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

N. J. Dimmock, *Neutralization of Animal Viruses*

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 intra-endosomal 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.