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

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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.