that each contributes to a different part of the same attachment process or that there is cooperativity in neutralization so that antibody to one protein abolishes (by steric or conformational influence) the activities of the others. The same argument applies to the dual functions of attachment and penetration performed by gB and gD; some evidence that gB has separate functions is provided by PEREIRA et al. (1989), who found that neutralizing anti-gB mabs react with the N-terminal domain or a mid-region domain. They suggest that the former is involved in low-affinity attachment to heparan sulphate (WUDUNN and SPEAR 1989) and the latter with penetration (HIGHLANDER et al. 1988).

The close packing of protein subunits of non-enveloped viruses offer opportunities for interactive events between consituent polypeptides which are less obvious in enveloped viruses, Poliovirus, for example, has three external polypeptides, VP1, VP2 and VP3. Most epitopes have a strong conformational element and the five antigenic sites involve VP1, VP2 and VP3 either alone or in permutation (see Table 11). There are a few mabs or sera which recognize isolated VP1, VP2 or VP3 and neutralize (see MINOR 1990), suggesting that some linear epitopes are also biologically active.

Adenovirus can be neutralized by IgG directed against any of the three constituents of the surface of the virion, the hexon, penton base and fibre proteins. In each case the mechanism of neutralization is different: none of the antibodies inhibited attachment, and anti-penton fibre actually increased attachment of neutralized virus to the cell; only anti-penton fibre inhibited internalization. After internalization, the genome of virus neutralized by anti-hexon and anti-penton base became DNase sensitive, as is normal with infectious virus, but most virus neutralized by anti-hexon remains in endocytic vesicles while only about 50% of that reacting with anti-penton base is so located. Anti-penton base is poorly neutralizing (to 45%), suggesting that it may block only one of several routes of entry into the cell (WOHLFART et al. 1985).

Rotaviruses have an outer shell composed of VP7 and the spike protein VP4 (until recently known as VP3). The latter has been visualized in combination with Fab by cryoelectronmicroscopy (PRASAD et al. 1990). IgG to both VP4 and VP7 is neutralizing but the situation is complex as some anti-VP7 mabs inhibit attachment and some do not, and some anti-VP4 mabs inhibit attachment and some do not (KUKUHARA et al. 1988; SABARA et al. 1985; RUGGERI and GREENBERG 1991).

Not all surface proteins function in neutralization—at least in vitro. Influenza virus was not neutralized by a mab prepared to a peptide of an external epitope of the M2 protein (ZEBEDEE and LAMB 1988), and antiserum to the HA2 transmembrane polypeptide of the major spike protein of influenza virus does not bind to infectious virus (GRAVES et al. 1983) or to virions budding from the surface of infected cells even though it inhibits haemag-

glutination by isolated HA (BECHT et al. 1984). Mabs to the transmembrane polypeptide GP-2 of LCMV do not neutralize (PAREKH and BUCHMEIER 1986; BURNS and BUCHMEIER 1991), but mabs specific to the analogous S2 protein of coronaviruses neutralize (LUYTJES et al. 1989; KOCH et al. 1990; WEISMILLER et al. 1990; ROUTLEDGE et al. 1991) or do not neutralize (LAUDE et al. 1986; CAVANAGH et al. 1986). Other membrane-anchoring proteins also function as neutralization antigens (gp41 of HIV-1, see Figs. 2,10; F1 of NDV, TOYODA et al. 1988). Their ability to mediate neutralization may depend on their immunogenicity and/or their accessibility to antibody once it is formed; alternatively they may have no role in neutralization at all. In this regard HIV-1 gp41 may be unusually accessible as spikes are relatively sparse (70–80 per virion compared with 500–1000 per virion for the similar sized influenza particle) and the distal element of the spike protein, gp120, is easily sloughed off (GELDERBLOM et al. 1987).

Conclusion. Most viruses have more than one outer protein which mediates neutralization, and this contributes to the complexity of neutralization. Often each has multiple neutralization sites and probably different mechanisms of neutralization. The efficiency of neutralization may vary between proteins.

13 Properties of Protein and Peptide Antigens Which Elicit Neutralizing Antibody

The contact area between the binding site of an antibody and the antigen (or footprint) is about 2.5 × 3 nm (AMIT et al. 1986; COLMAN et al. 1987) and, in the example of lysozyme, 16 amino acid residues of the epitope are involved in interaction with 17 of the paratope (AMIT et al. 1986). The conventional view of the antibody binding site being a cleft capable of accomodating about six amino acid residues does not explain the simultaneous binding of two different mabs to a peptide representative of influenza virus HA as their epitopes are separated by only three residues. In explanation, JACKSON et al. (1988) suggest that the binding site is a flat surface and that each mab binds to the opposite side of the peptide. Study of neutralization-escape mutants suggests that a change in one of the residues can abrogate the interaction, but this is a property of probably no more than four key amino acids (BROWN et al. 1990). For example, escape mutants of poliovirus or influenza virus usually have only one changed residue and this is the same residue, or one with similar properties, whenever the selection process is repeated (MINOR 1990; WEBSTER and LAVER 1980; LAVER et al. 1981; NEWTON et al. 1983). It is rare that an escape mutant requires substitutions of more than one amino acid at any one antigenic site, but double mutations have been found in escape mutants located at the NimIII site of rhinovirus 14 (SHERRY et al. 1986), in an escape mutant of human PIV-3 (COELINGH and TIERNEY 1989b) and in a strain of A influenza virus (YATES et al. 1990). It is not clear how the subsitution of a single residue prevents neutralization so emphatically, but presumably it causes a physical or conformational change within the epitope such that binding is abrogated. The situation in field strains of influenza virus is more complex and BROWN et al. (1990) discuss how amino acid changes outside the epitope apparently affect its conformational integrity. A mutation outside the neutralizing epitope is responsible for an escape mutant of HIV-1 (WILSON et al. 1990). BROWN et al. (1990) describe how an escape mutant may react with no change to other antibodies, while in other mutants these may not bind, may show a reduction in binding, or may even show an increase in binding. There may also be functional changes: escape mutants produced with mabs to sites AB, B and C of PIV-3 still bind mabs to other sites, but those to site A are no longer able to effect neutralization (COELINGH and TIERNEY 1989b).

There are two types of epitope in proteins: linear and non-linear. Linear epitopes may or may not have a conformational dimension, the latter usually involving a covalent disulphide linkage. Non-linear epitopes consist of amino

acid residues, non-contiguous in sequence, which are juxtaposed to form a site which is destroyed if the conformation is upset. These can be constituted by tertiary structure or the quaternary disposition of two identical or dissimilar polypeptides. In practice, the ability of a mab to bind to a sodium dodeylsulphate (SDS)-denatured, non-reduced polypeptide in a western blot is commonly used to indicate that the epitope is linear. Neutralization epitopes also are held to be largely non-linear but can be of either type. There is not the information to generalize about the relative proportion of non-linear to linear epitopes and this may vary between different viruses: for instance, all neutralization epitopes of the very well studied influenza type A virus HA are non-linear (reviewed by BROWN et al. 1990), most but not all poliovirus epitopes are non-linear (see Table 11 and MINOR 1990) and some epitopes of FMDV are linear (ACHARYA et al. 1989; KITSON et al. 1990; MATEU et al. 1990). Linear neutralization epitopes have also been identified on, for example, Japanese encephalitis virus (SRIVASTAVA et al. 1987), TBEV (HEINZ et al. 1983a; HEINZ 1986; GUIRAKHOO et al. 1989), HSV-1 gD and gB (MINSON et al. 1986; PEREIRA et al. 1989; MESTER et al. 1990), HCMV (UTZ et al. 1989; URBAN et al. 1992), rabies virus (BUNSCHOTEN et al. 1990; DIETZSCHOLD et al. 1990b), VSV (VANDEPOL et al. 1986), NDV (SAMSON et al. 1988), MHV-4 (TALBOT et al. 1984; LUYTJES et al. 1989; ROUTLEDGE et al. 1991), varicella-zoster virus (FORGHANI et al. 1990), rubella virus (WOLINSKY et al. 1991); rhinovirus 2 (SKERN et al. 1987; HASTINGS et al. 1990) and equine infectious anaemia virus (HUSSAIN et al. 1988). HIV-1 has both linear-conformational and discontinuous epitopes—which may have a linear component recognized by antibodies (see Sect. 25). Both are immunogenic in human infections. Currently more is known about linear epitopes since peptide immunogens and antigens have been used widely (Table 6).

It may be that some linear epitopes represent only a portion of the complete (non-linear) epitope (e.g. peptide 156–170 is part of the NimII antigenic site of rhinovirus type 2; SKERN et al. 1987) but the fact that 8–10% of the neutralizing activity of polyclonal animal (and possibly human) sera recognize this peptide indicates that the linear epitopes may be biologically significant (HASTINGS et al. 1990).

As the sequence of more viral proteins becomes known, there has been considerable interest in using defined synthetic peptides to investigate neutralization epitopes, often by blocking neutralization by antibodies raised against native virus or viral proteins. This is a less certain diagnosis of linear epitopes than other methods since a population of the peptide may at any one time be fortuitously in the correct conformation. However, putative neutralization peptides of influenza virus give no appreciable level of neutralizing antibody, confirming that the antigenic sites identified by WILSON et al. (1981) are indeed non-linear. Peptides to other regions also gave no neutralizing antibody (reviewed by WILSON and COX 1990), with one exception (MÜLLER et al. 1982). The problem here is not one of immunogenicity since peptides can be covalently linked to carrier proteins such as keyhole limpet haemocyanin.

Table 6. Examples of peptides which elicit neutralizing antibody

Family	Virus	Peptide and comments	References
Corona	TGEV		Posthumus et al. 1990
Herpes	HSV-1	gD, gB	Cohen et al. 1983; Dietzschold et al. 1984; Weijer et al. 1988; Mester et al. 1990
Picorna	FMDV	Mainly 141–160 of VP1; 201–213 less successful	Bittle et al. 1982; Pfaff et al. 1982; Strohmeier et al. 1982; DiMarchi et al. 1986; Francis et al. 1987a; Parry et al. 1988, 1989; Bolwell et al. 1992; Doel et al. 1992
	Bovine enterovirus	Peptides of VP2 and 3 gave better response than of VP1	Smyth et al. 1990
	Poliovirus 1	Priming response only	Emini et al. 1983d
	Poliovirus 3	Site 1, but peptides usually give poor responses	Ferguson et al. 1985
	Rhinovirus 2	VP2, 153–164, part of site NimII	Francis et al. 1987b; McCray and Werner 1987; Skern et al. 1987; Hastings et al. 1990
	Mengo virus	VP1, 259–277	Muir et al. 1991
Reo	Rotavirus	VP4, not VP7	Hansen et al. 1992
Retro	HIV-1	gp120, 254–274	Ho et al. 1988
		301–341 (V3 loop)	Ho et al. 1987; Goudsmit et al. 1988a; Palker et al. 1988; Javaherian et al. 1989; Langedijk et al. 1991; Laman et al. 1992
		426–437	Ho et al. 1987; Sun et al. 1989
		384–472	Michel et al. 1988
		458–484	Ho et al. 1987
		503–532	Chanh et al. 1986
		gp120, C-terminal half	Putney et al. 1986; Krohn et al. 1987
		gp41, 616–632	Ho et al. 1987; Schrier et al. 1988
		728–751	Chanh et al. 1986; Ho et al. 1987; Dalgleish et al. 1988; Evans et al. 1989
	Feline leukaemia virus		Elder et al. 1987
Rhabdo	Rabies virus		Dietzschold et al. 1990b

However, free peptide is only immunogenic for B cells if it has a second epitope which is recognized by helper T cells. Success with peptide 141–160 from VP1 of FMDV came fortuitously in this way with a good neutralizing response which was also protective in vivo (BITTLE et al. 1982; PFAFF et al. 1982; STROHMEIER et al. 1982). Part of the reason for the sucess of this peptide in raising neutralizing antibodies (PARRY et al. 1988, 1989) probably arises from its structure in the virion where residues 133–158 of VP1 form a disordered loop and linear epitope (ACHARYA et al. 1989). Significantly, a high proportion of anti-peptide antibodies recognize native FMDV particles (PARRY et al. 1988). These are also reactive against heterologous strains of virus (BOLWELL et al. 1992). Other neutralizing antibodies raised to peptides in various formats are listed in Table 6.

Although titres are not high, peptides from various regions of the envelope protein of HIV-1 have been remarkably successful (especially compared with influenza virus) in raising neutralizing antibody (Table 6). In fact, a single peptide formed of sequences from two different strains of HIV-1 elicited neutralizing antibodies against both (JAVAHERIAN et al. 1989). In general, neutralization titres obtained by immunizing with peptide antigens are low, but exceptionally can range over several orders of magnitude. It would be instructive to determine the affinity of antibodies made against peptides for virions; this is probably low compared with the real thing since the peptide will induce antibodies against conformations which are irrelevant to the native epitope. One pessimistic prediction is that since an anti-peptide serum is specific for a single antigenic site it would efficiently select escape mutants. Experimentally this did not occur with antibodies to the VP1 loop of FMDV (PARRY et al. 1989), all the more surprisingly since a single amino acid change of His to Arg at position 146 abolished reactivity (MATEU et al. 1990).

What is required is a technical advance which will enable a peptide to take up a required conformation. In practice this has been helped by cyclization through disulphide linking terminal cysteine residues; this reduces the structural freedom of non-cyclized peptides to fewer and hopefully more useful structures. A single terminal cysteine improved immunogenicity of a rhinovirus peptide, possibly by permitting formation of stable dimers (FRANCIS et al. 1987b). POSTUMUS et al. (1991) had only limited success in stimulating neutralizing antibody to TGEV with a single peptide containing the relevant regions of a non-linear neutralization epitope. Alternatively, peptides can be inserted in place of a natural sequence in another protein, in this case in one of the hypervariable loops of IgG (ZANETTI 1992). In another approach a peptide from the V3 region of HIV-1 gp120 was expressed in Ty virus-like particles and stimulated syncytium-inhibiting antibodies (GRIFFITHS et al. 1991); similarly, a peptide expressed on hepatitis B virus S antigen particles gave neutralizing antibody in one rhesus monkey (SCHLIENGER et al. 1992). The problems of peptide vaccines are discussed by SHINNINCK et al. (1983), ARNON et al. (1983) and FRANCIS and CLARKE (1989).

Of relevance to a discussion of the importance of the conformation of an epitope is its molecular environment. Experimentally this is found in chimaeras of poliovirus where the sequence encoding an epitope in a full length infectious DNA copy of the viral genome is replaced by the equivalent from another serotype. The new epitope is expressed in the virus particle and binds the relevant mab, but in some cases does not now effect neutralization (MINOR et al. 1990). The converse also hold and a non-neutralizing mab was able to neutralize the chimaera (MINOR et al. 1991). There are many difficulties still with achieving the original conformation of the epitope and site 1 transfers more readily than do sites 2,3 or 4 (MINOR et al. 1991). In rotavirus recombinants the expression of epitopes in VP4 depends on the particular VP7 with which that virion is constructed. For example, mab 2G4 recognizes VP4 of strain SA11 in association with PV7 of SA11 but not in association with the VP7 of strain B223 (CHEN et al. 1992). An analogous situation is found where escape mutants bind the non-selecting mabs but are no longer neutralized by them (Table 4A). There is a parallel with naturally occurring strains of a number of different viruses which can bind and are unaffected by a mab which is known to neutralize another strain (Table 4B). It is likely that the conformation of the epitope is affected by adjacent amino acid residues. Evidently binding is a crude parameter and neutralization itself a much more subtle process.

Conclusion. Interaction of a neutralization epitope with its cognate antibody depends on its sequence and/or its conformation and this is can be abolished by a single amino acid change within or outside the epitope.

14 Neutralization In Vivo

It is widely believed that antibody is important in prevention of reinfection and possibly in recovery from infection as well. However, it is far from easy to determine how such antibody acts. However, immunoglobulin-mediated immunity is easily demonstrated by adoptive transfer to naive animals. In this way it was shown that some monoclonal IgGs which neutralize in vitro confer protection in vivo and others do not; the latter include antibodies to mouse hepatitis virus TALBOT et al. 1984; BUCHMEIER et al. 1984, HSV-1 (RECTOR et al. 1982, KÜMEL et al. 1985), HSV-2 (BALACHANDRAN et al. 1982) and bovine coronavirus (DEREGT et al. 1989). Table 7 appears to show a fairly close correlation between these two parameters but this may be the result of a bias against reporting negative data in the literature; certainly it could never be assumed that a neutralizing antibody would protect in vivo. What properties make for protection are not known. There may be a huge range in efficacy: MATHEWS and ROEHRIG (1984) found that one neutralizing mab to Saint Louis encephalitis virus (SLEV) was 1000-fold more protective than other neutralizing mabs to the same protein. They concluded in an earlier study that avidity and topography of binding were important factors in protection by neutralizing mabs to Venezuelan encephalomyelitis virus (VEEV) (MATHEWS and ROEHRIG 1982). Administration of particular neutralizing mabs may prevent death but result in chronic disease (MHV-4, BUCHMEIER et al. 1984; Aleutian disease parvovirus, ALEXANDERSEN et al. 1989). The elimination of the immunoglobulin response in chickens by bursectomy led to the suggestion that endogenously produced antibody potentiates disease during infection by the infectious laryngotracheitis herpesvirus, possibly by increasing the viscosity of the tracheal exudate, leading to death by axphyxiation (FAHEY and YORK 1990). Bursectomy also exacerbated avian influenza (PORTNOY et al. 1973). The beneficial effects of adoptive transfer of immune serum to influenza virus-infected mice was first demonstrated by LOOSLI et al. (1953) and anti-HA is the most effective anti-viral antibody (VIRELIZIER 1975; VIRELIZIER et al. 1976; MCLAIN and DIMMOCK 1989). Inoculation of athymic mice with a type A influenza virus causes a persistent infection. Adoptive transfer of mab to the HA reduced shedding of virus and allowed resquamation of the trachel epithelium to occur, but the virus was not cleared and, as the antibody titre waned, re-desquamation took place (KRIS et al. 1988).

The complexity of neutralization in vivo (protection) was demonstrated by KÜMEL et al. (1985), who adoptively transferred complement-dependent

Table 7. Correlation between neutralization in vitro and protection in vivo

Family	Virus/protein	Ig[a]	Neutralization in vitro	Protection in vivo	References
Arena	LCMV/GP1	m	+	+	Wright and Buchmeier 1991;
		m	–	+	Baldridge and Buchmeier 1992
Corona	MHV-4/E2	m	+	–	Buchmeier et al. 1984
		m	+	+[b]	
		m	+	+	Talbot et al. 1984
		m	+	–	
	Bovine coronavirus/E2, E3	m	+	+/–[c]	Deregt et al. 1989
	LDV	m	–	+	Harty and Plagemann 1990
Flavi	Yellow fever virus/54K	m	+	+	Gould 1986
	/48K	m	–	+	
	SLEV/E1c	m	+	+	Mathews and Roehrig 1984
		m	+/–	(+)	
Hepadna	Hepatitis B virus/S	m	0	+	Iwarson et al. 1985
Herpes	HSV-1	m	+	+	Dix et al. 1981
		m	–	+	Balachandran et al. 1982
		m	+/–	+/–	Kumel et al. 1985 Rector et al. 1982; Lausch et al. 1990; Staats et al. 1991
Myxo	Influenza A virus/NA	s	+	+	Schulman et al. 1968
	/HA	s	+	+	Virelizier 1975; Virelizier et al. 1976
	/HA	m	+	+	McLain and Dimmock 1989
	Influenza C virus/HE	m	+	+	Takiguchi et al. 1992
Paramyxo	Murine CMV	m	+/–	+	Farrell and Shellam 1991
	Measles virus/H	m	+	+	Giraudon and Wild 1985
	/F	m	+	+	Malvoisin and Wild 1990
	Simian virus 5/F	m	+	+	Paterson et al. 1987
	RSV/G,F	m	–[f]/+	+	Walsh et al. 1984
	RSV	s	+	+	Prince et al. 1987
		s	+	+	Prince et al. 1990

	RSV/G,F	m	+	+	TAYLOR et al. 1984
		m	–	+	
	PIV-3/F,HN	m	+	+[e]	RYDBECK et al. 1988
	NDV/HN	m	+	(+)	UMINO et al. 1990b
	/F	m	+	+	
	Mumps virus/F	m	–	+	LOVE et al. 1986
	Sendai virus/HN	m/IgA	+	+	MAZANEC et al. 1987
		m	+	+	PIGA et al. 1990
Parvo	Aleutian disease virus	m	?	+[b]	ALEXANDERSEN et al. 1989
Picorna	FMDV	m	+	+	MCCULLOUGH et al. 1992
		s	+	+	BOLWELL et al. 1992
	TMEV	m	+	+	FUJINAMI et al. 1989
Reo	Rotavirus/VP4	m	+/–	+/–	OFFIT et al. 1986
	/VP7	m	+	+	MATSUI et al. 1988, 1989
	Bluetongue virus	m	+	+	LETCHWORTH and APPLETON 1983
	Reovirus/σ1	m	+	+	VIRGIN et al. 1988
		m	–	+	
Retro	HIV-1/gp120	m	+	+	EMINI et al. 1990, 1992
Rhabdo	Rabies virus/G	m	+	+	DIETZSCHOLD et al. 1990a
	VSV/G	m	–	+[d]	LEFRANCOIS 1984
		m	+	+	
	Egtved virus	m	+	+	LORENZEN et al. 1990
		m	–	+	
Toga	Sindbis virus	m	+	+	SCHMALJOHN et al. 1983
		m	–	+	
		m	+	+	LEVINE et al. 1991
		m	+	–	
	Semliki forest virus	m	+	+	BOERE et al. 1983, 1984, 1985
		m	–	+	
	Semliki forest virus (peptide)	s	–	+	GROSFELD et al. 1989
		s	–	+	SNIJDERS et al. 1991
	VEEV	m	+	(+)	MATHEWS and ROEHRIG 1982

[a] Immunoglobulin as antiserum (s) or monoclonal antibody (m).
[b] Against death, but chronic disease ensued; a mixture of four mabs was used; no data on neutralization.
[c] Possibly because different strains were used in vitro and in vivo.
[d] Fc region required; only $F(ab)_2$ were used.
[e] None protected individually but a mixture did protect.
[f] Neutralizes only with complement.
0, No in vitro assay available; (+), some mabs give better protection than others; +/–, some mabs neutralize and do not protect, others do not neutralize and do protect.

neutralizing mabs to HSV-1 gB, gC and gD envelope proteins into DBA-2 mice, which are deficient in C5. Some antibodies protected well but there was no correlation with any known parameter; indeed, mabs to different epitopes on the same protein which neutralized (with complement) to the same extent in vitro protected to different extents. Since the mice are complement-deficient, KÜMEL et al. (1985) suggest that protection is mediated by a mechanism other than neutralization of virions, but this ignores the interaction of virus–mab complexes with C1, C2, C4 and C3. Non-neutralizing mabs similarly protected C5-deficient AJ mice from a lethal dose of HSV-2 (BALACHANDRAN et al. 1982).

The mechanism of protection by antibody may also be complex. LEVINE et al. (1991) found that the adoptive transfer of certain neutralizing mabs to just two epitopes of the E2 protein of Sindbis virus clears virus from the central nervous system of persistently infected mice. A pulse of mab given at 2 days post-infection and removed after 4 days led to the clearance of infection even though large amounts of infectious virus were still being produced at the time antibody was removed. Intracellular markers of infection (viral plus strand RNA synthesis and cytopathic vesicles) were eliminated or reduced. The conclusion that antibody acted by affecting transcription or translation rather than by neutralizing virus per se recalls the modulation of the intracellular expression of the measles virus genome by antibody (Sect. 17).

Possible reasons for the inability of certain mabs to protect are many. Amongst them could be:

1. Cell-specific neutralization, as described by KJELLÉN and SCHLESINGER (1959), KJELLÉN and VON ZEIPEL (1984), KJELLÉN (1985), GRADY and KINCH (1985) and PHILPOTT et al. (1989); i.e. antibody neutralizes when infectivity is assayed on cultured cells in vitro but less effectively or not at all with the target cell in vivo (see Sect. 7).
2. Affinity of IgG: if this were too low the antibody might be ineffective in vivo.
3. The ability to activate complement, which in turn depends on the particular immunoglobulin isotype under study.

Antibody may act in vivo not by neutralizing virus infectivity but by interaction with viral antigens on the cell surface, and this can also involve non-neutralizing antibodies to internal virion and non-structural antigens. It is possible but not proven that such antibodies protect in vivo by activation of components of the complement system or by interaction with Fc receptors on cells (mostly of the monocyte/macrophage lineage) which then exert anti-infected cell activity (VEEV, MATHEWS et al. 1985; VSV, LEFRANCOIS 1984; yellow fever virus, GOULD 1986; dengue virues type 2, SCHLESINGER et al. 1987; Semliki forest virus, GROSFELD et al. 1989). There appears to be a varying requirement for intact antibody, rather than $F(ab)_2$, for protection but in most cases there was a requirement for a property other than that of complement activation. Neither neutralizing nor non-neutralizing $F(ab)_2$

protected mice from LCMV, and here C5-deficient animals were used to show that the full complement system was not required (BALDRIDGE and BUCHMEIER 1992); similarly only intact IgG protected mice against VEEV, again by a complement-independent mechanism, as shown by the use of C3- or C5-deficient mice (Mathews et al. 1985); and with FMDV, 10- to 500-fold more $F(ab)_2$ than IgG was required for protection of mice (McCULLOUGH et al. 1986). $F(ab)_2$ prepared from neutralizing or non-neutralizing monoclonal IgGs to Semliki forest virus protected mice poorly or not at all (Boere et al. 1985). However, $F(ab)_2$ prepared from a pool of human sera protected cotton rats against RSV infection, showing that the Fc region was not required (PRINCE et al. 1990). Neutralizing but not non-neutralizing $F(ab)_2$ protected mice from VSV but there are no data on the involvement of complement (LEFRANCOIS 1984).

In a review of the extensive work on paramyxoviruses, NORRBY (1990) concludes that antibodies to the HN/G envelope proteins give better protection than anti-F, but for RSV the converse holds true. Table 7 gives other references to protection by antibodies against paramyxovirus infections. UMINO et al. (1990b) comment that the best predictor of protection is not the neutralization titre but a high ratio of haemagglutination-inhibition: neutralization titre and/or the ability to inhibit plaque formation when antibody is incorporated into the overlay medium. Recent data on protection against the primate lentiviruses HIV-1 and SIV suggest that antibody mediates protection; this is shown most clearly from the passive transfer of HIV-1 gp120-specific neutralizing mabs into chimpanzees (EMINI et al. 1990, 1992). The, as yet, unresolved confusion concerning the relative contribution of anti-virus and anti-cell antibodies in protection of monkeys against SIV has already been discussed (Sect. 11). Passively transferred immune monkey serum protected cynomolgus monkeys from HIV-2 and SIV_{sm} (PUTKONEN et al. 1991).

Although outside the scope of this review, it should not go unmentioned that both neutralizing and non-neutralizing antibody are thought to enhance the pathogenic potential of some viruses in vivo by permitting virion–antibody complexes to gain entry to cells which they would not normally infect by binding to Fc receptors (see Sect. 8). Suspicions centre on viruses such as dengue (HALSTEAD 1988), rabies (SIKES et al. 1971; BLANCOU et al. 1980; PRABAKHAR and NATHANSON 1981), Japanese encephalitis (GOULD and BUCKLEY 1989), yellow fever (BARRETT and GOULD 1986; GOULD and BUCKLEY 1989) and latterly HIV-1 and SIV (TAKEDA et al. 1988, 1990; HOMSY et al. 1989, 1990; MONTEFIORI et al. 1990; ROBINSON et al. 1989, 1990a,b, 1991), but others are unconvinced (MORENS and HALSTEAD 1990).

In vivo a narrow range of antibody specificities may be produced to influenza virus and may be responsible for driving antigenic variation (drift) by selecting neutralizing antibody escape mutants (HAAHEIM 1980; NATALI et al. 1981; WANG et al. 1986). Neutralization escape mutants of a number of different viruses have reduced virulence (KÜMEL et al. 1985; ROOS et al.

1989; VAN HOUTEN et al. 1991; JOHNSON et al. 1990; KÖVAMEES et al. 1990; CECILIA and GOULD 1991) or cause a different type of disease (ZURBRIGGEN and FUJINAMI 1989). Escape mutants are found in vivo during infection with HIV-1 (ALBERT et al. 1990; EMINI et al. 1990; NARA et al. 1990; MONTEFIORI et al. 1991) and hepatitis B virus CARMAN et al. 1990).

Conclusion. Some neutralizing antibodies protect very effectively in vivo while others do not. This discrepancy is not understood and may be virus–antibody–target cell dependent, as it is in vitro but with the added complexity of the possible involvement of other elements of the defence system.

15 Complement and Neutralization

Complement has at least three roles involving neutralizing antibody and neutralization (COOPER 1987; HIRSCH 1982; COOPER and NEMEROW 1986, 1989): it can mediate the attachment of virus-antibody complexes to complement receptors on cells (see Sect. 8); it can enhance neutralization or bring about loss of infectivity with non-neutralizing antibody or sub-neutralizing amounts of neutralizing antibody; and it can bring about the solubilization of antigen–antibody aggregates (MILLER and NUSSENZWEIG 1975). Sindbis virus, NDV and some retroviruses are neutralized by the classical pathway independently of antibody (COOPER and NEMEROW 1986). C1 binds directly to viral protein, p15E in the case of retroviruses (WELSH et al. 1975; COOPER et al. 1976; BARTHOLOMEW et al. 1978). The alternative pathway can also be activated without the involvement of antibody (influenza virus, Sindbis virus, VSV and Epstein–Barr virus: COOPER and NEMEROW 1986). In addition, complement is activated by virus-infected cells which may or may not require antibody as an intermediary. The classical pathway is activated by IgM and, in order of complement-activating efficiency, human IgG3, 1 and 2 or mouse IgG2a, 2b, 3 and a subclass of 1 (EY et al. 1979; BURTON 1990; VALIM and LACHMANN 1991). Human IgG4 and IgA do not fix complement. Epitope density also influences the efficiency of complement fixation (VALIM and LACHMANN 1991). In the presence of specific antiviral antibodies enveloped virions are neutralized when they are permeabilized by insertion of a sufficient number of C9 pore structures, presumably allowing the entry of nucleases or intravirion ion concentrations incompatible with infectivity (avian bronchitis virus, BERRY and ALMEIDA 1968; gross leukaemia virus, OROSZLAN and GILDER 1970; equine infectious anaemia virus, RADWAN et al. 1973; HIV-1, SPEAR et al. 1990). However, COOPER and NEMEROW (1989) doubt that physiological concentrations of antibody are sufficiently high for this to be common in natural infection. Alternatively, complement proteins are deposited on the surface of enveloped or non-enveloped virions and interfere with infection (HIRSCH 1982; COOPER and NEMEROW 1986).

While in principle complement allows non-neutralizing IgG and IgM to neutralize infectivity (HSV-1, DANIELS et al. 1970; YOSHINO and ISONO 1978; influenza virus, BEEBE et al. 1983; equine arteritis virus, RADWAN and BURGER 1973; PIV, VASANTHA et al. 1988), studies with mabs show that this is not always so. Quantitative considerations are important as HSV-1 can form infectious complexes with critical amounts of virus– IgM–C1, C4, C2; increasing IgM or complement components leads to neutralization (DANIELS et al.

1970). The neutralizing capacity of neutralizing antibodies is not necessarily increased in the presence of complement. For example, complement did not increase neutralization of FMDV by a number of different mabs (McCULLOUGH et al. 1986). Human PIV-3 has seven antigenic sites, of which three (D, E, F) are non-neutralizing; these remained non-neutralizing and neutralization via the neutralizing site A was unchanged after the addition of complement, but the neutralizing activity of mabs to the other neutralization sites (B, C, AB) was increased 100-fold by complement (COELINGH and TIERNEY 1989a). Similarly, varicella-zoster virus has five antigenic sites; of these only mabs to site I and some mabs to site II exhibited complement-dependent neutralization (FORGHANI et al. 1990). There are similar data with mabs to other viruses (Epstein–Barr virus, mab C-3-2 to the membrane 236–212 kDa antigen, STRNAD et al. 1982; RSV, WALSH et al. 1984; GARCÍA-BARRENO et al. 1989; HSV-1, MINSON et al. 1986). WEISS et al. (1986) and HO et al. (1987) found no enhancement of HIV-1 neutralization with complement, but contradictory data are reported by SPEAR et al. (1990). A human IgG mab neutralized the RF strain of HIV-1 only in the presence of complement, although it could intrinsically neutralize strains MN and IIIB (LAKE et al. 1992). Lastly, there is a host dimension, as monkeys infected with HCMV synthesized mainly IgG which required complement for neutralization, whereas monkey CMV in the same host elicited complement-independent neutralizing antibody (GRAHAM et al. 1971).

In explanation it is likely, first, that some neutralizing mabs already give maximum neutralization which complement cannot improve on. Secondly, it is possible that the disposition of mabs to particular epitopes on the surface of virions controls the effectiveness of complement (VALIM and LACHMANN 1991). Activation of the first component of complement, C1q, requires that it binds to two or more Fc regions of a complement-fixing isotype simultaneously, so that if the disposition of a mab on the surface of a virion is such that this cannot physically occur complement will have no effect. However, high concentrations of virus or conditions which permit aggregation by antibody (Sect. 4) might permit activation. One would expect, therefore, that mixtures of mabs or antisera would interact with complement more efficiently.

One other advantage of complement is that it increases the versatility of neutralizing antibodies and only in this way is post-attachment neutralization seen with monkey CMV (FARRELL and SHELLAM 1990). There is complement-mediated neutralization with antibody to carbohydrate (influenza virus: HAUKENES 1977).

Complement-mediated neutralization is not thought to be of major importance to virus infections of man, as complement deficiencies are not associated with undue susceptibility to disease, although there are heightened problems with certain bacterial infections (HIRSCH 1982; MORGAN and WALPORT 1991). However, complement deficiency states in mice—whether congenital or induced—are correlated in experimental infections with Sindbis virus, LCMV, influenza type A virus and rabies virus with substantially increased accumu-

lation of infectivity and often mortality as well (HIRSCH et al. 1980; HIRSCH 1982). Some aspects of virus–IgM–complement interactions are discussed in Sect. 10.

Conclusion. Complement can enhance neutralization by neutralizing and non-neutralizing IgM and IgG and inactivate virus in the presence of subneutralizing amounts of these antibodies. The relevance of complement to neutralization of virions during natural infections is not clear although its absence can exacerbate experimental infections. Not all monoclonal IgGs with the potential to fix complement do so, possibly because of topographical difficulties in getting two molecules sufficiently close for both to simultaneously bind to C1q. This would be less of a problem with polyclonal IgG or mixtures of mabs.

16 Neutralization by Inhibition of Release of Progeny Virus from the Infected Cell

Newly synthesized influenza virus buds from the plasma membrane and immediately binds to NANA linked to cell surface molecules. Normally the virion NA cleaves off NANA so that virus is released. IgG directed against virion NA neutralizes free virions weakly by aggregating them (COMPANS et al. 1969; SETO and CHANG 1969; DOWDLE et al. 1974) but is more effective when acting upon newly budded virions. The question is, by what mechanism is this brought about? One view is that sialylation of nascent virions and their desialylation shortly after budding by the action of NA is a key event. Thus if NA is inhibited by antibody, virions bind to each other and also to NANA in the plasma membrane (SETO and ROTT 1966; WEBSTER et al. 1968; COMPANS et al. 1969; SETO and CHANG 1969; DOWDLE et al. 1974). Exactly the same accumulation of virions around the cell is seen with mutants which have a defective NA (PALESE et al. 1974) or in the presence of an inhibitor of NA (PALESE and COMPANS 1976). However, anti-NA Fab also inhibits NA activity very efficiently but does not affect the release of virus from infected cells or diminish plaque size when incorporated into the agar overlay (BECHT et al. 1971). This suggests that it is not inhibition of NA activity per se but aggregation which is responsible for neutralization by anti-NA. Anti-NA also confers protection in vivo, possibly by the effects of antibody, against the disease but not the infection (KILBOURNE 1976).

SAIRENJI et al. (1988) report that Epstein–Barr virus also is neutralized by antibody that inhibits its release from the cell.

What is perhaps surprizing is that viruses other than the Orthomyxoviridae, Paramyxoviridae and Coronaviridae, and particularly those of non-lytic character, have no receptor-destroying enzyme and do not appear to have a problem of binding to receptors on the surface of the cell which has just created them. Possible explanations include the down-regulation of receptor synthesis during infection and the removal of virus–receptor complexes by capping and patching. To get release it may even be necessary for nascent virions to saturate all available receptors—a simple although somewhat extravagent solution.

Conclusion. There is little evidence that prevention of virus release from the infected cell is a common mechanism of neutralization

17 Change in Virus Proteins and Virion Structure on Binding Antibody, Including Synergistic Neutralization

By analogy with interactions involving other macromolecules and their ligands it is possible that binding of antibody by virus brings about alterations in the structure of that virus by changing the conformation of its proteins (by allostery or distortion) or their association with each other or a third party. Unequivocal evidence to link this with neutralization is currently unavailable but it seems likely (to this reviewer) that at least some instances of neutralization involve antibody-induced changes in conformation. Lack of data is partly a technical problem since antibody itself is also a large and complex protein. Early data on non-viral systems are reviewed by CELADA and STROM (1972) and CELADA et al. (1983); they show that antibody releases heme from myoglobin (CRUMPTON 1966); and that antibody against the relevant native protein enhances activity of defective β-D-galactosidase (ROTMAN and CELADA 1968; ACOLLA et al. 1983; DUNCAN 1983), catalase (FERNSTEIN et al. 1971) and l-amino oxidase (ZIMMERMAN et al. 1971), presumably by stabilizing and/or altering conformation. Also, the equilibrium between monomers and dimers of the *E. coli* alkaline phosphatase is shifted towards the dimer by reaction with anti-dimer antibodies (LAZDUNSKI et al. 1975). The activity of mutant and wild-type enzyme is also enhanced by specific antibodies, and most spectacularly by a Fab fragment (PAGES et al. 1976). The NA activity of the HN protein of PIV-1 is enhanced by neutralizing antibody to site A (YEWDELL and GERHARD 1982). Recently, antibodies have been shown to exert enzymic activities themselves through specificity for one of the metastable states of the appropriate antigen (LERNER and TRAMONTANO 1987; IVERSON and LERNER 1989; TRAMONTANO and SCHLOEDER 1989; POLLACK et al. 1989).

More relevant to virus neutralization is the demonstration that only after binding antibody could one of the polypeptides of bovine enterovirus be labelled with iodine (CARTHEW 1976). Conformational changes which are reflected by a huge change in the pI of the particle or by instability to low pH at low ionic strength are associated with neutralization of poliovirus by some antibodies and of rhinoviruses by IgGs and their Fabs to all four major antigenic sites, but it is not known if these events are causal or epiphenomenological (Sect. 23.6). FMDV, another picornavirus, also undergoes profound conformational changes on binding one particular mab (4C9) and virion RNA is released. The virion dissociates to the extent that, within 30 s at

37°C, it becomes permeable to negative stains used in electron microscopy (McCULLOUGH et al. 1987). However, there is no change in polypeptide composition. Several molecules of mab per virion were required to effect the change since at high dilutions of antibody normal particles could be seen with antibody attached. At 10^{-3} dilution of mab there was 90% neutralization but no conformational change, suggesting that the latter was not the immediate cause of loss of infectivity. It may be that a similar mechanism operates with LDV, since there is loss of RNA from virions during neutralization (PLAGEMANN et al. 1992). Dissociation of proteins on binding antibody is also interpreted as the result of induced conformational changes: dissociation of a heterodimer containing the E1 protein of Sindbis virus (CLEGG et al. 1983) and of adenovirus penton base and fibre proteins on binding antibody to the fibre (BOUDIN and BOULANGER 1981). The gp120 of HIV-1 is released from virions by binding to its CD4 receptor (MOORE et al. 1991) and it may be that some antibodies have the same effect. More commonly observed is the synergistic binding of pairs of mabs—where more is bound than occurs when virus is reacted with either of the mabs singly (Table 8). With many of these there is enhanced neutralization by as much as 100-fold (SUÑÉ et al. 1990), and neutralizing activity can be conferred on non-neutralizing mabs (DUBUISSON et al. 1990). It is of interest that a non-neutralizing mab can enhance the binding and neutralization of neutralizing mabs (rotavirus, BURNS et al. 1988; HCMV, LUSSENHOP et al. 1988). The situation with mabs to the E1 envelope protein of rubella virus is even more extreme. Alone, none of the mabs to any of the five separate antigenic sites identified are neutralizing (although neutralizing antibodies have been found by others). However, in certain combinations the mabs became neutralizing: mabs to sites A, B or D with mabs to any of the other five sites were neutralizing, but not mixtures of mabs to site C and site E (GERNA et al. 1987). Non-neutralizing mabs to HCMV used in combination also became neutralizing and gave increased binding (LUSSENHOP et al. 1988). Enhancement is variously thought to result from conformational changes, with increase avidity (LUBECK and GERHARD 1982; HEINZ et al. 1984), speed of neutralization (probably also a measure of avidity: VOLK et al. 1982) and/or the number of antibody molecules binding (Table 8) through altering the structure, accessibility or availability of epitopes. HEINZ et al. (1984) suggest that site A of TBEV is only immunogenic when virus is complexed with antibodies to site B. The mechanism may well be complex as synergistic binding of a pair of antibodies to human chorionic gonadotropin was reduced if one of them was as an $F(ab)_2$, and abolished if it was an Fab (EHRLICH et al. 1982). There is enhanced neutralization in another context when two or more mabs or monospecific polyclonal antisera protected animals better than either did alone (PIV-3, RYDBECK et al. 1988; RAY et al. 1988; NDV, UMINO et al. 1990b), but it is not in fact clear if this effect is synergistic or additive; there is a similar situation with mabs to NDV in vitro (IORIO and BRATT 1984). In an analogous way there was improved neutralization with antiserum from rabbits immunized with pairs of

Table 8. Synergistic binding and neutralization by pairs of different monoclonal antibodies

Family	Virus	Measure of synergism[a]			Reference
		>Neutralization	>Avidity	>No. mabs bound	
Bunya	La Crosse virus	+	?+	?+	KINGSFORD et al. 1983, 1991; KINGSFORD and ISHIKAWA 1984; KINGSFORD 1984
Corona	TGEV	+			DELMAS et al. 1986
	TGEV	+			SUÑÉ et al. 1990
Flavi	Japanese encephalitis virus	+			KIMURA-KURODA and YASUI 1983
	West Nile virus	+			PEIRIS et al. 1982
	TBEV	+	+		HEINZ et al. 1984
	Dengue virus 2				HENCHAL et al. 1985
Herpes	Bovine herpes virus	+			DUBUISSON et al. 1990
	Varicella zoster virus	+			FORGHANI et al. 1990
	HCMV	+		+	LUSSENHOP et al. 1988
Myxo	Influenza A virus		+[b]		LUBECK and GERHARD 1982
Paramyxo	NDV	+			RUSSELL 1986
	RSV	+			ANDERSON et al. 1988
	RSV	+			GARCÍA-BARRENO et al. 1989
Rhabdo	VSV	NC	?		VOLK et al. 1982
	VSV				LEFRANCOIS and LYLES 1982a, b
Reo	Rotavirus VP4			+[b]	BURNS et al. 1988
	Rotavirus VP7			+[b]	Shaw et al. 1986
Retro	Bovine leukaemia virus			+[b]	BRUCK et al. 1982a
	HIV-1	+		+	TILLEY et al. 1992
	HIV-1	+			BUCHBINDER et al. 1992
Toga	Rubella virus	+			GERNA et al. 1987
	Semliki forest virus			+[b]	BOERE et al. 1984

[a] No entry indicates no information available; ?, a tentative conclusion; NC, no change.
[b] Not determined if increased binding gave increased neutralization.

peptides to gD of HSV-1, compared with serum obtained when peptides were injected singly (WEIJER et al. 1988).

Neutralization of the fowl plague strain of type A influenza virus by saturating amounts (but not by subsaturating amounts see Sect. 24.4) of IgG directed to the HA does not interfere with any of the early stages of virus–cell interaction and the virus is uncoated of its envelope together with attached neutralizing antibody. However, the genome, now free of antibody and ensconced in the nucleus, is not transcribed. It is now thought that it is not

the transcriptional process itself which is defective but uncoating of the core structure. This idea is supported by evidence which shows that while the genome of infectious virus becomes sensitive to ribonuclease, the genome of neutralized virus remains resistant (POSSEE et al. 1982; RIGG et al. 1989; OUTLAW and DIMMOCK 1991). The explanation postulated was that anti-HA antibody induces or prevents a change in the virion structure which is a necessary prerequirement for secondary uncoating of the core. Recent data obtained with neutralizing IgA have modified this conclusion to suggest that secondary uncoating (the escape of the viral ribonucleoprotein from within the M1 core) takes place, but that a novel tertiary uncoating stage (seen as an activation event affecting the ribonucleoprotein so that transcription can proceed) is inhibited (ARMSTRONG and DIMMOCK 1992). Anti-HA mabs can in principle prevent conformational changes: the electron spin resonance studies by KIDA et al. (1985) show that one such antibody prevented the low pH-induced conformational change of the HA of seal type A influenza virus. However, this would prevent primary, not secondary, uncoating. The effect was highly specific as other anti-HA mabs did not prevent such changes in HA structure. Inhibition of transcription in vitro was another indirect long-range effect of anti-HA IgG (SHIMIZU et al. 1985) which was suggested as a possible explanation of the data above by POSSEE et al. (1982) and then abandoned after closer examination (RIGG et al. 1989).

The foregoing implication that certain mabs are able to transduce a signal when they bind to a cognate transmembrane viral protein which is then transmitted across the plasma membrane is supported by studies of measles virus-infected cells. Application of antibodies to the HA protein to infected cells in vitro or in vivo reduces the transcription and expression not only of the HA but also of other virus-encoded proteins (FUJINAMI and OLDSTONE 1979, 1980, 1984; OLDSTONE et al. 1980; BARRETT et al. 1985; LIEBERT et al. 1990). That the signal is transmitted via the HA is shown by sequential depression and then elevation of HA, F, NP, P and M proteins in persistently measles virus-infected glioma C6 cells treated in vitro with a neutralizing anti-HA mab, the eventual rise in expression being associated with the failure of the mab to recognize the HA, due presumably to its mutation (ZINNHEIMER-DREIKORN and KOSCHEL 1990). While these studies are of infected cells they establish that in principle an antibody bound to the HA of a measles virion could deliver the same signal across the viral membrane to internal proteins with which it is in contact.

In the context of transmembrane cellular proteins it has long been known that antibodies and lectins can mimic some aspects of the normal signalling by their cognate ligands and result in the non-specific activation of B or T lymphocytes (see for example ALÉS-MARTÍNEZ et al. 1991; RETH et al. 1991; FINKEL et al. 1991; CAMBIER and CAMPBELL 1990; RUDD 1990). The mechanism of transduction across the plasma membrane is obscure; while long-distance transmission of conformational changes is a possibility (ALLEWELL 1991) the consensus today centres on changes in disposition of receptors resulting from dimerization or aggregation, or movements relative to each

other (see for example YARDEN and SCHLESINGER 1987; BÖNI-SCHNETZLER and PILCH 1987; WEINER et al. 1989; KLAUS 1990b; RUDD 1990; CUNNINGHAM et al. 1991; PAKULA and SIMON 1992). Specifically, the kinase associated with the epidermal growth factor receptor is activated by anti-receptor IgG, and it is of interest that activation requires bivalent binding as Fabs do not exert this effect (BÖNI-SCHNETZLER and PILCH 1987). It should not seem too surprising, therefore, when some viral antibodies behave likewise. Moving back to viruses, WEINMANN-DORSCH and KOSCHEL (1989) show that anti-measles virus IgG added to cells persistently infected with measles virus led to the activation of a G-protein-mediated induction of inositol polyphosphate and activation of phosphokinase C. A different effect of antibody is the membrane depolarization of macrophages seen on the addition of IgG specific for epitopes on the Fc receptor (YOUNG et al. 1983).

Antibodies are known to block conformational changes necessary for successful adenovirus infection: the proteolytic cleavage of a 15-kDa fragment from hexon protein which occurs in the low-pH environment of endosomes is inhibited by hexon-specific antibodies (WOHLFART 1988; VARGA et al. 1990). Neutralization of HIV-1 by anti-gp120 IgG has been postulated to be due to inhibition of cleavage of the V3 domain or of conformational changes which are necessary for infectivity (STEPHENS et al. 1990; CLEMENTS et al. 1991; Sect. 5.3).

Non-reciprocal interactions seen in competitive binding studies with PIV-3 are thought by COELINGH and TIERNEY (1989a) to result from induction of conformational changes.

Further evidence of conformational changes comes from the study of escape mutants. Usually there is a single amino acid change within the epitope recognized by the selecting antibody and that antibody no longer binds to the virion. However, occasionally mutations are located outside the antigenic site, implying that there is a conformational change such that neutralization can no longer be mediated by the selecting antibody. Here viruses still bind the selecting antibody, although this interaction is no longer neutralizing (Table 4). Presumably the mutation lies outside the neutralization epitope and results in conformational rearrangements which abrogate neutralization.

X-ray diffraction analysis of crystals of complexes of influenza virus NA with monclonal anti-NA Fab revealed that there were changes in conformation to both the viral protein and the antibody—a subtle re-ordering of juxtaposed groups likened by the authors to a handshake (COLMAN et al. 1987, 1989).

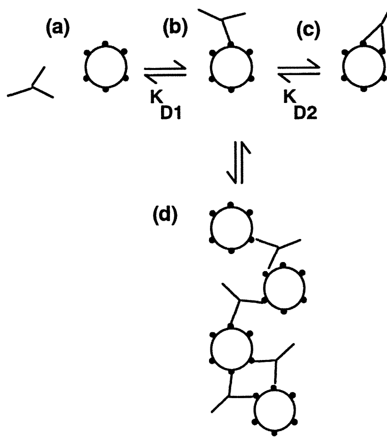
Other data show that antigen affects the conformation of bound antibody: IgG bound to virus is stabilized against denaturation by acid (KJELLÉN 1965b). Some non-viral examples are discussed by METZGER (1974, 1978), PECHT (1983) and ZAVODSKY et al. (1983).

Conclusion. The binding of some neutralizing, and some non-neutralizing, antibodies causes presumptive conformational changes to viral proteins. These can alter their interaction with other antibodies and may lead to increased neutralization. In enveloped viruses, antibody-mediated signals can be transduced across the viral envelope.

18 Reversibility of Neutralization

In practice reversibility of neutralization applies to the dissociation of virus–antibody aggregates into single virus particles bound to antibody and to the dissociation of antibody from the latter (Fig. 3); for further discussion see FAZEKAS DE ST. GROTH 1962; HORNICK and KARUSH (1972); BLANK et al. (1972) and DAY (1990). Reversibility depends upon the dissociation constant and the concentration of antibody. Many IgGs have high affinity and their bivalent character increases their avidity by 1000-fold; consequently, their rate of dissociation (K_{D2}) is low (poliovirus, MANDEL 1961; BRIOEN et al. 1985a; NDV, RUBIN and FRANKLIN 1957). Dilution dissociation of poliovirus–antibody complexes, after they were diluted 10,000-fold and held at 37°C for 5 h, was negligible for both antiserum and two mabs (BRIOEN and BOEYÉ 1985). This is in contrast with the ready dilution-dissociation of influenza virus–Fab complexes (LAFFERTY 1963b; Sect. 19). Antibody produced early in infection is of low avidity (WEBSTER 1968). Avidity increases as immunogen concentration falls due to the selective stimulation of B cells. However, ROSENSTEIN et al. (1971) found no difference in dissociation of Fabs from the bacteriophage ϕX174 whether they were derived from neutralizing early antiserum or late antiserum, although only Fabs from late antiserum were neutralizing. Reactivation can occur by adding an excess of inactivated homologous virus. This probably results from the collision of a neutralized virus–antibody complex with inactivated virus which allows transfer of antibody and restoration of infectivity (influenza virus, ISAACS 1948; western equine encephalitis virus, DULBECCO et al. 1956). Most published work (reviewed by SVEHAG 1968) precedes the advent of mabs and therefore has the intrinsic uncertainties inherent in the heterogeneous nature of a polyclonal antibody preparation. Poliovirus infectivity was recovered when virus–antiserum mixtures were treated with fluorocarbon (SVEHAG 1963) or by freezing and thawing (MANDEL 1973). Recovery of infectivity decreased with time of incubation of virus and antibody, suggesting that more stable molecular interactions were being formed. Incubation with a monoclonal IgG gave no reactivation of infectivity (ICENOGLE et al. 1983). In principle, dissociation from virus will be proportional to the avidity of individual mabs. However, criteria used in screening may tend to select mabs with high affinities (around $10^9\,M^{-1}$); even so, mabs to influenza virus used by BROWN et al. (1990) ranged in value from $10^{6.8}$ to $10^{9.5}\,M^{-1}$. Interestingly, they found no correlation between affinity and neutralization. The infectivity of diluted TGEV–mab complexes was dramatically increased by 10^3–10^6-fold after holding at 4°C for 3 h, presumably by dissociation (SUÑÉ et al. 1990).

A. low ratio of IgG : virus



B. High ratio of IgG : virus

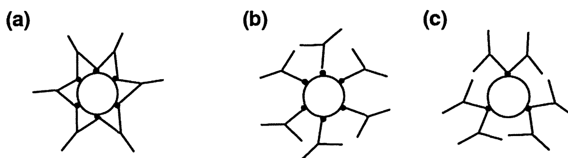


Fig. 3A, B. Diagrammatic representation of the association and dissociation of virions with a monoclonal IgG. Aggregation is favoured by a relatively low ratio of IgG : virus (**A**) which permits crosslinking of two or more virions (**A**,*d*). At higher IgG : virion ratios epitopes are saturated and none is free to permit crosslinking and aggregation (**B**). Bivalent binding depends on the neutralization protein (●) having at least two identical epitopes, as in **B**,*a* but not in **B**,*b*. Bivalent binding does not occur, even if the neutralization epitope has two or more identical epitopes, when the proteins are situated too far apart to be bridged by the IgG (**B**,*c*). Bivalent binding will be more stable than monovalent since complete dissociation requires both paratopes to dissociate simultaneously.

Some reactivation is seen when mab-neutralized poliovirus is electrofocused, and this is probably helped by the conditions of low ionic strength and high electrical potential (BRIOEN et al. 1985a). Acid- or alkali-stable viruses offer an ideal system for study since most antigen–antibody complexes can be dissociated at extremes of pH. This was exploited by GRANOFF (1965), who demonstrated very clearly that infectivity of NDV is restored at a pH of 12.1, which would be expected to dissociate antibody. Many workers have similarly retrieved infectivity from acid-stable picornaviruses at low pH. Here it is relevant to note that antibody was stabilized against low-pH denaturation when bound to adenovirus (KJELLÉN 1965b). One reason for the loss of neutralizing activity by monovalent Fab fragments is dissociation inherent in the reduction of avidity by three orders of magnitude compared with the

bivalent molecule, but intra- or intervirion crosslinking is also of potential importance in mediating neutralization (especially of poliovirus, see Sect. 23.5).

Dissociation of virus and antibody is of interest as it offers the opportunity to investigate if neutralization results in irreversible changes to the virus particle (for example, the inhibition of the presumed tertiary uncoating of influenza virus: ARMSTRONG and DIMMOCK 1992). Little work has been done in this area, presumably because early experiments were phrased and interpreted within the hypothesis that antibodies only neutralize by sterically interfering with attachment to the cell receptor.

Virus aggregation by low concentrations of antibody is inherently unstable since only one paratope is involved and the dissociation constant K_{D1} is about 10^3-fold greater than K_{D2} (Fig. 3; THOMAS et al. 1985). In this situation neutralization by mabs that act purely by aggregation (poliovirus, see Sect. 23.5; MOSSER et al. 1989) will be highly prone to reversal by dissociation. This is also seen with type A influenza virus and IgG, IgA and IgM (TAYLOR et al. 1987; OUTLAW et al. 1990; OUTLAW and DIMMOCK 1990; ARMSTRONG et al. 1990). IgM is a special case: its affinity is intrinsically low since IgM-secreting B cells are stimulated during infection under conditions of high antigen concentration and its binding sites undergo no refinement as a result of somatic mutation. Thus, although multivalent binding compensates for the low affinity, aggregates are inherently unstable since they necessarily involve few paratope–epitope interactions (Dugbe virus, GREEN et al. 1992; influenza virus, ARMSTRONG et al. 1990).

For an insight into early work the reader is referred to the comprehensive review of SVEHAG (1963).

Conclusion. There is little spontaneous dissociation of virus–polyclonal antibody complexes although this would be expected at very low antibody:virion ratios. The situation with mabs is highly variable but has not been widely investigated.

19 Neutralization by Fragments of Antibody

Experiments to determine whether or not the whole antibody molecule is necessary for neutralization were made possible by digestion with the proteolytic enzymes pepsin, which releases the Fc region leaving an $F(ab)_2$ structure, or papain, which results in a monovalent antigen-binding fragment (Fab). Most data relate to IgG. Some work suggests that $F(ab)_2$ is not as efficient at neutralizing as the whole IgG (poliovirus, KELLER 1966; R17 phage, KLINMAN et al. 1967; visna virus, JOLLY et al. 1989) or does not neutralize at all (adenovirus, KJELLEN 1965a). However, $F(ab)_2$ made from monoclonal IgG showed no reduction of neutralization (VEEV, MATHEWS et al. 1985; influenza virus HA, R.J. Rigg and N.J. Dimmock, unpublished data). Coversely, $F(ab)_2$ to Semliki forest virus neutralized 100-fold more poorly than whole IgG (BOERE et al. 1985). This is difficult to explain unless interaction of Fc regions with amino acid residues of the antigen which is a factor in precipitation by antibody (STEENSGAARD 1984) is important in neutralization of Semliki forest virus by this mab. Obviously $F(ab)_2$ cannot carry out those activities which depend on signals integral to the Fc region such as binding to cellular Fc receptors or to complement, but $F(ab)_2$ to RSV reduced lung virus titres in cotton rats by 100-fold (PRINCE et al. 1990).

A clear answer to the question of the ability of monovalent Fab to neutralize virus is only just emerging. Difficulties of this experimental system include the inevitable loss of avidity, of the order of 10^3-fold, when the bivalent immunoglobulin is converted to monovalent Fab, the need to determine accurately the specific activities of the immunoglobulin with the derived Fab, and the problem of trace amounts of immunoglobulin or $F(ab)_2$, which may have a specific activity of an order of magnitude or more than Fab. Even taking all these into account, it may be no more possible to make a generalization about Fab neutralization than it is about IgG neutralization. However, it can reasonably be predicted that Fabs derived from any IgG, which can neutralize with only monovalent binding, will be active. The converse may not hold as some Fabs are more efficient at neutralization than the IgG from which they were derived (ASHE et al. 1968). A decrease in avidity was demonstrated when the infectivity of Fab-neutralized influenza virus increased by dilution-induced dissociation of Fabs (LAFFERTY 1963a). However, if the affinity of the original IgG is sufficiently high the loss of avidity on conversion to Fab may have no practical effect on neutralization; poliovirus Fabs showed no dilution dissociation (VOGT et al. 1964). This was also the interpretation of the improvement of neutralization of ϕX174 by Fab derived from serum

obtained several months after immunization compared with that from an early bleed (ROSENSTEIN et al. 1971). However, this interpretation may be over-simplistic as there is no evidence that the early and late antibodies were directed against the same epitopes.

Although equivocal there is evidence from many different virus families that some Fabs are neutralizing, though often with reduced efficiency (T2 phage, STEMKE and LENNOX 1967; R17 phage, KLINMAN et al. 1967; T1, T6 phages, GOODMAN and DONCH 1964; influenza virus, LAFFERTY 1963a; KIDA et al. 1985; YODEN et al. 1985; vaccinia virus, LAFFERTY 1963a; western equine encephalitis virus, CREMER et al. 1964; Venezuelan equine encephalitis virus, MATHEWS et al. 1985; rhinovirus, COLONNO et al. 1989; poliovirus, CREMER et al. 1964; VEEV, ROEHRIG et al. 1988; VOGT et al. 1964; KELLER 1966). Fabs made from monospecific antibody to the HN of SV5 were only marginally less neutralizing than IgG in MDBK cells but fell to 30% in CV1 cells, although the 50% neutralization titre was the same in all cases (MERZ et al. 1981). Similarly, anti-F Fabs were neutralizing, but again less so in CV1 cells. For anti-HN this may reflect the relative abilities of IgG and Fab to block attachment to different length cell receptor units: the prediction is that these are longer on CV1 than MDBK cells, so that the smaller Fab molecule is less effective at blocking attachment. Consistent with this is the finding that IgG and Fabs were equally effective at blocking attachment to rbcs—probably to glycophorin A, a small molecule which carries most of the rbc's sialic acid (Sect. 7). On the other hand, some Fabs derived from neutralizing antibodies do not themselves have that activity: poliovirus type 1 was not neutralized by a 10^5-fold excess of Fab (THOMAS et al. 1985) and adenovirus was not neutralized despite the fact that its haemagglutinating activity was inhibited (KJELLÉN 1964). At the other extreme are anti-HSV-1 Fabs which neutralize 10-fold better than the IgG from which they were derived (ASHE et al. 1968). This may be due to reduction in steric hindrance to binding compared with IgG, as found recently with influenza HA (JACKSON et al. 1991). The same argument may explain how Fab neutralized the persistent fraction of HSV-1 pre-neutralized with IgG (ASHE and NOTKINS 1967). Anti-influenza virus polyclonal Fabs neutralized without loss of activity provided that there was no dilution dissociation, as already mentioned (LAFFERTY 1963b). More recently KINGSFORD et al. (1991) have shown that monoclonal Fabs to La Crosse virus neutralize well. Fabs to sites A, D, E and G required nearly four fold higher concentrations to achieve the same degree of neutralization as the IgG from which they were derived, but two mabs to sites C and B had a higher specific neutralizing activity and also a greater rate of neutralization than their IgGs. Fabs to VP8* of rotavirus were also neutralizing (RUGGERI and GREENBERG 1991). Finally, it has been demonstrated unequivocally using HIV-1 gp120-specific Fabs produced by recombinant DNA technology that some, but not all, Fabs have neutralizing activity (BARBAS et al. 1992); also a recombinant DNA-generated Fab to rabies virus has been produced (CHEUNG et al. 1992). All Fabs were of high affinity.

Another approach has been to use papain to convert IgG already bound to neutralized virus to Fab and then determine if there was still neutralization. Very early work demonstrated that infectivity of coliphage neutralized with 'low' amounts of IgG was restored in this way (KALMANSON and BRONFENBRENNER 1943) but the interpretation is ambiguous as no evidence was presented that Fab remained attached to the virus. Certainly, when the phage was initially neutralized with higher amounts of antibody papain did not restore infectivity. Later KELLER (1968) and EMINI et al. (1983b) found similar data with poliovirus. Other also report that papain digestion restored infectivity to neutralized poliovirus (ICENOGLE et al. 1983; THOMAS et al. 1985, 1986). All demonstrate qualitatively that Fabs remained attached to virus by causing re-neutralization with anti-mouse IgG but none quantitated the amount of Fab remaining after papain treatment. Thus these data are not sufficient to demonstrate that Fab is non-neutralizing as neutralizing IgG can bind to virions in low amounts without causing neutralization (ICENOGLE et al. 1983; MOSSER et al. 1989). Fabs from mab 35-1f4 added de novo to poliovirus type 1 did not neutralize even in 10^5-fold molar excess. This is consistent with this mab neutralizing primarily by crosslinking virions (THOMAS et al. 1985) but at odds with the fact that single neutralized virions are produced at high IgG concentrations. EMINI et al. (1983b) find that papain does not restore infectivity of virus neutralized with antiserum to VP3, but Fabs from other sera are neutralizing (CREMER et al. 1964; VOGT et al. 1964; KELLER 1966, 1968). All this suggests that some poliovirus Fabs are neutralizing and some are not. It may be significant that the strongly neutralizing Fabs are directed against type 2 virus and, like IgG, neutralize by inhibiting attachment (KELLER 1966), whereas most mabs so far studied neutralize by a more complex post-attachment mechanism (Sect. 23). The recent and careful study of COLONNO et al. (1989) on another member of the Picornaviridae demonstrates that Fabs derived from IgG2a mabs to all four antigenic sites of rhinovirus 14 were neutralizing although the specific neutralizing activity was reduced from 13- to 61-fold. All caused the same downshift in pI as the IgG, from 6.7 to 1.8-3.2, the significance of which is discussed in Sect. 23.6. The Fabs, like the IgG, neutralized by inhibiting attachment. This is in contrast with the situation with poliovirus above, where no monoclonal Fabs neutralize. That a molecule as small as a Fab can block attachment suggests that it binds close to the virus attachment site. The reason no poliovirus type 1 mab Fabs neutralize may be that none of the IgGs block attachment; this can be tested with mabs to type 2 and 3 which do.

Papain digestion of IgG bound to VP7 or to the VP5* fragment of VP4 of rhesus rotavirus abrogated neutralization, and this was restored by the addition of anti-mouse IgG. In contrast, papain had no effect on neutralization by mabs to the VP8* fragment of VP4 (RUGGERI and GREENBERG 1991). Since anti-VP7 neutralized by a post-attachment mechanism and anti-VP4/VP8* by inhibiting attachment, the papain data may indicate that while Fabs to VP4/VP8* can still block attachment, neutralization by VP7 or VP5* requires

a bivalent molecule. Neutralization by free Fab was not tested. Cryo-electron-microscopy has resolved the binding of Fabs to rotavirus VP4; the latter are visualized as spikes, each of which binds two Fabs at its distal end. Oddly, the angle subtended by each Fab is different (PRASAD et al. 1990). Although papain digestion reduced neutralization by Fabs made from mab 2G4 to 40% (RUGGERI and GREENBERG 1991), Fabs alone are neutralizing (M.K. Estes, personal communication).

Conclusion. It seems likely that some Fabs are neutralizing and some are not, an ability probably dependent on the mechanism of neutralization of the relevant IgG. This somewhat confused area is overdue for re-examination using high- and low-affinity mabs and efficient separation of Fab from IgG and $F(ab)_2$, or Fabs made by recombinant DNA technology.

20 Quantitative Aspects of Neutralization

Insight into the mechanisms and efficiency of neutralization requires knowledge of the number of immunoglobulin molecules necessary to neutralize one virus particle. For example, if only one immunoglobulin molecule per virion causes loss of infectivity it is unlikely that the immunoglobulin is interfering with attachment—unless the virus has only one attachment site. Early work is difficult to interpret because polyclonal antibody recognizing an unknown number of antigenic determinants was used (see DELLA-PORTA and WESTAWAY 1978) but mab solves those problems at a stroke. The situation is not simple since, paradoxically, neutralization, as evidenced by much early work, is a single-hit process but several immunoglobulin molecules are bound per virion.

If every immunoglobulin molecule in a population is equally capable of neutralization and causes neutralization with equal probability when it binds to a virus particle, statistics predict that when there is 1/e (63%) neutralization there will, on average, be one immunoglobulin molecule bound per virion. This has been found for polyclonal antibody (presumably IgG) to adenovirus hexon protein (WOHLFART 1988) and to poliovirus (WETZ et al. 1986). Others find that poliovirus binds 4 to ≥ 15 IgG molecules per virion depending on the mab (ICENOGLE et al. 1983; MOSSER et al. 1989; D. Leippe and R.R. Rueckert, personal communication) and that type A influenza binds about 70 monoclonal IgG molecules per virion (TAYLOR et al. 1987). The fact that viruses can bind a certain number of molecules of neutralizing monoclonal immunoglobulin and suffer no detectable loss of infectivity was confirmed by direct labelling with colloidal gold (IgG, OUTLAW et al. 1990; IgA, ARMSTRONG and DIMMOCK 1992; IgM, ARMSTRONG et al. 1990) and by the enhancement of infectivity when Sindbis (CHANAS et al. 1982) and West Nile (PEIRIS et al. 1982) viruses were titrated on cells carrying Fc receptors on their surface.

The rate of neutralization is initially linear but residual virus is often inactivated more slowly and gives rise to a biphasic inactivation curve. Such kinetic data have been extensively discussed in earlier reviews (see Sect. 1, especially FAZEKAS DE ST GROTH 1962). The 'persistent fraction' of infectious HSV could be neutralized by the addition of Fab derived from the neutralizing IgG (ASHE and NOTKINS 1967), suggesting that neutralization efficiency was reduced by steric effects between IgG molecules. This has been explored more recently with influenza virus by POUMBOURIOS et al. (1990) and JACKSON et al. (1991). Indeed, the biphasic neutralization of La Crosse virus by IgG became monophasic when Fabs were used (KINGSFORD et al. 1991).

Kinetic studies with mabs show very clearly that neutralization of influenza virus by IgG and IgA is single-hit (OUTLAW and DIMMOCK 1991; M.C. Outlaw, unpublished), confirming earlier data with polyclonal antibodies (poliovirus; DULBECCO et al. 1956; MANDEL 1985). There is also very good agreement between predicted and observed neutralization when the concentration of IgG is varied. The conclusion is that a virus particle is neutralized by one molecule of IgG. The apparently conflicting conclusions derived from biochemical and kinetic studies can be reconciled by hypothesizing that neutralization is mediated only through critical antigenic sites. It follows that there are antigenically identical non-critical sites which are irrelevant to neutralization. Thus, the difference lies not in antigenicity but in the relationship of the protein carrying the critical site to other structures in the virion. Binding of IgG to a critical but not to a non-critical site renders the virus particle non-infectious by causing a change in the structure of the virion or preventing a change necessary for infection from taking place. Thus, for influenza virus where neutralization requires an average of 70 IgG molecules per virion of mab to sites A or B of the HA, the ratio of critical:non-critical sites is 70:1 and since the virus has 1000 HA spikes there are 14 critical sites per virion. It follows that if the first IgG molecule binds to a critical site the virus will be neutralized, but on statistical ground 70 IgG molecules bind before a critical spike is hit (TAYLOR et al. 1987). These data and conclusions are essentially similar to those for polyclonal antibody of AMELUNXEN and WERDER (1960). However the observed kinetics of inactivation which result from the binding of more than one molecule of antibody can also be satisfied by the interpretation than each molecule of antibody causes a fractional loss of infectivity (see ICENOGLE et al. 1983 for a fuller explanation). In contrast, adenovirus is neutralized by 1.4 anti-hexon IgG molecules per virion (WOHLFART 1988), suggesting that all hexon sites are critical for neutralization. Furthermore, since each virion has 240 hexons, each a trimer, the binding of one IgG molecule can only neutralize by triggering the loss of infectivity.

At saturation type A influenza virions bind about one polyclonal or monoclonal IgG molecule per HA spike (which is a trimer) instead of the potential maximum of three (FAZEKAS DE ST. GROTH and WEBSTER 1963; TAYLOR et al. 1987). This was demonstrated for mabs to sites A, B and D. However, virus or free HA attached to a plastic matrix bound three molecules per HA spike of mabs to sites A and B. Using another influenza strain, POUMBOURIOS et al. (1990) found that free HA in solution bound only one IgG to site B or B/D, showing that the constraint is intrinsic to the HA spike, rather than between interactions of adjacent spikes, but mabs to sites A or E bound three molecules per spike. Mixtures of mabs to sites A or E with B and B/D bound additively to free HA up to maximum of four per spike. Poliovirus was saturated with 30 molecules of F7.12 (now mab 1), suggesting that there was bivalent binding to all the 60 antigenic sites present on the virion (ICENOGLE et al. 1983).

The 'percentage law' of neutralization was propounded for phage by ANDREWES and ELFORD (1933) and has been shown to hold for animal viruses with polyclonal (FMDV: CAPSTICK et al. 1959) and monoclonal antibodies (poliovirus: BRIOEN and BOEYÉ 1985). It still defies explanation. The law states that regardless of virus concentration (up to 10^8 pfu/ml), a constant percentage will be neutralized by a fixed amount of antibody. At virus concentrations over 10^8 pfu/ml neutralization is proportional to virus or antibody concentration.

As the antibody response matures there is a change in kinetics of neutralization or an increase in affinity of antibody (poliovirus: OZAKI 1968; influenza virus: WEBSTER 1968). A source of more recent references to this general area is FOOTE and MILSTEIN (1991). BROWN et al. (1990) sought a relationship between affinity and neutralization for 12 mabs to influenza virus HA and failed to find it. Mabs had values ranging from 10^6 to $10^{9.5}$ M^{-1} and represented extremes of neutralization. Mabs having an affinity of 10^6 and $10^{7.5}$ M^{-1} were equally neutralizing and mabs having the same affinity of $10^{8.5}$ M^{-1} showed a 10-fold range in neutralization. However, LANGEDIJK et al. (1991) found that mabs to peptides representing the V3 region neutralized HIV-1 in proportion to their affinity. STEWARD (1983) has edited a general review on the biological significance of affinity.

It is selfevident that affinity can, under some circumstances (e.g. very low values), affect neutralization, but as yet there is no direct evidence of proportionality and within limits other factors appear to be more important. However, it is likely that antibodies which neutralize by inhibiting attachment (Sect. 2) will be those most affected by affinity since they act in competition with the cell receptor unit; this may help explain why some mabs to the binding region of HIV-1 (LASKY et al. 1987; DOWBENKO et al. 1988; SCHNITTMAN et al. 1988) or to rabies virus G protein (BRACCI et al. 1988; CUSI et al. 1991) are non-neutralizing (Table 2).

Conclusion. All neutralization follows apparent single-hit kinetics and yet at 1/e (63% neutralization) different viruses bind anything from 1 to 70 molecules of mab per virion instead of the one molecule expected. For virions binding more than one mab molecule, it is hypothesized that they have a minority of antigenic sites which are critical for neutralization. Critical and non-critical sites are envisaged as being identical antigenically and differ only in their relationship with other virion components.

21 Unconventional Neutralization

21.1 Genetic Engineering of Antibodies and Viruses

With growing understanding of neutralization and with recent technical advances it is now possible to select or design antibodies (LERNER 1984), including those having physical attributes and binding sites which do not exist in nature. Heavy chain gene switching in hybridomas or recombinant DNA technology allow the variable region to be expressed in conjunction with any or all of the immunoglobulin isotypes, e.g. for the NP hapten (BRÜGGEMANN et al. 1987). These have been used to study their ability to activate complement via the classical or alternate pathways where the only variables were the immunoglobulin heavy chain constant region and the epitope density (VALIM and LACHMANN 1991). Sets of neutralizing immunoglobulin isotypes with identical paratopes have not yet been made and studies, for example on the efficacy at neutralization in vitro and in vivo, are anticipated with interest; it can be envisaged, for example, that the different flexibility of arms or elbows of antibody (see BURTON 1990) may permit bivalent binding in one isotype which is not possible in another. This is of particular relevance to neutralization of poliovirus (Sect. 23.5). In vivo a particular isotype may offer advantages which would favour protection such as tissue distribution, complement fixation or the ability/inability to bind to Fc receptors. More radical is the report that antibodies can be made to enter cells by altering their solubility (KABANOV et al. 1989a,b). If confirmed this opens up the possibility of reaction with new epitopes, on immature virion proteins, on internal virion proteins and on non-virion proteins. A hybrid antibody with two different specificities can be made fusing two hybridomas (SURESH et al. 1986), and this offers the possibility of bivalent binding to heterologous epitopes when the homologous epitopes are an unfavourable distance apart.

Through the manipulation of recombinant DNA encoding the light and heavy chains of antibodies it is possible to reduce the binding element in size, with possible advantages accruing from the reduction of autointerference in binding and improvement in tissue penetration. The activity of Fabs is discussed in Sect. 19 but still further reduction in size is possible (see Fig. 4 and WINTER and MILSTEIN 1991). Fv consists of the noncovalently linked V_H and V_L domains; these may be unstably associated but can be joined by introducing, through genetic manipulation, a flexible peptide from the C terminus of one to the N terminus of the other to form a scFv. Some single V_H domains, known as dAbs, surprisingly recognize and bind antigen, but tend to have

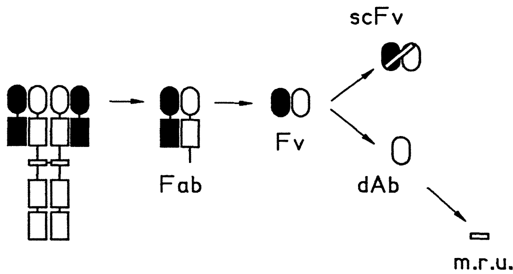


Fig. 4. Antibody fragments produced by recombinant DNA technology. Each *box* represents a domain. Single-chain Fv fragments (*scFV*) in which V_H and V_L domains are linked by a peptide have been made. Single V_H domains (*dAbs*) and single complementarity determining regions (or minimal recognition units or *m.r.u.*) have been identified with antigen-binding activities. Site-directed mutagenesis of antibodies and fragments can also be used to alter effector functions and improve affinities. (From WINTER and MILSTEIN 1991)

undesirable hydrophobic properties which have to be engineered out. This discovery was anticipated in the work of GOODMAN and DONCH (1965), who found that the isolated L chain had neutralizing activity for T1 and T6 phages. The humanization of mouse or rat mabs, in the absence of an efficient system of stimulating or immortalizing human B cells, is another improvement of passive immunization. Such humanized antibodies to HSV-1 gB and gD proteins neutralized with unaltered affinity (CO et al. 1991).

WINTER and MILSTEIN (1991) also discuss various ways of bypassing conventional hybridoma technology by cloning V_H and V_L genes at random and allowing these to reassociate at random, giving novel paratopes which are screened for the required activity (see also MCCAFFERTY et al. 1990; MARKS et al. 1991; CLACKSON et al. 1991 and for Fabs to HIV-1 and rabies virus (BARBAS et al. 1992; CHEUNG et al. 1992). The affinity of these molecules would probably be low but could be improved by in vitro mutagenesis (ROBERTS et al. 1987; RODWELL 1989). For virus neutralization this offers the opportunity to explore the neutralization potential of normally immunogenically recessive parts of the virion surface. Coupled with the reduction in size of the antigen-binding region discussed above, it might be possible to find antibodies which can recognize and bind to virus attachment sites which are normally hidden in depressions on the virion surface and are either non-immunogenic or unavailable to immunoglobulin-sized molecules. Antibodies which bind to attachment sites are relatively uncommon in nature (Table 1) and may not neutralize anyway (Table 2).

Conversely, it is now possible to create chimaeric viruses by engineering new epitopes into infectious virus particles and to use these to study neutralizing epitopes. Various epitopes have been inserted into poliovirus (HIV-1, EVANS et al. 1989; FMDV, KITSON et al. 1991) and from other poliovirus serotypes (MINOR et al. 1990; MURDIN et al. 1992); also, an HIV-1 epitope has been inserted into non-infectious particles of hepatitis B S antigens and

used to raise neutralizing antibodies in vivo (SCHLIENGER et al. 1992). However, immunogenicity may be weak (MURDIN et al. 1992). This approach will permit analysis of neutralizing epitopes of influenza virus. This has not been possible previously as epitopes are all non-linear and antisera raised to peptides have no neutralizing activity. Now LI et al. (1992) have replaced a peptide from one type A influenza strain with that of another. This chimaera can be neutralized by antibodies specific to either strain and elicits antibodies to both. A similar approach to achieving the required conformation is to insert the peptide of interest into the hypervariable loops of antibody (ZANETTI 1992).

21.2 Anti-idiotype Antibodies and Neutralization

Antibodies to the idiotype of neutralizing antibody have been proposed as vaccines (GAULTON and GREENE 1986; KÖHLER et al. 1989; RIMMELZWAAN et al. 1989) and are used to study neutralization itself (Fig. 5). Anti-idiotype antibodies have neutralizing activity only if the original neutralization epitope is involved in the interaction with the cell receptor, and if the original

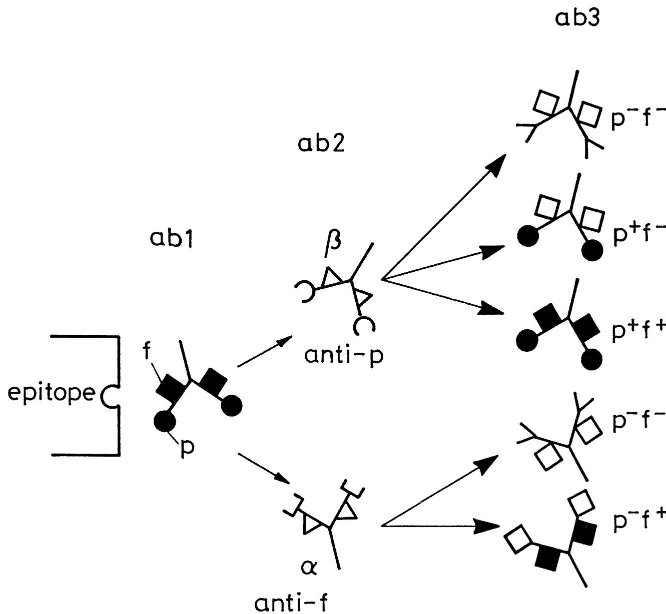


Fig. 5. Anti-idiotypic antibodies. These may recognize idiotopes associated with the paratope (*p*) or framework region (*f*) of *ab1*. Thus there are two types of anti-idiotypic antibodies, *ab2α* and *ab2β*, which recognize the paratope (p^+) and framework (f^+) idiotopes, respectively. Only ab2β stimulates anti-anti-idiotypic antibodies (*ab3*) which can recognize the same epitope as ab1, and possibly be neutralizing (*ab3*, p^+f^+ or p^+f^-). Other ab3 specificities are possible, as shown. (From RIMMELZWAAN et al. 1989)

neutralizing antibody (ab1) interferes in this interaction (see Table 1). Anti-idiotype antibody (ab2), which has to be to the 'internal image' of the binding site of ab1, may 'neutralize' by binding to the cell receptor and preventing interaction with the virus attachment sites. Anti-anti-idiotypic antibody (ab3) may neutralize by binding to the original neutralization epitope. Structurally, both ab1 and ab2 may be a poor imitation of the epitope (BENTLEY et al. 1990; DAVIS et al. 1992), but nevertheless good enough to stimulate neutralizing ab3. 'Neutralization' by ab2 has been demonstrated for HIV-1 (BRODEUR et al. 1991), TGEV (SUÑÉ et al. 1991), Sindbis virus (UBOL and GRIFFIN 1991; WANG and STRAUSS 1991; WANG et al. 1991) and bovine herpes virus type 1 (ABDELMAGID et al. 1992). An ab3 was synthesized naturally when mice were immunized with measles virus (KRAH and CHOPPIN 1988). Neutralization by ab3 is reported for a number of viruses (e.g. rabies virus, REAGAN et al. 1983; reovirus SHARPE et al. 1984; GAULTON et al. 1986; poliovirus type 2, UYTDEHAAG and OSTERHAUS 1985; influenza virus, ANDERS et al. 1989; HCMV, KEAY et al. 1988; RSV, PALOMO et al. 1990; TGEV SUÑÉ et al. 1991). Ab3 also protects on passive transfer in vivo (HSV-1, KENNEDY et al. 1984; hepatitis B virus, KENNEDY et al. 1986; poliovirus type 2, UYTDEHAAG and OSTERHAUS 1985; reovirus, GAULTON et al. 1986). Ab3-containing antisera can be highly specific, as they neutralized influenza virus via the antigenic site of the H2 haemagglutinin to which the original ab1 was directed; conversely, they also contain heterotypic antibodies reacting with H1 and H3 haemagglutinins which were not recognized by ab1 (ANDERS et al. 1989).

The downside of anti-idiotypic antibodies is that they can block the action of neutralizing antibody or protection in vivo (e.g. VLASPOLDER et al. 1990). As immunogens, in addition to stimulating ab3, they can elicit potentially immunopathological reactions such as delayed-type hypersensitivity (RIMMELZWAAN et al. 1989). In a mirror image of this situation, anti-antibody (ab2) to the *cell receptor* can neutralize virus by binding to the virus attachment site (e.g. where ab1 is Leu-3a to the CD4 receptor of HIV-1: CHANH et al. 1987).

A different strategy is to use molecules other than immunoglobulins to neutralize infectivity. One way is to block attachment with cell receptor molecules, expressed from recombinant DNA, which have been solubilized by deletion of their transmembrane region. Prominent in this category is soluble CD4, the major receptor of HIV-1, HIV-2 and SIV (WEISS 1988a,b; CLAPHAM et al. 1989; LAYNE et al. 1991). A second generation of molecules addresses the problem of the innate instability of soluble CD4 in vivo; these are fusion proteins consisting, for example, of the terminal domain of CD4 which binds HIV fused with the constant region of an immunoglobulin. This potentially has advantageous antiviral properties of fixing complement, binding to Fc receptors and crossing the placenta (CAPON et al. 1989; BYRN et al. 1990). The multivalent CD4–μ fusion was particularly effective in preventing syncytium formation (TRAUNECKER et al. 1989). Similarly, soluble recom-

binant CR2 (CD21) and soluble ICAM-1 have been used to inhibit attachment to cells of Epstein–Barr virus (NEMEROW et al. 1990) and rhinovirus (MARLIN et al. 1990), respectively. Poliovirus is neutralized by incubation with its cloned receptor during which process it is converted from a 160*S* to a 135*S* particle (KAPLAN et al. 1990).

There are also synthetic molecules which allow attachment but prevent uncoating. Rhinoviruses have a deep depression (a 'canyon') on their surface which is believed to interact with the cell receptor. One inhibitor, WIN 52084, fits into the canyon and prevents conformational changes which occur during the uncoating process (ROSSMANN et al. 1987; ROSSMANN 1988).

Conclusion. With the realization that antibodies frequently act in more subtle ways than merely blocking attachment to receptors comes the possibility of modifying antibodies, adapting natural ligands or synthesizing ligands (not necessarily peptide in nature) de novo which will mimic and extend the effects of existing neutralizing antibodies.

22 The Evolutionary Significance of Neutralization Sites

22.1 Why Do Viruses Have Neutralization Sites?

Neutralization sites are regions on the surface of a virus particle which on binding antibody result in the loss of infectivity. Neutralizability is a particular property since many viruses have other, different sites which are immunogenic and bind antibody but do not result in neutralization (see below). Nearly all viruses have neutralization sites and, to the knowledge of this reviewer, only African swine fever virus (HESS 1981; VIÑUELA 1985) and Marburg and Ebola viruses of the Filoviridae (REGNERY et al. 1981) lack the ability to be neutralized. Non-neutralizing virus-specific antibodies are made during infection, or by immunization with these viruses, and it is assumed that infection is controlled by cellular immunity. Loss of neutralizability is known to occur in vivo: after neuroadaptation Sindbis virus retains the ability to bind antibody that neutralizes the original virulent strain but is itself no longer neutralized (STANLEY et al. 1985); the same occurs with poliovirus after attenuation, and reversion is accompanied by a return to neutralizability (CRAINIC et al. 1983). The existence of epitopes which are neutralizing in one strain and non-neutralizing in another has already been mentioned (Sect. 17; Table 4), and may be a reflection of the same phenomenon.

Neutralization sites, the ability to be neutralized and the mechanism of neutralization may confer an evolutionary advantage. Such an idea is reinforced by the evolutionary plasticity of viral antigens, in vitro and in vivo. It argues that neutralization sites provide viruses with a selective advantage; that is, viruses require to be neutralized, for example, to avoid killing the host or disabling it to the extent that it can no longer produce the new generation of susceptibles on which survival of the virus depends. It may be to ensure neutralizability that viruses have multiple neutralization sites—there are four or five sites on most picornaviruses (MINOR et al. 1990; UHLIG et al. 1990) and influenza type A HA also has four or five sites (WILSON et al. 1981; CATON et al. 1982). The nature of the neutralization sites and the mechanism of neutralization is a separate issue: such sites may be unique to the neutralization process or the neutralization process may use sites which are essential to the process of infection and antibodies may compete, block or trigger prematurely a process enacted by a natural ligand.

In general neutralization sites are readily mutable and mutants resistant to the selecting monoclonal antibody arise with ease in vitro. This is not particularly surprising with RNA viruses, which have an error rate in copying

their RNA which lies between 100 000 and 10 000 000 times that of DNA (REANNY 1984). However, many RNA viruses are antigenically stable in nature. Measles virus, for example, is monotypic. Study of island populations indicated that measles becomes extinct unless sufficient new susceptibles are produced, and survival of the virus requires a minimum of about half a million people (BLACK 1966). Evidently, measles is a virus which cannot evolve to escape the protective immune response. Polioviruses types 1, 2 and 3 are others which have remained antigenically stable in the face of immunity generated by a vaccine which has shown no loss of effectiveness over a 30-year period (MINOR 1990). Despite this severe evolutionary pressure there has been (with one exception: see below) no outbreak of escape mutants, implying that these either do not arise or arise but are not selected for in vivo. The former may be the case since the frequency with which escape mutants arise in response to antibody against one antigenic site depends on the mutation rate (around 10^{-5}), but if antibody against two or more sites is present the mutation rate becomes vanishingly small (for antibody against two sites about 10^{-10}; three sites 10^{-15}, etc). Thus escape mutants arise only if the antibody response is to a single site (quasi-monoclonal) or if polyclonal immunity is weak (influenza virus, LAVER and WEBSTER 1968; FMDV, HYSLOP 1965; HYSLOP and FAGG 1965; FAGG and HYSLOP 1966). Alternatively, evolution may follow a relatively rapid circular path so that site X in the presence of anti-X immunoglobulin evolves to site Y, which under the influence of anti-Y evolves back to X. The latter was seen in the poliovirus type 3 outbreak in Finland in 1986, where initial isolates were found to have asparagine in the major neutralization site of VP1 instead of the usual arginine, but within 2 weeks the asparagine reverted to a lysine, a conservative change suggesting that there are strong evolutionary pressures which act to restore the status quo (MINOR et al. 1987; HUOVILAINEN et al. 1987). Possible evidence of circularity in evolution of epitopes is the acquisition by a strain of FMDV of the ability to be neutralized by a mab to a previously existing subtype (HERNÁNDEZ et al. 1992).

Clearly there are evolutionary pressures for conservation of neutralization sites. Contrast this with viruses such as type A and B influenza (WEBSTER et al. 1982; AIR et al. 1990; NOBUSAWA et al. 1991; BEAN et al. 1992), FMDV (HYSLOP 1965; MATEU et al. 1988), HIV-1 (PUTNEY and MCKEATING 1990; ALBERT et al. 1990; SIMMONDS et al. 1990) and other lentiviruses (CLEMENTS et al. 1988; PEDERSEN 1989; RWAMBO et al. 1990) which have neutralization sites that evolve continuously. While the neutralization sites of these viruses vary, other parts of the virion (notably their attachment sites) are necessarily invariant (see below).

22.2 Strategies Which Avoid or Minimize Expression of, or Response to, Neutralization Sites

Such strategies relate to the interaction between the virus particle and the immune system, but for convenience a division is made here between properties which can be attributed to the particle itself and those which primarily concern the immune response.

22.2.1 Relating to the Virus Particle

The persistence of a virus in a individual host or a population in vivo depends in part on its ability to escape the attention of neutralizing antibody. It can do this in a variety of ways so that either no antibody is generated or if antibody is formed it is unable to bind to its epitope (Table 9). This has parallels with the relationship between eukaryotic parasites and their hosts (DAVID 1990).

A virus can lose a neutralization site by a mutation which permits it to be glycosylated. The site is then no longer immunogenic or recognized by existing antibody. For influenza virus, which causes a non-persistent infection, this is seen as an evolutionary device for preserving the integrity of an antigenic site, so that the period over which antigenic variation and survival of the virus in the population is extended (SKEHEL et al. 1984), while for HIV-1 changes in the glycosylation pattern may contribute to spread within an individual host (DAVIS et al. 1990). Loss of a glycosylation site permitted escape from neutralization by NDV (GOTOH et al. 1988) and mumps virus (KÖVAMEES et al. 1990 and see Sect. 12.1).

Aleutian disease virus (Parvoviridae) causes a life-long persistent infection of mink which mount only a feeble neutralizing response. Virus-specific antibody is produced but virus—antibody complexes are infectious (PORTER and CHO 1980). Recently, STOLZE and KAADEN (1987) found that neutralization sites are masked by phospholipids which may interfere with the generation and/or action of neutralizing antibody. In the persistent infection caused by LDV (Coronaviridae) infectious virus persists in the circulation in the presence of neutralizing antibody (ROWSON et al. 1966; NOTKINS et al. 1966; CAFRUNY and PLAGEMANN 1982a) protected by an excess of non-neutralizing antibody. This virus–antibody complex can be neutralized by the addition of anti-IgG (NOTKINS et al. 1968). Presumably non-neutralizing sites of LDV are immunodominant. However, it seems that the species immunized influences the spectrum of antibodies synthesized since rabbits make conventional neutralizing antibody (CAFRUNY and PLAGEMANN 1982b). Neutralizing murine mabs can be made by immunizing with isolated VP3 polypeptide (COUTELIER and VAN SNICK 1988). Non-neutralization sites appear to be destroyed by formaldehyde, since after such treatment neutralization site(s) become immunogenic in mice (HARTY and PLAGEMANN 1988). In another form of

Table 9. Examples of strategies for avoiding or minimizing the expression of neutralization or potential neutralization sites which relate to the virus particle

Strategy	Virus	References
Camouflage by:		
carbohydrate	Rabies virus	Wunner et al. 1985
	Influenza virus	Skehel et al. 1984
	Mumps virus	Kövamees et al. 1990
	HIV-1	Davis et al. 1990
phospholipid	Aleutian disease virus	Stolze and Kaaden 1987
non-neutralizing antibody	LDV	Rowson et al. 1966; Notkins et al. 1966, 1968; Cafruny and Plagemann 1982a
	Mouse mammary tumour virus	Massey and Schochetman 1981b
	TBEV	Heinz et al. 1983b
non-viral protein	HCMV	McKeating et al. 1987
hypermutable epitopes	influenza virus	Wiley et al. 1981; Colman et al. 1983
	FMDV	Fox et al. 1989
	HIV-1	Putney and McKeating 1990; Albert et al. 1990; Nara et al. 1990; Simmonds et al. 1990
	Lentiviruses	Clements et al. 1988
small size	FMDV	Fox et al. 1989
Concealment	Rhinovirus[a]	Rossmann et al. 1985, 1987
	Mengo virus[a]	Luo et al. 1987
	LDV	Harty and Plagemann 1988
Silence	HSV-1	Mester et al. 1990
Destruction by protease	Poliovirus type 3	Icenogle et al. 1986; Minor et al. 1987
Deletion	MHV-4	Parker et al. 1989; Gallagher et al. 1990
	RSV	García-Barreno et al. 1990; Rueda et al. 1991
Loss of a glycosylation site	NDV	Gotoh et al. 1988
Immunodominance	Poliovirus type 3	Minor 1990
	FMDV	
Mimicry of self	FMDV	Fox et al. 1989
'Original antigen sin'	influenza, paramyxo viruses, togaviruses, enteroviruses, HIV-1	Francis 1953; Fenner et al. 1974; Fazekas de St Groth 1969; Nara and Goudsmit 1991; Nara et al. 1991
Detachment	HIV-1	McKeating et al. 1991
Absence	African swine fever virus	Hess 1981; Viñuela 1985
	Marburg virus, Ebola virus	Regnery et al. 1981

[a] Concealment of virus attachment site within a structural depression of the virion.

concealment, HCMV recovered from urine is coated with β_2-microglobulin; such virus resists neutralization by polyclonal and monoclonal antibodies, with or without the assistance of complement (McKEATING et al. 1987).

COLMAN et al. (1983) have suggested that regions of proteins such as the attachment site which are not immunogenic and which must be conserved to provide the means of infection can be hidden amongst hypermutable epitopes. This could apply to influenza virus or FMDV. In influenza virus antigenic sites A, B and D surround the pocket which binds NANA (Fig. 1, WILEY et al. 1981). Paradoxically, the disorganized loop of FMDV consisting of residues 141–160 of VP1 serves as both attachment site and hypervariable region. Possibly the small size of the FMDV attachment site (three amino acids, Arg-Gly-Asp, RGD: FOX et al. 1989) is an adaption to minimal immunogenicity. Alternatively, attachment sites may be concealed in a pit or canyon on the surface of Mengo virus or rhinovirus (LUO et al. 1987; ROSSMANN et al. 1987). This does not appear to be the situation in FMDV, which can be neutralized by antibodies to peptides containing the RGD motif (F. Brown, unpublished, in FOX et al. 1989). Alternatively, as RGD is a common motif in cellular proteins (PIERSCHBACHER and RUOSLATI 1987) the immune system may be tolerized to it and antibodies not synthesized. In effect this is also the situation with HSV-1, although the mechanism by which it is effected is not known: it has an immunologically silent neutralization site on glycoprotein gB but antibodies made against a 20 mer peptide are both neutralizing and protective in vivo (MESTER et al. 1990).

Another device which allows FMDV to escape from neutralization but maintains the sequence of the attachment loop is demonstrated by mab 24.31 which binds to residues 146–150 within the loop and also to residues 200–213. The only amino acids altered in escape mutants were residues 43, 48 and 49 of VP1 but nonetheless the selecting mab no longer bound to the virion (PARRY et al. 1990). Thus even the 'disordered' sequence of 141–160 is affected by mutations elsewhere.

Poliovirus type 3 has a single immunodominant site situated at the pentameric apex of the particle (site 1, Fig. 6) composed of various juxtaposing residues of VP1 (MINOR 1990); 96% of mabs are made to site 1 in mice or rats and only 4% to the other three antigenic sites. [The situation is similar in FMDV, and only recently has it become apparent that there are four antigenic sites rather than just the one neutralization site composed of residues 141–160 of VP1 (KITSON et al. 1990; MINOR 1990).] The type 3 poliovirus immunodominant site is destroyed in the gut, its main site of multiplication, by trypsin acting on a highly conserved arginine residue. Site 1 is then neither immunogenic nor reacts with antibody to the intact site, and concomitant with its destruction, sites 2, 3 and 4 become immunogenic. The significance of the destruction of this neutralization site is not understood but, as mentioned above, its trypsin sensitivity is conserved (MINOR et al. 1987; HUOVILAINEN et al. 1987). A neutralization site can be lost as occurred when about 130 amino acid residues from the N-terminal half of the envelope protein of the

neurotropic coronavirus MHV-4 were deleted (GALLAGHER et al. 1990). The same effect was achieved by a frameshift and subsequent loss of a 25 amino acids from the C terminus of the G protein of RSV (GARCÍA-BARRENO et al. 1990; RUEDA et al. 1991). HIV-1 loses neutralization sites when it sloughs off gp120 but, conversely, such depleted virions are more susceptible to neutralization; free gp120 may, but does not always, interfere with neutralization (MCKEATING et al. 1991).

The lentiviruses all cause persistent infections and interest in their neutralization has surged with the HIV pandemic (see CLEMENTS et al. 1988; PEDERSEN 1989). Equine infectious anaemia virus elicits narrowly reactive neutralizing antibody and escape mutants are found in vivo (RWAMBO et al. 1990). These in turn generate a broadly reactive antibody response which results in a very low level persistent infection. Some neutralizing mabs (IgG and IgA mabs) have been isolated but these act only inefficiently (HUSSAIN et al. 1988). In addition, there can be high titres of antibody to the glycoprotein which neutralize poorly, suggesting that this may have non-neutralizing/blocking activity (O'ROURKE et al. 1988). Caprine (goat) arthritis and encephalitis virus stimulates either no or very little neutralizing antibody but there is a large amount of antibody specific for the envelope proteins. This is presumed to be directed against non-neutralizing epitopes and may block neutralization. Synthesis of neutralizing antibodies in persistently infected goats can be stimulated by immunization with inactivated *Mycobacterium tuberculosis*, which induces a novel population of macrophages (NARAYAN et al. 1984). Visna-maedi virus of sheep occupies an intermediate position and stimulates some neutralizing antibody which results in the formation of some escape mutants. Over a period of years neutralizing antibody is formed to the mutants but it is ineffective in vivo, possibly because the time it takes to neutralize virus (about 15 min) is longer than that needed to infect a cell. Antibody is of low affinity and its presence does not correlate with disease either way (TORFASON et al. 1992).

Lastly, there is the phenomenon of original antigenic sin; this is a term coined for the immune response to influenza virus infections wherein there is a stronger response to (related) antigens experienced at an earlier time than to the antigens being currently experienced (FRANCIS 1953; FAZEKAS DE ST GROTH and WEBSTER 1964, 1966; FAZEKAS DE ST GROTH 1969). This has been observed with togaviruses, paramyxoviruses, enteroviruses (HEARN and RAINEY 1963; VAN DER VEEN and SONDERKAMP 1965; MIETENS et al. 1964) and most recently with HIV (NARA and GOUDSMIT 1991; NARA et al. 1991). Conjecturally, this may be seen as a strategy which diminishes the homologous antibody response and hence avoids neutralization.

22.2.2 Relating to the Immune System

The antibody response to a neutralization site can be one of the targets of immunosuppression or immunostimulation that many viruses influence during infection (NOTKINS et al. 1970; SPECTER et al. 1989). This can be generalized

or specific, and ranges from complete ablation of the antibody response (as in infection with bursal disease virus of chickens) to virus-, protein- or epitope-specific suppression. It may originate with ablation of the helper function of $CD4^+$ T cells, with the B cells themselves or with accessory cells; modulation of the expression of MHC antigens is another possibility. The effect may be directly the result of infection of lymphocytes or indirectly by, for example, affecting cytokine production by other cells. An example of stimulating neutralizing antibodies to caprine arthritis and encephalitis virus by boosting the immune system with killed tubercle bacilli has already been mentioned (NARAYAN et al. 1984). A wide-ranging discussion of mechanisms is outside the scope of this review and the reader is referred to MCCHESNEY and OLDSTONE (1987), SPECTER et al. (1989), RINALDO (1990) and MAUDSLEY and POUND (1991). They are, however, an important dimension of the neutralization process and the example of influenza virus will be discussed briefly below and in Table 10. Immunomodulation by mostly type A influenza virus was reviewed by ROBERTS and DOMURAT (1989). Some of the effects on the immune system require infectious virus but many do not. The effects are usually transient.

In an in vitro system infectious or non-infectious UV-irradiated or heat-inactivated influenza virus inhibited the generation of an antibody response to sheep rbcs, i.e. non-specifically and by an unknown mechanism. The production of antibody to viral antigens was not examined (DANIELS and MARBROOK 1981). Usually, influenza virus infection of lymphocytes, monocytes and macrophages is non-productive and non-lytic; synthesis of viral proteins is dependent upon the degree of activation of the cell and viral proteins are detected only after activation of cells by lectins (BROWNSON et al. 1979; ROBERTS and HORAN 1985). Thus any deficiency in antibody production may result from subtle effects on cell function, including the production of cytokines (PATHKI and POLASA 1987; TINSLEY et al. 1987; HOUDE and ARORA 1989; LARRICK 1989; VACHERON er al. 1990; DEL GOBBO et al. 1990; JAKEMAN et al. 1991). Conversely, the HA of certain subtypes of influenza A virus is a potent non-specific B-cell mitogen (BUTCHKO et al. 1978; ANDERS et al. 1984, 1986) through binding to the MHC class II I–E antigen. Infectivity is not required. Influenza virus has effects upon a wide range of cells within the immune system which could conceivably affect antibody synthesis: polymorphonuclear leucocytes (MARTIN et al. 1981; ABRAMSON et al. 1982; CASSIDY et al. 1988), monocytes (KLEINERMAN et al. 1975; PIKE et al. 1977; ROBERTS and STEIGBIGEL 1978; MATHIES and HOGG 1989), macrophages (KLEINERMAN et al. 1975; PIKE et al. 1977; ROBERTS and STEIGBIGEL 1978), T cells (BLOOMFIELD and MATEER 1919; REED et al. 1972; SCHEINBERG et al. 1976; DEL GOBBO et al. 1990; LAMB et al. 1983; FFRENCH et al. 1989; MORGAN and DIMMOCK 1992) spleen cells (MASIHI et al. 1984) and B cells (splenic, CASALI et al. 1984; lung, MORGAN and DIMMOCK 1992).

Influenza virus also exerts antigen-specific effects on the production of antibody to heterologous viral antigens and on the isotype of immunoglobulin. If an animal is primed and then restimulated by the same virus, the antibody

Table 10. Summary of some effects of type A influenza viruses or constituents there of on various components of the immune system

Cell	Effects	Reference
Polymorphonuclear leucocyte	Abortive virus multiplication, chemotaxis ↓, Phagocytosis ↑	MARTIN et al. 1981; ABRAMSON et al. 1982; CASSIDY et al. 1988
Monocyte	chemotaxis ↓, procoagulant activity ↓, abortive multiplication	KLEINERMAN et al. 1975; PIKE et al. 1977; ROBERTS and STEIGBIGEL 1978; ROBERTS and HORAN 1985; MATHIES and HOGG 1989
Macrophage	Abortive multiplication, chemotaxis ↓, phagocytosis ↓, lung macrophages more susceptible	KLEINERMAN et al. 1976; ROBERTS and STEIGBIGEL 1978; ROBERTS 1982; JENNINGS et al. 1984; RODGERS and MIMS 1981, 1982
'Spleen cell'	Chemiluminescence ↓	MASIHI et al. 1984
'Lymphocyte'	Abortive multiplication	BROWNSON et al. 1979; ROBERTS 1982; ROBERTS and HORAN 1985
T cell	Delayed-type hypersensitivity ↓	BLOOMFIELD and MATEER 1919; REED et al. 1972; KANTZLER et al. 1974
	Suppression ↑	DEL GOBBO et al. 1990
	HA peptide-induced anergy	LAMB et al. 1983; FFRENCH et al. 1989
	Lymphopenia	SCHEINBERG et al. 1976
	Mitogen responsiveness: lung T cells in vivo and in vitro ↓	MORGAN and DIMMOCK 1992
	Neuramidase-specific helper cells	JOHANNSON et al. 1987a, b
B cell	Mitogen-induced antibody synthesis: (spleen and lung) ↓	CASALI et al. 1984; MORGAN and DIMMOCK 1992
	Cell division ↑	BUTCHKO et al. 1978; ANDERS et al. 1984, 1986
	Antibody to sheep rbc ↓	DANIELS and MARBOOK 1981
NK cell	No known effect in vitro or in vivo	CASALI et al. 1984; MORGAN and DIMMOCK, unpublished
Various	IL-1, IL-2, IL-6, TNF, EP	JENNINGS et al. 1984; ROBERTS et al. 1986; PATHKI and POLASA 1987; TINSLEY et al. 1987; HOUDE and ARORA 1989; VACHERON et al. 1990; DEL GOBBO et al. 1990; JAKEMAN et al. 1991

IL, interlenkin; TNF, tumour necrosis factor; EP, erythropoietin.

response to the viral NA is markedly suppressed compared with that to the HA, by HA suppressing the generation of NA-specific T-helper cells (KILBOURNE et al. 1987; JOHANSSON et al. 1987a, b). The precise mechanism is not known. Also, detergent-disrupted virus suppresses the antibody response to whole virus vaccine (JENNINGS et al. 1987). In relation to the immunoglobulin isotype response to HA in mice, HOCART et al. (1988, 1989a, b) found variation according to whether the immunogen was virulent or attenuated virus or purified protein, and according to the route of inoculation, strain of mouse and whether the antibodies were collected from the lung or serum. Dimmock and coworkers found that after intranasal infection of mice there is suppression of the anti-HA response in the lungs but not in the systemic compartment: in the serum there is HA-specific IgG, which is both haemagglutination inhibiting and neutralizing, and anti-NA specific IgG, whereas locally in the lung there is anti-NA but no detectable antibody to the HA (DIMMOCK et al. 1986; MCLAIN and DIMMOCK 1989). This failure in the mouse of serum antibody to penetrate into the lungs illustrates very well how the immune response can sometimes be compartmentalized. Under conditions where infection is modulated by the homologous defective interfering virus, HA-specific antibody suppression is partially relieved: haemagglutination-inhibiting IgG appears in the lung but there is no neutralizing antibody. Presumably this situation reflects control of antibody synthesis at the epitope level. A biased response to different neutralizing epitopes of the HA is also seen in natural human infections (HAAHEIM 1980; NATALI et al. 1981; WANG et al. 1986) and this may be of evolutionary significance in the selection of new strains. In mice, epitope dominance is genetically linked to the MHC haplotype (THOMAS et al. 1987; SMITH et al. 1991).

Conclusion. There appears to be a balance between the ability of virus to be neutralized and its evolutionary survival and this depends upon the nature of the virus–host relationship. Influenza (and other viruses) can influence cognate and other antibody responses in a variety of ways.

23 Neutralization of Poliovirus and Rhinovirus: A Summary

23.1 Introduction

Data relating to the IgG neutralization of poliovirus are discussed in this separate section since they are complex, unclear and sometimes apparently conflicting despite the wealth of knowledge about the antigenic properties of the virus (MINOR 1990) and its structure at the atomic level (HOGLE et al. 1985; FILMAN et al. 1989). Rhinovirus data are from COLONNO et al. (1989). The few data on IgA neutralization (PAGE et al. 1988; MOSSER et al. 1989) and IgM neutralization (CHESEBRO et al. 1968; MANDEL 1967a; SVEHAG and MANDEL 1964; SVEHAG et al. 1967; THOMAS et al. 1986) of poliovirus are discussed in Sects. 9 and 10, respectively. The problems with IgG neutralization are various:

1. Poliovirus has four well-described antigenic sites and recently a fifth has been discovered (UHLIG et al. 1990) (Table 11); all have a strong conformational element and involve residues on more than one of the three major viral proteins VP1, VP2 and VP3. Despite the way in which viral polypeptides interlock, poliovirus antigenic sites have a considerable degree of independence from each other, since exchange of part of a site for a foreign peptide does not alter the antigenicity of other sites (MINOR et al. 1991). Other picornaviruses may have a different antigenic structure as Mengo virus appears to have a single composite site (BOEGE et al. 1991); however, this may be a matter of the degree to which 'separate' sites converge. Poliovirus and other picornaviruses also have linear epitopes which are by definition VP specific (poliovirus, CHOW and BALTIMORE 1982; BLONDEL et al. 1982; DERNICK et al. 1983; EMINI et al. 1983c; WIEGERS and DERNICK 1983; VAN DER MAREL et al. 1983; FMDV, MELOEN et al. 1979; Coxsackievirus B3, BEATRICE et al. 1980). Antigenic sites are located on the surface of the virion as shown in Fig. 6 (HOGLE et al. 1985; ROMBAUT et al. 1990). Interestingly, it seems that it is the molecular context of the site which is important in neutralization rather than the site itself, since poliovirus into which the V3 sequence from gp120 of HIV-1 is engineered (at site 1 of poliovirus type 1) can be efficiently neutralized by the appropriate HIV-1 mabs (DEDIEU et al. 1992).
2. There are at least three different neutralization mechanisms.
3. Sufficient attention has not always paid to quantitation, in particular to (a) the amount of antibody used to bring about neutralization, or (b)

Table 11. Mutations in antigenic variants selected with monoclonal antibodies from poliovirus types 1, 2 and 3 (after Table 1 of MINOR 1990)

Antigenic site[a]	Mutated residue found in[b]		
	Type 1	Type 2	Type 3
1	1097, 1099, 1100, 1101	1093, 1094, 1095, 1096, 1097, 1098, 1099, 1100, 1101, 1105	1089, 1091, 1093, 1095, 1097, 1098, 1099, 1100, 1166
	1144	1174	1253
2	1221, 1222, 1223, 1224, 1226		
	2164, 2165, 2166, 2168, 2169, 2170		2164, 2166, 2167, 2172
3(3A)			1286, 1287, 1288, 1290
	3058, 3059, 3060, 3071, 3073		3058, 3059
4(3B)	2072		
	3076		3077, 3079
5[c]		2039, 2240, 2241, 2242, 2243, 2244, 2245	
		3195, 3196, 3197, 3198, 3199, 3200, 3201, 3202, 3203, 3204, 3205, 3206, 3207	

[a] The former antigenic site nomenclature is in parenthesis.
[b] The initial digit indicates the virus protein and the next three digits the amino acid residue, e.g. 1097 is VP1, residue 97.
[c] A new site reported by UHLIG et al. (1990).

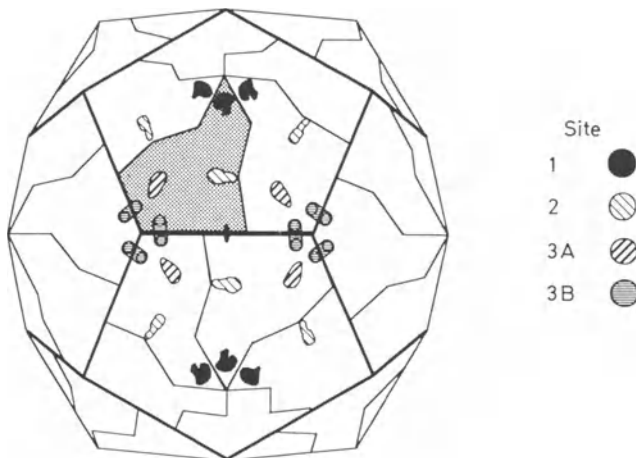


Fig. 6. The approximate location of escape mutations defining nutralization epitopes of poliovirus type 1. Pentamers are drawn with a *bold line* (From ROMBAUT et al. 1990).

whether or not the extent of a functional defect fully accounts for the degree of neutralization observed.

4. There are differences in antigenicity and neutralization between the three serotypes (MINOR 1990; P.D. Minor, unpublished data and see below).
5. Differences in neutralization may depend on the minimum and maximum distance apart of antigenic sites. This may determine whether or not IgG can crosslink sites within one virion or between two different virions (ICENOGLE et al. 1983; MOSSER et al. 1989).
6. The affinity of neutralizing antibodies may be important in determining the nature of aggregation, as suggested by THOMAS et al. (1985), but there are no data.

23.2 Attachment

The ability of mab-neutralized poliovirus to attach to cells is serotype dependent. No mab to serotype 1 inhibits attachment to an extent which accounts for the degree of neutralization observed (EMINI et al. 1983a; P.D. Minor, unpublished data). Mab ICJ27, claimed by EMINI et al. (1983a) to do just that, in fact inhibited attachment by 80% while neutralizing by 99% and even in saturating amounts still allowed 9% of virus to attach. There appears to be a fundamental difference between mabs to poliovirus type 1 and types 2 and 3. Although there are many mabs to types 2 and 3 which do not inhibit attachment, others show a close accord between inhibition of attachment and neutralization (P.D. Minor, unpublished data, and Fig. 7). The reasons for this difference between serotypes is not clear:

- It is not site specific since there are mabs to each of the four antigenic sites of type 3 virus which inhibit attachment.
- It is not amino acid residue-specific as type 3 attachment-permitting mabs and attachment-inhibiting mabs are site 1 specific and select the same mutation in escape mutants (PAGE et al. 1988).
- There is no clear correlation with isotype of IgG.

Antiserum to type 1 also does not inhibit attachment either (HOLLAND and HOYER 1962; MANDEL 1967a; EMINI et al. 1983a), whereas antiserum to types 2 or 3 is attachment inhibiting (KELLER 1966; P.D. Minor, unpublished data, and Table 5). IgG mabs block attachment of rhinovirus 14, and this is effected through each of the four major neutralization sites (COLONNO et al. 1989; see Sect. 2).

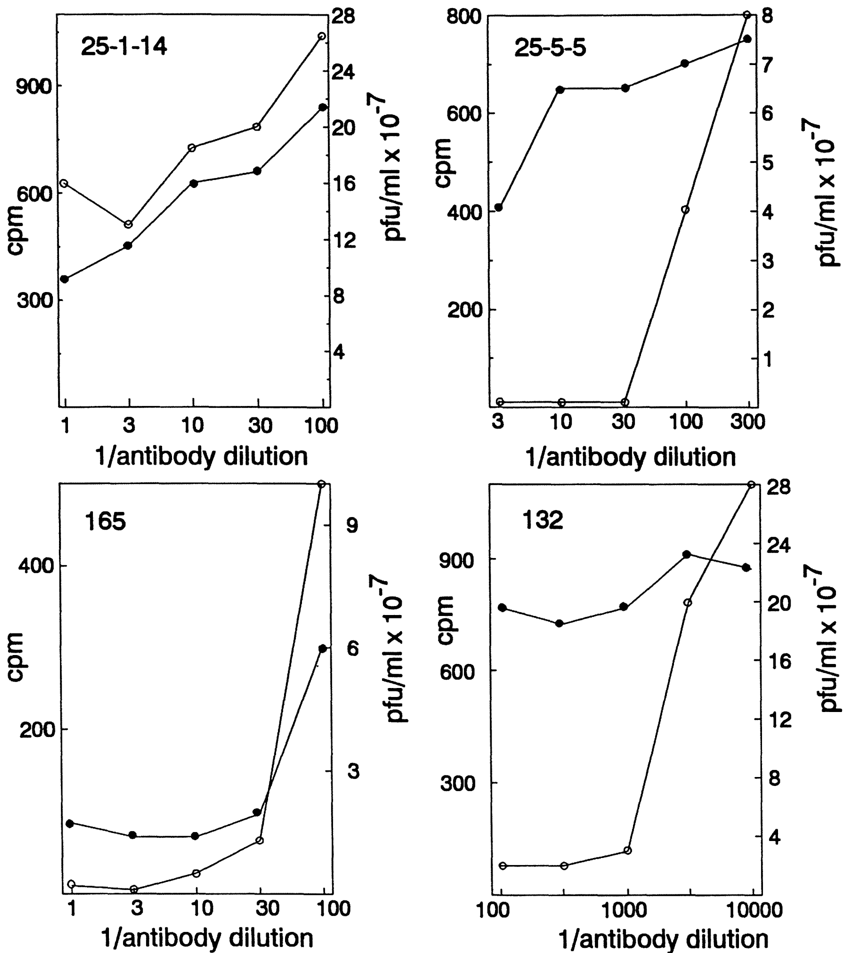


Fig. 7. Some monoclonal antibodies (25-1-14, 165) to poliovirus type 3 neutralize by inhibiting attachment to HeLa cells while others do not 25-5-5, 132). All recognize antigenic site 1. Mabs 132 and 165 were made in mice and 25-1-14 and 25-5-5 in rats. There are mabs to each of the four major sites of polioviruses types 2 and 3 which inhibit attachment to host cells. No mab to poliovirus type 1 is known to inhibit attachment. ●-●, attachment of ^{35}S-methionine-labelled virus to HeLa cells; o-o, neutralization. (Unpublished data of P.D. Minor)

23.3 Internalization

For internalization and later stages of virus entry there are data relating to type 1 virus only. Entry of virus into the cell is difficult to study since a substantial proportion (about 50%) of attached non-neutralized virus spontaneously elutes. This eluted particle is non-infectious and has undergone biochemical (loss of VP4) and antigenic changes. MANDEL (1967b) found

that a smaller proportion of neutralized virus (25%) elutes than of non-neutralized virus (about 50%) and less of it was sedimentable, suggesting that it was more degraded than non-neutralized eluted virus. More intact RNA from neutralized virus ends up inside the cell than from non-neutralized virus. Mandel concludes that antiserum has little effect on entry of the virus into cells. In similar experiments but using urea to strip non-internalized virus from cells EMINI et al. (1983a) find that 32% of the uridine label from attached non-neutralized virus is not internalized. This increases about two fold (63%–77% released) with attached neutralized virus, nowhere near enough to account for 99% neutralization. Interpretation of this result is not straightforward as, after attachment at 5°C, cells were incubated at 37°C before stripping with urea but no measure of virus eluting at 37°C was made and a result like that of MANDEL (1967b) would make the difference between non-neutralized and neutralized virus even less significant. The same set of experiments confirms earlier work by HOLLAND and HOYER (1962) showing that antiserum does prevent entry. Surprisingly, their attempt to mimic this presumably polyclonal effect with a mixture of five functionally distinct monoclonal or monospecific antibodies was not successful.

23.4 Post-internalization

Little has been reported on post-internalization. It would be useful to know if the neutralized virion was uncoated, and what its fate in the cell was. There is no transcription (EMINI et al. 1983b).

23.5 Aggregation

At relatively low IgG:virus ratios many, if not all, mabs aggregate particles, and this alone appears to be responsible for neutralization since monomerization of attached IgG by digestion with papain disaggregates the virus and restores infectivity while leaving Fab still attached (ICENOGLE et al. 1983; THOMAS et al. 1985, 1986). However, there are no quantitative data on the amount of Fab which remains attached after papain digestion and it is not excluded that there is a reduction in Fab to a level which would not be significantly neutralizing even if it were IgG (depending on the mab, 4–15 IgG molecules per virion give 63% neutralization: ICENOGLE et al. 1983; D. Leippe and R.R. Rueckert, personal communication) but would still be enough to react with anti-IgG. This merits consideration as avidity falls 10^3-fold when bivalent IgG is converted into monovalent Fab and some of the mabs are thought to be of low affinity (THOMAS et al. 1985). However, isolated Fab fragments (up to 10^5 per virion) did not neutralize without added anti-immunoglobulin (THOMAS et al. 1985). In contrast, Fabs prepared to each of the four major sites of rhinovirus 14 gave 95% neutralization at 13- to

61-fold greater concentration than their respective IgGs (COLONNO et al. 1989). EMINI et al. (1983b) find that papain digestion restored infectivity after neutralization at low, but not high, antibody concentration; they do not discuss the possibility of incomplete digestion of the IgG. Papain digestion did not restore infectivity neutralized by an antiserum to VP3. Aggregation in itself does not appear to hinder interaction with cells as those IgGs which permit attachment of type 3 virus cause substantial aggregation (P.D. Minor, unpublished data). However, it is conceivable that aggregation leads virus into a degradative pathway after it enters the cell. Care must be taken in extrapolating from the work cited above as most experiments were done with a very low degree of neutralization. There are also conceptual problems with aggregation as the sole reason for loss of infectivity: a priori it seems possible to aggregate 100 virions into a single infectious unit and hence get 99% neutralization, but there is no experimental proof of this and aggregates are variously defined by THOMAS et al. (1986) as '6-mers or greater' or 'virus–antibody complexes sedimenting at 10 000 *g*'. Neutralization of low concentrations of virus (10^3 pfu/ml) by aggregation seems to take place efficiently (THOMAS et al. 1985). However, it seems unlikely that aggregates of 10^4 or 10^5 particles would be sufficiently stable to get the corresponding degree of neutralization, although this is inferred by BRIOEN et al. (1985a). It seems likely that this problem does not arise as particles become monodisperse at high IgG:virus ratios (ICENOGLE et al. 1983; THOMAS et al. 1986) when there are no epitopes available for cross linking particles. Again, this is not a simple situation as mabs differ considerably in this ability (THOMAS et al. 1986): mab 1c disaggregated virus after a very small increase in antibody concentration but mab 1h2 did not disaggregate virus with over a 100-fold increase. Of particular interest are mabs (3, 5 and 8 directed against site 2) which neutralize to a greater extent at low concentration than high concentration: for example 10–100 IgG molecules added per virion mab 5 cause over 99% neutralization but at about 1000 IgG molecules added per virion there is barely any neutralization (MOSSER et al. 1989). However, other site 2 mabs (2, 4 and 7) showed no reduction of neutralization when their concentration was increased. This theme of aggregation efficiency of mabs is taken up again below.

Recent sequence data taken in conjunction with the three-dimensional structure of the virion (HOGLE et al. 1985) have clarified some aspects of the neutralization problem. The majority (38/40) of type 1 mabs directed to sites 2, 3A and 3B neutralize to about 99% and addition of more antibody does not increase neutralization. This is in contrast with mab 1 (previously called F7.12 in ICENOGLE et al. 1983) and mab 9 to site 3A which, under similar conditions, give 99.99%–99.999% neutralization. MOSSER et al. (1989) argue that the distance apart of 3a sites permits IgG to crosslink within one virion. They quote IgG as being able to span 9–15 nm and unable to bind to sites 6 nm or less apart. Paradoxically, both the weak and strong 3A-specific mabs can cause the same escape mutation of Lys to Glu at residue 3060 (PAGE et al. 1988). MOSSER et al. (1988) explain that only if a mab binds in

Table 12. IgG neutralization of type 1 poliovirus

Attachment to virion	Ratio of IgG:virus	Theoretical maximum IgG/virion	Aggregation	pI shift[a]	IgG + papain			IgG + papain + anti-IgG	
					Aggregation	Infectivity	pI shift	Infectivity	pI shift
Mainly univalent, e.g. 35-1f4[4,5],	Low	NA	++[4]	–[4]	–[5,6]	+[5,6]	NA	–[5]	–[5]
site 2	High	60	–[6] to ++[4,6]	–[4]			NA		
Mainly bivalent, e.g. F7.12[3]	Low	NA	±[3,6]		–[3]	+[3]	NA	–[2,3]	+[2]
(now mab 1) site 3	High	30[3]	–[1,3,6]	+[1,(3),4]	NA	+[b]	–[2]	–[b]	

[1] Emini et al. 1983a; [2] Emini et al. 1983b; [3] Icenogle et al. 1983; [4] Brioen et al. 1985a; [5] Thomas et al. 1985; [6] Thomas et al. 1986. Parenthesis around a reference number indicates uncertainty; blanks indicate no information available.

[a] mab 36-5h2 also binds to site 2 (B. Rombaut, personal communication) but has properties intermediate between 35-1f4 and F7.12: it binds stably to single virions, aggregates virus efficiently and causes a pI shift[5,6].

[b] Reference 2 (Emini et al. 1983b) states otherwise, but see text.

NA, not applicable.

a particular orientation is it able to crosslink within one virion; with an alternative orientation this is unlikely and it will have a Fab free to attach to a second particle. Properties bearing on this include affinity, flexibility at hinge and elbow and span of the antibody as well as the topography of the epitope. This then agrees with earlier work which noted the varying abilities of mabs to mediate aggregation (THOMAS et al. 1986; TANIGUCHI and UROSAWA 1987), and THOMAS et al. (1986) noted that mab F7.12 (now mab 1) aggregates virus relatively poorly and forms monomeric complexes at high concentration which saturate at 30 molecules per virion (i.e. all 60 antigenic sites would be occupied). In contrast, THOMAS et al. (1985) suggest that an efficient aggregating mab like 35-1f4 preferentially crosslinks virions. They hypothesized that it is of low affinity and that two virions must be crosslinked by two molecules of mab to create a stable aggregate. THOMAS et al. (1986) supported this suggestion by analysing virus aggregated at a low IgG:virus ratio and showed that while putative oligomers contained both virus and antibody, single virions had no antibody attached. Note, however, that mab F7.12 (now mab 1) bound stably to single virions (ICENOGLE et al. 1983), as did polyclonal IgG (WETZ et al. 1986). Also, at high concentrations of mab 35-1f4 (site 2: B. Rombaut, personal communication) virus only reluctantly dissociates into monomeric complexes (THOMAS et al. 1986; BRIOEN et al. 1983), although this mab can neutralize very efficiently to 99.98% (BRIOEN et al. 1985a). Table 12 summarizes currently available data. Further, there is a difference in the number of IgG molecules bound per virion: with an average of one molecule of IgG mab F7.12 (now called mab 1) per virion there is no significant neutralization and with four to six molecules of mabs 1 or 9 per virion there was 63% (1/e) neutralization (ICENOGLE et al. 1983; D. Leippe and R.R. Rueckert, personal communication). ICENOGLE et al. (1983) also showed that virus populations with an average of two or more IgGs per virion were aggregated in proportion to the loss of infectivity. Thus aggregation contributes at least to the initial loss of infectivity even with this type of antibody. With the majority of mabs (38/40) neutralization to 1/e requires 15 or more molecules per virion (D. Leippe and R.R. Rueckert, personal communication).

23.6 Conformational Changes on Binding Antibody

MANDEL (1976) first demonstrated that neutralizing antisera cause poliovirus to undergo a 1000-fold change in pI from around 7 to a non-infectious form with a pI of about 4. He suggested that the capsid was metastable and was held in the pH4-form after binding antibody. Later, neutralizing, but not non-neutralizing mabs were shown to reproduce this phenomenon (EMINI et al. 1983b) but neither quantitated the relationship between the pI shift and neutralization. Digestion with papain, which restores the infectivity of neutralized virus (see above), also returned the pI to neutrality. Addition of

anti-IgG restored neutralization and a pI of 3 (Table 12; EMINI et al. 1983b; ICENOGLE et al. 1983; THOMAS et al. 1985). However, the general situation is far from clear as a neutralizing anti-VP3-specific serum (confusingly called anti-VP4G) caused no pI shift (EMINI et al. 1983b). Later, BRIOEN et al. (1985a) examined the quantitative relationship between neutralization and pI shift with various antisera and mabs and found the following:

1. A guinea pig antiserum gave a good correlation.
2. Neutralization by mab 35-1h2 (to site 3B: B. Rombaut, personal communication) ran ahead of the pI shift so that with 96% neutralization there was no change in pI but with 99.99% neutralization all virus had a pI of 4.5.
3. Neutralization by mab 35-5h2 (to site 2: B. Rombaut, personal communication) of 10% of virus ran behind the pI shift (64% of virus).
4. Mab 35-1f4 (to site 2: B. Rombaut, personal communication) and a rabbit antiserum gave no pI shift even with 99.98% neutralization. Thus one can conclude that some neutralizing antibodies do not cause a pI shift and that most others only do so only at high IgG:virus ratios.

It could be argued that the pI shift depends on crosslinking certain critical epitopes within a single virion and/or on crosslinking a sufficient number of them. This could be tested by determining if anti-IgG crosslinking of a complex of virus + Fabs derived from a non-pI-shifting mab like 35-1f4 altered the pI (as suggested in Table 12). No pI shift would indicate that the phenomenon was epitope dependent. It would also be of interest to know if the pI shift, where it occurs, shows multiple-hit kinetics or the single-hit kinetics of neutralization. The pI shift is not dependent on isotype since 35-1h2 and 35-1f4 mentioned above are both IgG2a. A neutralizing monoclonal IgM also shifted the viral pI to an acid value (THOMAS et al. 1986).

By contrast, the situation with rhinovirus 14 is clear: neutralizing IgG2a's to each of the four sites acid-shifted the pI from 6.7 to below 3.6. Fabs to each of the four sites also shifted the pI and neutralized, so no crosslinking is required in this system; the quantitative relationship between the pI shift and loss of infectivity was not determined. Papain digestion of IgG2a-neutralized virus restored infectivity and the pI but infectivity of virus neutralized by the mab to site NimII was only partially restored (COLONNO et al. 1989).

It might help to understand the antibody-induced pI shift if the virion underwent a pI shift as part of the normal infectious process. One speculation based on intravirion crosslinking is that the pI shift occurs when the particle binds a sufficient number of cell receptor units. The acid pI conformation of neutralized virions must surely have unique epitopes against which mabs could be raised and used to investigate cells infected with non-neutralized virus. Finally, although mab 35-1f4 gives no pI shift it causes a different putative conformational change which can be detected by incubating neutralized virus in low ionic strength solution at pH 2: virions lose attached anti-

body, develop new epitopes and sediment at 80*S* instead of 160*S*. Non-neutralized virions are stable under these conditions (BRIOEN et al. 1985b). Somewhat similar conformational changes are found when another picornavirus, FMDV, reacts at neutral pH with a mab. These involve permeabilization to negative stain and loss of RNA (MCCULLOUGH et al. 1987).

Conclusion. There are at least three categories of poliovirus neutralizing antibody. By far the commonest aggregates virus efficiently (category 1) and appears to be of low affinity (A.A.M. Thomas, personal communication), but for most of these aggregation is unlikely to be the mechanism of neutralization as virus does not regain infectivity when it is disaggregated at high antibody concentrations. However, a minority of mabs in this category does render virus infectious under these conditions, suggesting that aggregation per se is the mechanism of neutralization. Two mabs only are known which neutralize strongly with little aggregation (category 2). Some mabs inhibit attachment (category 3) but these are type 2 or type 3 virus-specific and not known for type 1; most do not, and neutralization is therefore a post-attachment event. The binding of some antibodies causes profound conformational changes to poliovirus particles which register as changes in their isoelectric point, but at this time the relevance of this phenomenon to any of the three categories of neutralization is not understood. MOSSER et al. (1989) wisely prefer to suspend judgement, commenting that the technology for measuring the pI change is suspect as it requires highly unphysiological conditions of low ionic strength, unusual dipolar ions, an absence of ions usually present in physiological fluids and a strong electric field.

24 Neutralization of Type A Influenza virus by Immunoglobulins M, A and G: A Summary

24.1 Introduction

Influenza virus provides a good system for study of the neutralization of enveloped viruses since the structure of major neutralization antigen, the haemagglutin in (HA), is known at the 2.8 Å level and there are many mabs, the epitopes of which have been located on the HA by escape-mutant mapping (WILSON et al. 1981; WILEY et al. 1981; WILSON and COX 1990). The following discussion is confined to the HA and its antibodies. The NA is covered in Sect. 16. The HA consists of two disulphide-linked polypeptides which are formed by protease cleavage from a single precursor: the transmembrane HA2, which is not involved in neutralization and in the virion is unable to react with antibody (BECHT et al. 1984), and the distal HA1. The HA is a homotrimer with the original C terminus inside the virion. There are five potential neutralization sites per monomer (A–E on H3), although D is partly formed by the adjacent interfaces of the monomer. All are located on the globular head formed by HA1 (Fig. 1). There are fourteen subtypes of type A HA (H1–H14; KAWAOKA et al. 1990) and the crystal structure of H3 only is known. How closely the arrangement of antigenic sites is conserved between subtypes is uncertain but there are significant differences between H3 and H1 (CATON et al. 1982). Strict division into five antigenic sites is not always possible (BROWN et al. 1990) and some would prefer to regard HA1 as an antigenic continuum. All epitopes are discontinuous and few anti-peptide sera have neutralizing activity even though they can react to very high titre by enzyme-linked immunosorbent assay (ELISA). Each HA monomer has an attachment site (i.e. three per trimer) for terminally linked N-acetyl neuraminic (sialic) acid; this site is not immunogenic and no antibodies are made against it. According to estimates with different strains, type A influenza has between 400 and 1000 HA trimers per virion, roughly 10 times more than HIV-1 (Sect. 25).

Type A influenza viruses normally infect mucosal surfaces of the respiratory tract (mainly of man, horse, pig, birds) and the gut (birds). Some avian strains cause generalized infections and are lethal. Secretory IgA predominates here but substantial amounts of IgG and IgM, all potentially neutralizing, are also found in these locations. There may also be a viraemic phase where serum antibody is important. IgM, IgA and IgG have all been studied in regard to their neutralizing properties. Data are summarized in Table 13 and, as the effects of relatively low or high concentrations of immunoglobulin are substantially different, these categories are dealt with separately.

Table 13. Mechanisms of neutralization of type A influenza virus[a]

	None	IgM		IgA[i]		IgG	
		Low[b]	High[b]	Low	High	Low	High
Host cell	BHK, MDCK	BHK	BHK, TOC	BHK, MDCK	BHK, TOC	BHK	BHK, CEF, TOC
Aggregation	–	+[c]	–	+[c']	–	+[c]	–
Inhibition of attachment	–	–	±[d]	–	+	±	–
Inhibition of entry	–	–	+	–	NA	–	–
Inhibition of fusion[e]	–	–	NA	+	NA	+	–[f]
Inhibition of primary uncoating[g]	–	–	NA	+	NA	+	–[f]
Inhibition of secondary uncoating[g]	–(–)[f,h]	–	NA	–(–)[h]	NA	NA	–
Inhibition of tertiary uncoating[f]	–(–)[f]	–	NA	NA(+)	NA	NA	+
Inhibition of transcription	–(–)[f]	–	NA	NA(ND)	NA	NA	+
Inhibition of acquisition of RNase resistance of genome in the cell	–(–)[f]	–	NA	NA(ND)	NA	NA	+
Infectivity	+(+)	+	–	–(–)	–	–	–

[a] See text for references; virus incubated with Ig for 1 h at 25°C.
[b] Relative amount of Ig/virus particle; for IgG 'high' is a saturating concentration.
[c] But only at a narrow window of Ig:virus.
[d] Not sufficient to account for the amount of neutralization observed.
[e] By fluorescence dequenching of R18 inserted into the viral membrane.
[f] Inferred.
[g] By immunofluorescence; after secondary uncoating. NP protein and/or virion RNA are located in the nucleus.
[h] Parenthesis: virus artifically fused to the plasma membrane with polyethylene glycol.
[i] IgA used here is the polymeric form.
BHK, baby hamster kidney; CEF, chicken embryo fibroblast; Madin–Darby canine kidney; TOC, tracheal organ culture from mouse
NA, not applicable; ND, not done.

24.2 IgM Neutralization

Relatively high concentrations of polyclonal IgM neutralize partly by inhibiting attachment to host cells; some neutralized virus attaches to but does not enter the cell (TAYLOR and DIMMOCK 1985a; OUTLAW and DIMMOCK 1990; Sect. 10). Electron microscope studies show that 'low' concentrations of IgM causes aggregates containing a maximum of 4.6 virions, but this occurs only over a narrow ratio of IgM:virus (about seven IgM molecules per virion). However, aggregates break down during pipetting and cause no neutralization. The latter also implies that this amount of IgM, which is sufficient to cover about 10% of the surface area of a virion, is not intrinsically neutralizing (ARMSTRONG et al. 1990). At low concentrations IgM attaches to the surface of virions in the 'staple' or 'crab' conformation. Data show that about 72 molecules of polyclonal IgM per virion is saturating but causes only 66% neutralization. At higher concentrations of applied IgM, more IgM molecules bind to each virion and electron microscope observation suggests that these are in a planar conformation which permits closer packing. At intermediate concentrations there is a mixture of crabs and planar conformations, and it seems likely that this (polyclonal) IgM preparation is not intrinsically neutralizing in the crab conformation, and that virus is neutralized only by bound planar IgM molecules. IgM crabs project no more than 10 nm from the surface of the virus but planar molecules can project up to 30 nm (Table 5); thus the latter have a considerably greater potential for inhibiting attachment by steric hindrance, as experimentation has confirmed (ARMSTRONG et al. 1990). The planar conformation does not fix complement. Since inhibition of attachment requires a certain critical number of IgM molecules per virion, IgM neutralization is expected to be a multiple-hit process, and preliminary work suggests that this is indeed the case (M.C. Outlaw and N.J. Dimmock, unpublished data). So far all IgM neutralization studies have been conducted with polyclonal antibody, and they need repeating with monoclonal IgMs.

24.3 IgA Neutralization

IgA neutralization is more complex and neutralization by polymeric monoclonal IgA depends on the ratio of IgA:virus (Sect. 9). At high ratios of IgA:virus there is no aggregation and attachment to BHK cells is inhibited; attachment to ciliated epithelial cells on organ cultures of trachea is enhanced, presumably through the presence of Fcα receptors, though infectivity is not restored (OUTLAW and DIMMOCK 1990). Inhibition of attachment is a property of polymeric IgA, since virus neutralized with monomeric IgA (produced by reduction of the polymeric IgA) attached to BHK without hindrance (TAYLOR and DIMMOCK 1985a). At a particular window of IgA:virus in the 'low' IgA concentrations category there is substantial attachment of neutralized virus to the host cell, as judged by electron microscopy. The mab used is polymeric,

specific for A/PR/8/34 (H1N1) and binds to site Sb, which equates to site B of the H3 haemagglutinin. Such neutralized virus is internalized by the cell, probably by endocytosis, but is not uncoated. These and other data (OUTLAW and DIMMOCK 1993) suggest that IgA-neutralized virus is unable to undergo primary uncoating by fusion (ARMSTRONG and DIMMOCK 1992). While this appears to be the major mechanism of neutralization under these conditions, it is not the only virus function inactivated by the IgA. This was demonstrated by artificially fusing virus with the plasma membrane of the cell, a procedure which gives normal yields of infectivity from non-neutralized virus. Artificially fused IgA-neutralized virus does not regain infectivity but does undergo secondary uncoating, and its M1 and NP antigens become located in the cytoplasm and nucleus, respectively, as they do when cells are inoculated with infectious virus. Thus, despite the fact that neutralizing antibody bound to the virion is left on the outside of the cell when virus is fused with the plasma membrane, and that the ribonucleoprotein of IgA-neutralized virus is located in the nucleus, no infection is initiated. It appears, therefore, that the IgA is able to affect the function of the viral ribonucleoprotein indirectly. It may inhibit a tertiary 'uncoating' process by which ribonucleoprotein attains the conformation required for transcriptional activity (ARMSTRONG and DIMMOCK 1992). Conceivably, IgA could prevent a signal which is necessary for infectivity from being relayed across the viral envelope, or alternatively transduce a signal via the HA which prevents the infectious process from proceeding (Sect. 17). There is no evidence available at present to decide between these possibilities.

24.4 IgG Neutralization

There are major differences in the mechanism of IgG neutralization at low or at saturating concentrations of IgG. Contrary to intuitive expectation, saturating amounts of IgG to sites A, B or D have no effect on the attachment of A/fowl plague/Rostock/34(H7N7) or its entry into the cell (POSSEE et al. 1982; TAYLOR and DIMMOCK 1985a; OUTLAW et al. 1990); furthermore, the RNA of neutralized virus appears in the nucleus at the same rate and in the same amount as that of infectious virus. Thus, it may be that other processes including uncoating and transport to the nucleus are equally unaffected, but this has not yet been ascertained directly. However, no virion RNA-directed transcription takes place; virion RNA remains resistant to RNase whereas transcriptionally active RNA from non-neutralized virus is RNase sensitive (RIGG et al. 1989). Recent data showing that M1 is removed from the internal core of the virion before the virion ribonucleoprotein can enter the nucleus (MARTIN and HELENIUS 1991a, b) suggest that IgG neutralization does not inhibit secondary uncoating (RIGG et al. 1989) but may prevent tertiary uncoating, as described above in connection with low-concentration IgA neutralization.

Very recent studies suggest that a number of different mechanisms contribute to neutralization by relatively low concentrations of IgG. Data are similar, although not identical, with IgG directed against antigenic sites A, B and D. First, as IgG concentration is increased, there is a small amount of aggregation, but this occurs only occurs at a narrow ratio of IgG:virus (OUTLAW et al. 1990). There is also some inhibition of attachment. With the limited number of mabs tested so far this varies with the strain of virus and with the mabs used: there was more inhibition of attachment of A/PR/8/34 than of A/fowl plague virus/Rostock/34, and A/X31 (H3N2)-specific mabs HC19 and HC100 caused 50% and 95% inhibition of attachment, respectively (OUTLAW and DIMMOCK 1993). Finally, IgG inhibits acid-dependent endocytic fusion of neutralized virus internalized by BHK cells, these data being inferred from the dequenching of fluorescence from octadecyl rhodamine B chloride (R_{18}) inserted into the lipid envelope of the virion (OUTLAW and DIMMOCK 1993). Alone none of these mechanisms can account for the observed neutralization. In the study by OUTLAW et al. (1990) the theoretical amount of neutralization of A/FPV resulting from aggregation and inhibition of attachment added together (assuming that these act independently), accounted for the observed neutralization of only one of the three mabs investigated (HC61 to site D) and only when the virus was neutralized by 90% or less. A similar theoretical calculation summing data on inhibition of attachment and inhibition of fusion could account for up to 63% neutralization with all virus strains and mabs studied (OUTLAW and DIMMOCK 1993). With higher amounts of mab and more neutralization, loss of infectivity far exceeded that accountable for by effects on aggregation, attachment and fusion for seven of the nine combinations of virus strain and mab investigated, while with the other two combinations, fusion fell below a level that could be quantified. Thus it is suggested that at least one other mechanism of neutralization, such as that described by RIGG et al. (1989), is operating simultaneously.

24.5 Discussion

Even with this body of knowledge there remains much which is still not understood about neutralization of type A influenza viruses. For example:

1. Despite the differences between the mechanisms of IgG-, IgM- and IgA-neutralization listed above and in Table 13, we cannot be sure that these reflect unique properties of the immunoglobulin isotypes since the immunoglobulins differ also in paratope. We need to compare neutralization by families of immunoglobulin isotypes each with the identical paratope (Sect. 21.1).
2. With IgM, IgA and IgG they are major differences in the apparent mechanism of neutralization at 'low' and 'high' amounts of immunoglobin but what, if anything, is the biological significance of this? Intuitively, it

would seem that low-dose neutralization is most likely to occur under physiological conditions, but it is conceivable that a virion remaining for sufficient time in a low concentration of high-affinity antibody would be neutralized, by what is defined in Table 13, as high-dose neutralization.

3. Experimental evidence shows clearly that high concentrations of IgG do not inhibit attachment of neutralized virus to most types of cell, and that low concentrations of IgG do inhibit attachment to some extent. Low concentrations of IgG may be more effective in blocking attachment because individual IgG molecules have more space between them and hence more rotational freedom, whereas at high concentration they are packed together in a paracrystalline array; this hypothesis requires testing experimentally.
4. It can be concluded tentatively that low, but not high, concentrations of IgG inhibit fusion of virion and endocytic membranes. Perhaps the discussion in point 3 provides a clue as to why this is so.
5. An alternative explanation to the paradox presented in point 3 may reside in the fact that at low concentrations of IgG it is likely that molecules of IgG will bind bivalently to the virion. IgG probably crosslinks HA trimers as bivalent attachment of IgG to sites within a single trimer of purified HA rosettes is never seen (WRIGLEY et al. 1977). The ability of a mab to crosslink HA trimers will depend on the position of the epitope and the flexibility of the IgG isotype (WILEY et al. 1981; BURTON 1990). There appears to be steric hindrance of IgG attachment at high concentrations as saturation is achieved with one IgG molecule per HA trimer (TAYLOR et al. 1987).
6. With regard to point 5 there is little information about the ability of Fab fragments to neutralize influenza virus. Fab fragments from a polyclonal antiserum were neutralizing but underwent rapid dilution dissociation (LAFFERTY 1963b). It would be of interest to determine the mechanism of neutralization by Fabs derived from a high-affinity mab, where dissociation would be minimal.
7. A problem of generalizing about neutralization, even with viruses as similar as those of influenza type A, is that virus strains differ, at least in degree, with regard to the effects of a particular isotype of neutralizing antibody (OUTLAW and DIMMOCK 1993).
8. Several mechanisms of neutralization can operate simultaneously; even with monoclonal IgG these include aggregation, inhibition of attachment of the host cell, inhibition of fusion and inhibition of tertiary uncoating. Of these aggregation and inhibition of attachment are immunoglobulin concentration dependent and hence transient. Figure 8 gives a hypothetical picture based on neutralization by aggregation, inhibition of attachment and inhibition of tertiary uncoating; inhibition of fusion is omitted only for the sake of simplicity and does in fact contribute to neutralization (OUTLAW and DIMMOCK 1993).

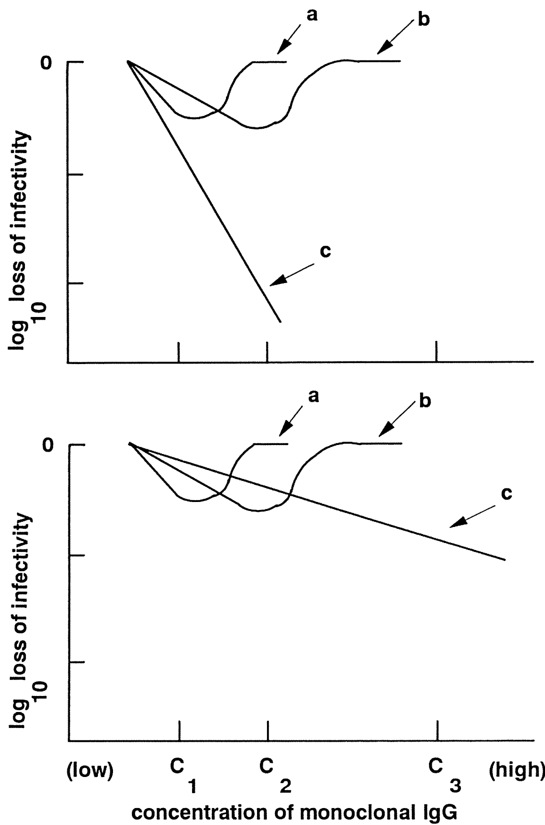


Fig. 8. Hypothetical examples of how type A influenza virus could be affected by different concentrations of neutralizing monoclonal IgG. Loss of infectivity is due to: (*a*), aggregation of virions, which is transient and disappears at higher IgG concentrations (OUTLAW et al. 1990); (*b*), inhibition of attachment, which is also transient and disappears at higher IgG concentrations (OUTLAW et al. 1990); and *c*, inhibition of tertiary uncoating (Sect. 17; RIGG et al. 1989). In the *upper panel* at IgG concentration C_1, aggregation, inhibition of attachment and inhibition of tertiary uncoating contribute to neutralization, and will be cumulative but possibly not strictly additive, Neutralization at IgG concentration C_2 is due to inhibition of attachment and inhibition of uncoating; at IgG concentration C_3, neutralization is solely due to inhibition of tertiary uncoating. In the *upper panel* the overall neutralization curve would be determined primarily by curve *c* and the effect of the other inactivation processes would be minimal.

In the *lower panel* inhibition of tertiary uncoating has a smaller inhibitory effect on infectivity; neutralization at C_1 is due primarily to aggregation and at C_2 to inhibition of attachment, and only at IgG concentration C_3 does tertiary uncoating become the sole cause of neutralization. The simplest view is that the overall neutralization curve would be determined by the sum of the losses of infectivity resulting from the various inactivation processes.

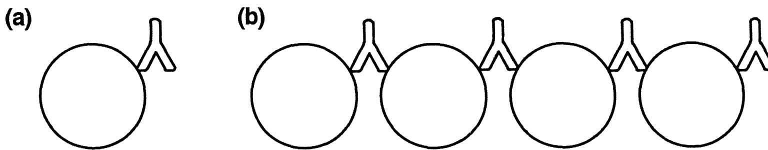


Fig. 9a,b. Neutralization caused by aggregation of virions by amounts of immunoglobulin which are not intrinsically neutralizing. **a** No neutralization when an immunoglobulin molecule binds to a non-critical site; **b** 75% neutralization when four infectious virions are aggregated to form one infectious unit. Although a bivalent antibody is shown the mechanism is equally applicable to polymeric imunoglobulins

9. The kinetics of neutralization present another problem: neutralization follows pseudo-first-order kinetics where one antibody molecule per virion is potentially neutralizing (OUTLAW and DIMMOCK 1991) and any theory of neutralization has to explain how one molecule of antibody can cause virus to lose infectivity. One IgG molecule per virion could reduce infectivity by aggregation, providing each IgG molecule cross-linked a second virion (Fig. 9). However, it is not clear how one molecule of IgG per virion, which is slightly smaller than an HA spike, could inhibit attachment to a cell of a virus which has 1000 HA spikes, each with three attachment sites. A priori it would seem that inhibition of attachment is a multiple-hit phenomenon. Reconciliation of these conflicting data could be achieved if the predicted shoulder of the multiple-hit attachment-inhibition curve was concealed by the loss of infectivity caused by different but simultaneous neutralization reactions. However, despite the one-hit nature of neutralization, neutralizing IgG can attach to virions without causing neutralization (to 'non-critical' or 'non-neutralization' sites, see Sect. 20), and when neutralization is at 1/e there are around 70 IgG molecules per virion (TAYLOR et al. 1987). Inevitably this will facilitate a multiple-hit process like inhibition of attachment. There are similar problems in accounting for neutralization by inhibition of fusion since each HA trimer or possibly each monomer has fusion activity. Thus, one IgG molecule can bind to only two spikes and, as there are about 1000-spikes/virion, it will inhibit at most 2/1000 or 0.2% of total fusion activity. Neutralization by fusion cannot therefore be a single-hit process, and the argument above in regard to inhibition of attachment could be applicable here.
10. Neutralization by one molecule of IgG per virion can be explained if IgG acts as a trigger, and it has been suggested that this is how influenza is neutralized by high concentrations of IgG (POSSEE et al. 1982; RIGG et al. 1989; see the discussion of ARMSTRONG and DIMMOCK 1992). If, in fact, this occurred at all concentrations of antibody, it would be the primary mechanism of neutralization, and on this other ways of inactivating virus infectivity (aggregation, inhibition of attachment, inhibition of fusion) are superimposed (see Fig. 8a). There are a number of possible scenarios:

for example, while the 'trigger' mechanism of inhibition of tertiary uncoating is single hit and takes place at all antibody concentrations, other mechanisms may neutralize to a greater extent at a given concentration of antibody (aggregation or inhibition of fusion in Fig. 8b), and aggregation at least is dependent on antibody concentration and has no effect at higher antibody concentrations. Thus, the different mechanisms of neutralization may be cumulative if, for example, some virus–antibody complexes are not intrinsically neutralized by the trigger mechanism but become part of an aggregate. Alternatively, a virion neutralized by the trigger mechanism may also be prevented by antibody from fusing with a cell and is thus neutralized 'twice over'. It can be shown experimentally by artifically fusing the virus to the cell that inhibition of fusion is not the sole cause of the loss of infectivity (ARMSTRONG and DIMMOCK 1992), but it is not a simple matter to determine which mechanism is *primarily* responsible for the loss of infectivity.

25 Neutralization of HIV-1: A Summary

The pressing need to devise a vaccine against HIV-1 fuels an intensive and ongoing study of its neutralization. The envelope protein has been sequenced and a structure computed (RATNER et al. 1985; MODROW et al. 1987; LEONARD et al. 1990) but understanding of functional relationships awaits its crystallization and determination of its atomic structure (see reviews by MCKEATING and WILLEY 1989; PUTNEY and MCKEATING 1990). The envelope protein is heavily glycosylated (about 50% of its mass is carbohydrate) and is post-translationally cleaved into the anchor, gp41, and a non-covalently linked distal segment, gp120. Two or four of these units form the mature spike protein (THOMAS et al. 1991) and there are 70–80 spikes per virion (GELDERBLOM et al. 1987). By comparing amino acid sequences of seven different strains, MODROW et al. (1987) identified five variable (V) and six constant (C) regions interspersed throughout gp120 and gp41. These and some information relevant to neutralization are shown in Figs. 2 and 10. The use of synthetic peptides to elicit or identify reactive antibody has located 12 possible antigenic sites, ten of which are neutralizing, although some of these are probably part of the same discontinuous site (Table 14; Fig. 10). Non-neutralization sites are also known on gp120 and gp41 (e.g. BANAPOUR et al. 1987; LASKY et al. 1987; LINSLEY et al. 1988; KINNEY-THOMAS et al. 1988; ARDMAN et al. 1990; LARCHER et al. 1990; TEEUWSEN et al. 1990; ROBINSON et al. 1991; XU et al. 1991) and include epitopes on the neutralization immunodominant V3 loop (LANGEDIJK et al. 1991; LAMAN et al. 1992). Attention in regard to neutralization has concentrated on the V3 loop (Fig. 2b) which is concerned with the entry process and, in particular, the fusion of viral and cellular membranes. Antibodies to V3 do not inhibit attachment to CD4, the cell receptor (see Sect. 3), although V3 peptides bind to a site adjacent to the main high-affinity binding site on CD4 (AUTEIRO et al. 1991), but may prevent fusion and protease cleavage of V3 (Sect. 5.2). V3 is also immunogenic in human infection and has been implicated in protection against infection in primates (EMINI et al. 1990; BERMAN et al. 1990; GIRARD et al. 1990). However, V3 peptides do not absorb most of the neutralizing antibodies from human sera (JAVAHERIAN et al. 1990). It is striking that so many sites can be defined by peptides whereas in influenza virus, which is also enveloped, no neutralization sites have been so identified. In addition to these at least partially linear sites, there are conformational sites (Table 14). Mabs to at least one of these epitopes competes with the binding of gp120 to neutralizing, attachment-inhibiting antibodies found in human post-

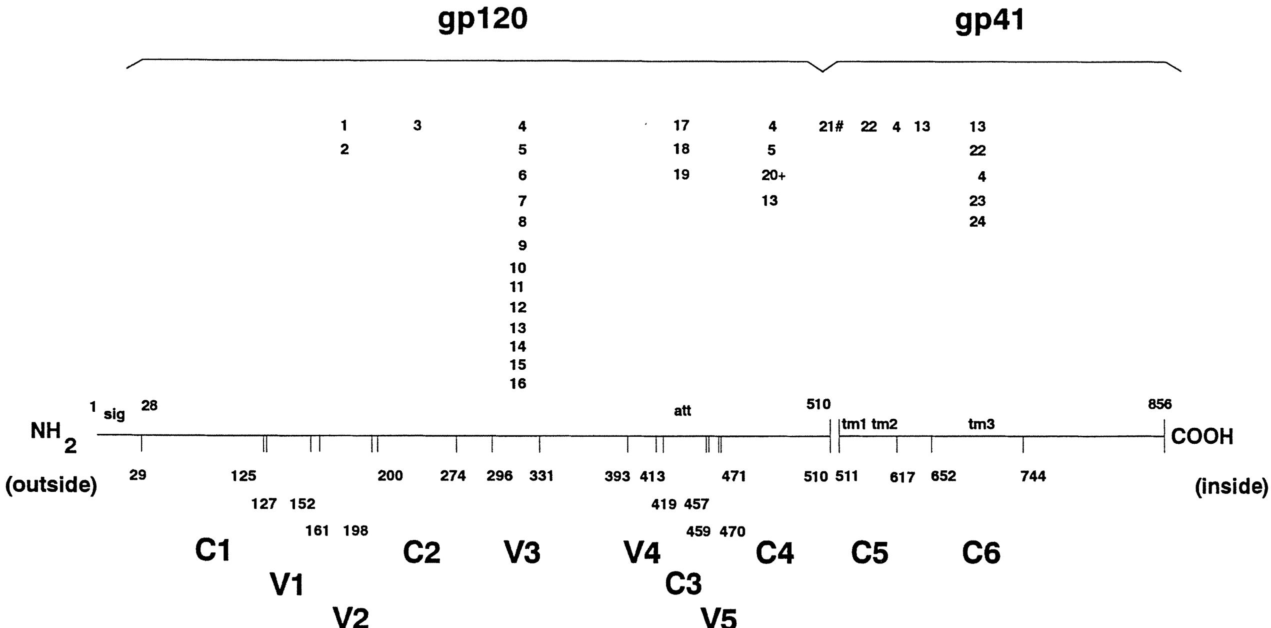


Fig. 10. Representation of the linear, or partly linear, neutralization sites of HIV-1 gp120–gp41, based on the data of RATNER et al. (1985) and MODROW et al. (1987). +, refers to peptide 342–510, i.e. from V3 to the end of C4. #, refers to peptide 503–532 which overlaps the protease cleavage site but is mainly in gp41. *sig*, N-terminal signal sequence 1–28; *att*, part of attachment site; *tm1–3*, putative transmembrane anchoring regions; *V, C*, regions of variable and constant amino acid sequence, respectively. Numbers in columns refer to references which describe neutralizing antibodies specific for the sequence of gp120-gp41 immediately below the column.
1, FUNG et al. 1992; *2*, HO et al. 1991b; *3*, HO et al. 1988; *4*, HO et al. 1987; *5*, KINNEY-THOMAS et al. 1988; *6*, LINSLEY et al. 1988; *7*, GOUDSMIT et al. 1988c; *8*, MATSUSHITA et al. 1988; *9*, SKINNER et al. 1988; *10*, FUNG, unpublished in SUN et al. 1989. *11*, MCKEATING et al. 1989; *12*, NARA et al. 1990; *13*, BROLIDEN et al. 1990; *14*, HU et al. 1991; *15*, BROLIDEN et al. 1992; *16*, LAMAN et al. 1992; *17*, MICHEL et al. 1988; *18*, SUN et al. 1989; *19*, CORDELL et al. 1991; *20*, BERKOWER et al. 1991; *21*, CHANH et al. 1986; *22*, SCHRIER et al. 1988; *23*, EVANS et al. 1989; *24*, DALGLEISH et al. 1988

Table 14. Summary of the currently known antibodies which recognize conformation-dependent[a] neutralization sites of the HIV-1 envelope protein

Polypeptide	Designation	Other information	Reference
gp120	Human anti-serum	Abs not stimulated by hyperimmunization	HAIGWOOD et al. 1990
	ICR39.13g, 39.4b	Rat mabs; compete with 15e	CORDELL et al. 1991
	ICR41.1i	Rat mab	J. Cordell (personal communication)
	15e	Human mab; inhibits binding of soluble gp120 to CD4	HO et al. 1991a
	G3–4	Mouse mab to part of V2; does not compete with 15e; discontinuous	HO et al. 1991b
	F105	Human mab; discontinuous epitope includes aa's 256, 257 + 368–370 + 421 + 470–484	POSNER et al. 1991; THALI et al. 1991
	S1-1	Human mab	LAKE et al. 1992
gp41	5–21–3	Mouse mab; continuous epitope	HUNT et al. 1990

[a] Epitopes which are destroyed if protein is denatured and/or reduced; note that other epitopes, notably V3, have a conformational element but antibodies to these recognize peptides also.

infection sera (P.J. Klasse and J.A. McKeating, unpublished data, reported in CORDELL et al. 1991). HAIGWOOD et al. (1990) also found neutralizing antibodies to a conformational site in human sera. Other attachment-inhibiting antibodies are found in human and animal sera (HO et al. 1988; FIELDS et al. 1988). The best interpretation of these data at the time of writing is that the CD4 attachment site is conformational (OLSHEVSKY et al. 1990) and most mabs made so far do not affect attachment. Amino acids 426–437 are part of the attachment site (LASKY et al. 1987) but this region is not strongly immunogenic during human infection (SUN et al. 1989); mabs which can be made against it are mostly non-neutralizing (Sect. 2), although one neutralizes to over 80% at 200 ng/ml (SUN et al. 1989). Vaccines which induce attachment-inhibiting antibodies are an urgent priority, but reports that HIV-1 infects cells via receptors other than CD4 (MCKEATING and WILLEY 1989) make it essential to ascertain if the virion has other attachment sites which might require antibodies of a different specificity (see Sect. 7). Neutralization by gp120-specific Fabs implies that crosslinking or aggregation of virions is not essential for the activity of the original antibodies (BARBAS et al. 1992). Complement is reported to enhance or not to enhance neutralization (Sect. 15). The conformational site studied above by HO et al. (1991a) and CORDELL et al. (1991) is dependent on glycosylation for its integrity; at least some other (linear) sites are not and non-glycosylated forms of gp120

induce (PUTNEY et al. 1986) or recognize (KINNEY-THOMAS et al. 1988) neutralizing antibody (Sect. 12.1). On the other hand, it is suggested that mutational acquisition or loss of a glycosylation moiety can, respectively, hide or reveal antigenic sites (Sect. 22.2.1; DAVIS et al. 1990). Synergistic neutralization by mabs to different sites on gp120 has been reported (TILLEY et al. 1992; BUCHBINDER et al. 1992; Sect. 17) and may be of importance to vaccine design. In addition, there is concern that non-neutralizing antibody and subneutralizing amounts of neutralizing antibody could facilitate infection (Sect. 8). gp41 anchors the envelope protein in the viral membrane and has five possible neutralization sites: there is uncertainty about the structural organization of gp41 since the sequence on the C-terminal side of the third transmembrane region is most likely inside the virion, according to MODROW et al. (1987), but contains a neutralization site identified by a number of different antibodies (see Figs. 2, 10). These antibodies also give cell-surface labelling (CHANH et al. 1986). Either part of the C-terminal tail is outside the virion or it is in equilibrium between the two orientations (MODROW et al. 1987). There is evidently sufficient space between spikes for this site to bind bulky IgM molecules (Sect. 10; DALGLEISH et al. 1988). There are also conformational neutralization sites in gp41 (HUNT et al. 1990).

Yet another neutralizing epitope on gp120 mimics an epitope common to HLA-A, -B and -C. This HLA epitope is present on the cell surface in a cryptic form until cells are activated; reaction with the mab (M38) inhibits antigen presentation by human peripheral blood mononuclear cells (GRASSI et al. 1991). The significance of this cross-reaction to neutralization, immunosuppression and AIDS remains to be elucidated. Antibodies to the core protein, p17, neutralize free virus, suggesting that an element of p17 is exposed to the exterior surface of the particle (PAPSIDERO et al. 1988, 1989; SHANG et al. 1991). This recalls earlier data on neutralization of defective spleen focus-forming virus by antiserum to $p45^{gag}$ (ANAND et al. 1981).

Conclusion. The distal part of the HIV envelope protein, gp120, has several neutralization sites, with the site on the V3 loop being immunodominant. Antibodies to V3 block fusion of viral and cellular membranes. The membrane-anchored envelope polypeptide gp41 possesses conserved neutralization sites but the mechanism of neutralization is not known. At present there are difficulties in raising antibodies to the attachment site which can prevent virions from binding to CD4 receptors; such antibodies are found in post-infection sera and would ideally be those induced by a vaccine.

26 Conclusions

1. There is no unifying mechanism of neutralization that is applicable to all viruses.
2. Each virus is neutralized by a mechanism which is determined by the permutation of components relevant to the situation under study. This will involve at least some of the following: the virus, the neutralization antigen, the epitope, the paratope, the isotype of immunoglobulin, the number of immunoglobulin molecules per virion and the cell receptor. Ideally, a neutralization mechanism should be defined with respect to all these parameters.
3. The primary mechanism of neutralization should be ascertained by determining the quantitative relationship between loss of infectivity and increasing antibody concentration.
4. However, there may be no single neutralization mechanism and the observed neutralization may result from several simultaneous antibody-induced inactivation processes acting additively.
5. The mechanism(s) of neutralization may change solely as a function of the anitbody:virus particle ratio. Thus, it should not be assumed that the same mechanism operates at both low and high ratios and it is necessary to examine the situation over a full range of values, using very low to saturating concentrations of antibody.

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