

# High and low efficiency neutralization epitopes on the haemagglutinin of type A influenza virus

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The relationship between the efficiency of the neutralization process and the affinity of five monoclonal IgG antibodies specific for the haemagglutinin of type A influenza virus has been investigated by determining their neutralization rate constants ( $K_{\text{neut.}}$ ) and affinities ( $K_{\text{dissoc.}}$ ). We addressed the hypothesis that if antibody affinity alone determined the efficiency of neutralization, then the  $K_{\text{neut.}}:K_{\text{dissoc.}}$  ratio would be the same for all antibodies. However,

we found that the  $K_{\text{neut.}}:K_{\text{dissoc.}}$  ratio varied by up to 125-fold, suggesting that properties unique to the epitope are of major importance in determining the efficiency of neutralization. These data suggest that vaccines should preferentially stimulate antibodies to epitopes that mediate the most efficient neutralization, and that a high  $K_{\text{dissoc.}}$  should be an important but secondary consideration.

## Introduction

Type A influenza virus causes near-annual epidemics of respiratory disease in the human population which result in severe economic losses and serious mortality in certain high-risk groups (mainly the over-65s and those suffering chronic conditions of the heart, lungs and kidneys). The frequency of influenza epidemics arises from the continual evolution of the major virus surface glycoproteins, the haemagglutinin (HA) and the neuraminidase (NA), under the selective pressure of neutralizing antibody which renders immunity, gained from infection, ineffective within about 4 years. In addition, there are irregular pandemics of influenza resulting from the emergence of new sub-types of virus having an HA (and sometimes NA) never before experienced by the current human population. There were pandemics of H1N1 virus in 1918, H2N2 virus in 1957 and H3N2 virus in 1968. The spread of the virus in an immunologically virgin population causes widespread illness which, in the 1918 pandemic, reached cataclysmic proportions and killed 20 million people worldwide in 1 year. The main reservoir of influenza A viruses is not man but aquatic birds,

and these are host to 126 subtypes, most of which have not yet crossed the species barrier into man. In addition, influenza viruses are an important economic threat to the poultry and horse-racing industries; they also infect pigs, which may act as a half-way house in the transfer of influenza virus genes into the human population (Kilbourne, 1987; Webster *et al.*, 1992).

Major protective immunity to influenza is thought to be mainly mediated through neutralizing antibody specific to the viral HA, although CD8<sup>+</sup> T cell immunity is probably important in recovery from infection (Wells *et al.*, 1981; Kilbourne, 1987; Askonas *et al.*, 1988; Webster *et al.*, 1992). Wiley *et al.* (1981) defined five neutralization antigenic sites on the crystal structure of the HA of an H3 subtype virus, but it is uncertain if these are discrete sites or represent the immunodominant regions of an antigenic continuum. Current prophylaxis is via an inactivated vaccine which is administered parenterally. This gives 60–90% protection through stimulating systemic neutralizing antibody (Potter, 1994). A vaccine which gives better protection is clearly desirable, and this is likely to be achieved by stimulating a local respiratory tract immunity which preferably gives some measure of cross-protection.

One of the current problems in assessing antibody immunity is that there is no information on the efficacy of neutralization epitopes and little on the importance of antibody affinity. However, it is widely assumed that neutralization epitopes are of equal efficiency, and that antibody affinity is the

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major determinant of neutralization. Here we have investigated the relationship between neutralization and affinity by determining and comparing the ratio of the neutralization rate constant ( $K_{\text{neut.}}$ ) (used here as a measure of neutralization efficiency) and the affinity ( $K_{\text{dissoc.}}$ ) of each of a panel of five neutralizing MAbs directed to three of the antigenic sites present on the haemagglutinin. If efficiency of neutralization is determined solely by antibody affinity, then the ratio of neutralization:affinity should be constant for all antibodies. Our data showed that although the best neutralizing antibodies had the highest affinity, the ratio of  $K_{\text{neut.}}:K_{\text{dissoc.}}$  varied by 125-fold, suggesting that in this system the epitope itself is the pre-eminent determinant of neutralization, and that affinity of the antibody is an important, but secondary, consideration.

## Methods

■ **Virus and cells.** Influenza virus A/fowl plague/Rostock/34 (H7N1) was grown in embryonated chicken's eggs. Virus was purified from allantoic fluid by differential centrifugation and rate zonal centrifugation on gradients of sucrose (Kelly & Dimmock, 1974). Aliquots were stored at  $-70^{\circ}\text{C}$ . Infectivity was determined by plaque assay under agar on monolayers of MDCK cells.

■ **Antibodies.** Five neutralizing mouse monoclonal IgG antibodies were used. All have been mapped to three antigenic sites by serology of antibody escape mutants and by sequencing the HA gene of the mutants (Sugrue *et al.*, 1990). These sites correspond exactly with those proposed for the H3 crystal structure: antigenic site A, amino acid 128 (MAb HC3W; IgG2b) and amino acid 144 (MAb HC2; IgG2a); site B, amino acid 161 (MAb HC10; IgG2a); site D, amino acid 198 or 250 (MAb HC58; IgG2a) and amino acid 210 (MAb HC61; IgG2a). Hybridomas were grown using pretested batches of foetal calf serum which contributed less than 15% to the total immunoglobulin present. MAbs were purified from tissue culture fluid on protein A (Ey *et al.*, 1978) covalently bound to acrylic beads (Sigma) and the protein concentration was measured by a dye-binding assay (Bio-Rad).

■ **Rate of neutralization.** The rate of neutralization was determined by assaying the loss of infectivity over time (Dulbecco *et al.*, 1956). Virus (800 p.f.u./ml) and MAb were incubated together at  $37^{\circ}\text{C}$  for up to 2 h, and samples taken initially at 5 min intervals and then every 15 min. The rate of neutralization was derived from the slope of the line from a plot of  $\log_{10} V_t/V_0$  versus time ( $t$ ), where  $V_0$  is the infectivity titre at time zero, and  $V_t$  is the titre after incubation for time  $t$  at  $37^{\circ}\text{C}$ . The neutralization rate constant ( $K_{\text{neut.}}$ ) was calculated using equation 1:

$$K_{\text{neut.}} = -[(\log_{10} V_t/V_0)/t]2.3D \quad (1)$$

where  $D$  is the reciprocal of the molar concentration of antibody.

■ **Antibody affinity.** Antibody affinity was represented by the antibody dissociation constant ( $K_{\text{dissoc.}}$ ), and was determined by ELISA (Friguet *et al.*, 1985) using whole virus particles. In brief, a known amount of antibody was allowed to react in solution to equilibrium with a range of antigen concentrations (16 h at  $20^{\circ}\text{C}$ ). For the capture phase, this equilibrium mixture was incubated for 90 min at  $37^{\circ}\text{C}$  with a virus-coated microtitre plate to determine the unbound fraction of antibody. Antigen ( $8 \times 10^{-9}$  M) was in at least 100-fold molar excess over antibody

(e.g. HC2 =  $1.2 \times 10^{-11}$  M maximum). The plates were then washed and the amount of MAb bound to the plate was determined by ELISA. The relationship between antibody concentration and the ELISA absorbance was linear (data not shown).  $K_{\text{dissoc.}}$  is perceived as a better measure of affinity than association, since only  $K_{\text{dissoc.}}$  takes no account of whether or not the antibody binds bivalently, a property that depends on the spatial separation of epitopes as well as the angle of the epitope to the paratope.  $K_{\text{dissoc.}}$  values were derived from Scatchard plots modified according to Stevens (1987):

$$\frac{\sqrt{\frac{A_0 - A}{A_0}}}{a_0 - i_0 \times \sqrt{\frac{A_0 - A}{A_0}}} = \frac{1}{K_{\text{dissoc.}}} \times \left(1 - \sqrt{\frac{A_0 - A}{A_0}}\right) \quad (2)$$

where  $A_0$  is the maximum ELISA absorbance of input antibody on the virus-coated microtitre plate, and  $A$  is the ELISA absorbance of the unbound fraction of antibody in the equilibrium reaction. The values for  $a_0$  and  $i_0$  are, respectively, the concentrations of input antigen and input antibody (M) in the equilibrium reaction. For the plot, the ordinate is the left-hand side of equation 2 (abbreviated as  $\Upsilon$ ), and the abscissa is  $\sqrt{A_0 - A/A_0}$ .

## Results

### Rate of neutralization

Two examples of the determination of the rate of neutralization are shown in Fig. 1. The rate of neutralization was proportional to antibody concentration (data not shown). Neutralization rate constants ( $K_{\text{neut.}}$ ) for five MAbs were calculated using equation 1 and are shown in Table 1. These values range by 38-fold, from a highest value of  $1.5 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$  for MAb HC61 to a lowest value of  $4.0 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$  for MAb HC10.

### Antibody affinity

Fig. 2 shows data obtained for a representative MAb, HC2. The  $K_{\text{dissoc.}}$  obtained from the slope of the line, was  $4.9 \times 10^{-10}$  M. Values for the  $K_{\text{dissoc.}}$  of each of the five MAbs are shown in Table 1. Affinities ranged from a high value of  $4.1 \times 10^{-10}$  M for MAb HC61 to the lowest of  $1.4 \times 10^{-9}$  M for MAb HC10.

### Neutralization and antibody affinity are not directly proportional

Data in Table 1 allowed us to determine the relationship between affinity and the efficiency of neutralization. In the first place, ranking the antibodies by  $K_{\text{neut.}}$  or  $K_{\text{dissoc.}}$  places them in approximately the same order, showing that, broadly speaking, the rate of neutralization and antibody affinity are directly related. However, our data permit a more exact analysis through calculation of the  $K_{\text{neut.}}:K_{\text{dissoc.}}$  ratio. In this way, if antibody affinity alone determined the rate of neutralization, all the ratios would be the same. In Table 1 the

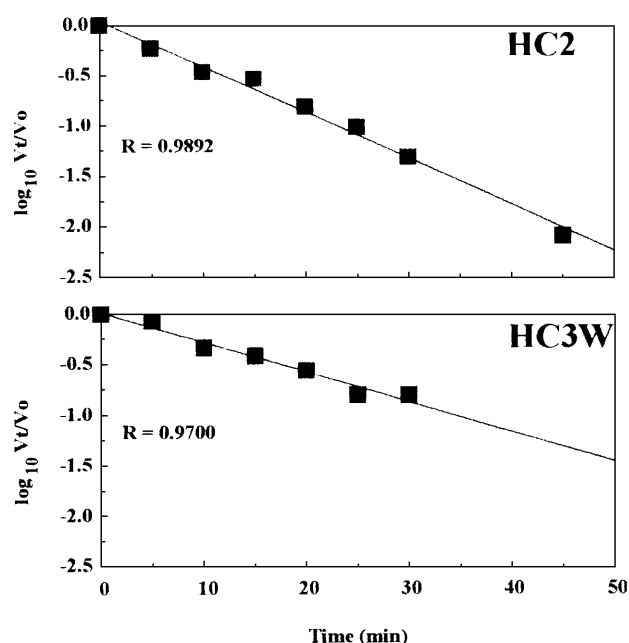


Fig. 1. Kinetics of neutralization of influenza virus A/fowl plague/Rostock/34 by MAbs HC2 and HC3W. Virus and MAb (HC2: 0.21 µg/ml; HC3W: 0.11 µg/ml) were incubated together at 37 °C for the times indicated.  $V_t/V_0$  represents the loss of infectivity, where  $V_0$  is the titre of virus at time zero, and  $V_t$  is the titre of virus after incubation for time  $t$ .  $R$  is the correlation coefficient. The SD was within the size of each datum point.

ratio pertaining to HC61 (that has the highest  $K_{\text{neut.}}$  and  $K_{\text{dissoc.}}$ ) has been defined as unity and all other values normalized to this. The Table shows that the ratios for MAbs HC2 and HC3W were similar (varying by up to 2.7-fold), while the ratio for HC58 differed by 23-fold, and that of HC10 by 125-fold. Since the rate of neutralization and antibody affinity do not increase in proportion, we conclude that factors other than antibody affinity must contribute to the efficiency of neutralization. These are likely to represent intrinsic (and as yet unknown) properties of the epitope (see Discussion).

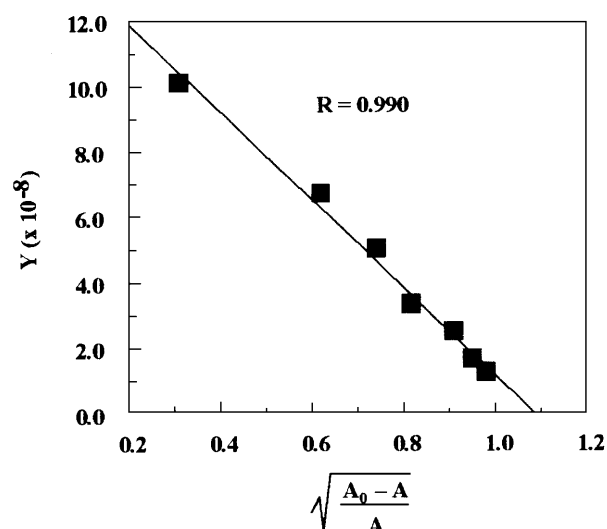


Fig. 2. Determination of affinity of MAb HC2: a Scatchard analysis of the binding of the MAb is shown. Dilutions of A/fowl plague/Rostock/34 and MAb were incubated to equilibrium (16 h at 20 °C), then incubated on microtitre plates coated with virus. The concentration of the unbound fraction of antibody was determined and entered into equation 2.  $K_{\text{dissoc.}}$  is derived from the slope of the line.  $R$  is the correlation coefficient.

## Discussion

In this paper we have determined the  $K_{\text{neut.}}$  and  $K_{\text{dissoc.}}$  for five monoclonal IgGs specific for the HA of type A influenza virus. Few data on  $K_{\text{neut.}}$  are available, but our values are within the range published for other viruses. For example, the  $K_{\text{neut.}}$  of a poliovirus MAb (Icenogle *et al.*, 1983) was 6-fold lower than that of our highest MAb HC61, while the  $K_{\text{neut.}}$  value of MAb b12, one of the most effective HIV-1 gp120-specific MAbs, derived from a human combinatorial library (Burton *et al.*, 1994; Moore *et al.*, 1995; Parren *et al.*, 1995; McInerney *et al.*, 1997) was nearly 5-fold lower than that of HC61, and ICR39.3b, a rat MAb (McLain & Dimmock, 1994) was 2400-fold lower than that of HC61.

All our MAbs had a relatively high affinity by the equilibrium method used in this report ( $K_{\text{dissoc.}} < 10^{-9}$  M).

Table 1. Summary of the properties of anti-influenza A virus monoclonal IgGs

Antibody	Rate of neutralization $K_{\text{neut.}}$ ( $\text{M}^{-1} \text{s}^{-1}$ )	Affinity $K_{\text{dissoc.}}$ (M)	Ratio $K_{\text{neut.}}:K_{\text{dissoc.}}$
HC2	$-1.1 \pm 0.3 \times 10^6$ (2)*	$4.9 \pm 0.5 \times 10^{-10}$ (2)	0.63†
HC10	$-4.0 \pm 2.1 \times 10^4$ (5)	$1.4 \pm 0.7 \times 10^{-9}$ (5)	0.008
HC3W	$-7.1 \pm 0.1 \times 10^5$ (3)	$5.3 \pm 1.3 \times 10^{-10}$ (4)	0.36
HC58	$-8.0 \pm 1.0 \times 10^4$ (4)	$5.0 \pm 0.5 \times 10^{-10}$ (3)	0.044
HC61	$-1.5 \pm 0.7 \times 10^6$ (1)	$4.1 \pm 2.2 \times 10^{-10}$ (1)	1.0

\* Numbers in parentheses show the ranking order of antibodies with respect to the property listed at the head of the column. (1) denotes highest activity.

† The ratio for HC61 is arbitrarily defined as 1.0, and all other ratios are calculated relative to this.

Values determined by surface plasmon resonance (SPR) were lower as is usually the case ( $0.1\text{--}14 \times 10^{-9}$  M), but ranked the MAbs similarly (Schofield & Dimmock, 1996). No data were obtained with HC58 by SPR, possibly because of the orientation of its binding on the virion surface, although it ranked third in Table 1. Values obtained by equilibrium filtration with polyclonal anti-influenza virus antibody averaged  $10^{-9}$  M (Fazekas de St Groth & Webster, 1963), while data obtained by radioimmunoassay with MAbs gave values up to  $10^{-10}$  M (Frankel & Gerhard, 1979) and for 12 different MAbs averaged  $6 \times 10^{-9}$  M (Brown *et al.*, 1990). Values for neutralizing IgG antibodies specific to viruses other than influenza, and assayed by ELISA with virions or virus proteins ranged from  $1.2 \times 10^{-10}$  M for rabies virions (Ueki *et al.*, 1990) down to  $2.7 \times 10^{-9}$  M obtained for HIV-1 recombinant (r) gp120 (Nakamura *et al.*, 1993). Other  $K_{\text{dissoc.}}$  values for HIV-1-specific IgGs and rgp120 protein were calculated from rate constants determined by SPR. These ranged from  $< 10^{-10}$  M to  $6 \times 10^{-9}$  M (Burton *et al.*, 1994; Conley *et al.*, 1994; VanCott *et al.*, 1994). Values for Fab fragments overlap this range at  $7.7 \times 10^{-10}$  M to  $1.2 \times 10^{-8}$  M (Barbas *et al.*, 1994; Roben *et al.*, 1994; Ditzel *et al.*, 1995). However, the highest value reflects an 8-fold increase engineered into the Fab through modification of the amino acid sequence of one of the complementarity determining regions (Barbas *et al.*, 1994). The degree of oligomerization of gp120, which is so crucial to its interaction with antibody (Moore *et al.*, 1994; Stamatatos & Cheng-Mayer, 1995; Sattentau & Moore, 1995), is not stated in these reports, so the relevance of these data to the interaction of antibodies with HIV-1 virions is not known.

We do not understand how the epitopes of the HA of influenza A virus differ so that neutralization but not affinity is affected. In general, neutralizing antibodies vary in a number of ways, which include the order of the kinetics of neutralization, the number of antibody molecules per virion required for neutralization, and the mechanism by which neutralization is mediated (Dimmock, 1993, 1995). All MAbs used in this study neutralize influenza virus infectivity at  $37^\circ\text{C}$  by pseudo-first order kinetics (Fig. 1 and data not shown), in agreement with earlier data (Taylor *et al.*, 1987; Outlaw & Dimmock, 1991). At  $1/e$  (63%) neutralization 67 and 76 antibody molecules of HC2 and HC10 respectively were bound per virion (Taylor *et al.*, 1987), showing that kinetics cannot be directly interpreted in terms of molecules of antibody. The mechanisms of neutralization of MAbs HC2, HC10 and HC61 have been investigated in some detail. The picture is frustratingly complex and the antibodies do not behave identically. However, as a generalization it appears that, at relatively low levels of neutralization, infectivity is reduced by aggregation of virions, some inhibition of attachment of neutralized virus to the target cell, and some inhibition of fusion of viral and cell membranes. None of these alone is sufficient to account for the loss of infectivity observed, but if added together could account for much of the neutralization (Outlaw *et al.*, 1990; Outlaw &

Dimmock, 1993). It is difficult to know if such a calculation is justified. A clearer picture emerges when HC2 and HC61 were used at concentrations that give high ( $> 99\%$ ) levels of neutralization. Under these conditions aggregation no longer takes place, and inhibition of attachment and of fusion are insignificant compared with the loss of infectivity. Thus another mechanism of neutralization must be operating. Further studies with highly neutralizing amounts of these antibodies and with neutralizing IgA (Armstrong & Dimmock, 1992) concluded that neutralized virions went through all stages up to and including secondary uncoating (dissociation of the M1 protein from the viral core structure) normally, and RNA from neutralized virus moved to its correct location in the nucleus. However, no transcription ensued. Thus it appears that these antibodies all inhibited a post-entry step in infection. It was postulated that these neutralizing antibodies caused a defect in tertiary uncoating, suggested to be the adoption by the viral RNP of a conformation consonant with the needs of transcription (Rigg *et al.*, 1989; Outlaw & Dimmock, 1991; Armstrong *et al.*, 1992). However no detailed information about this stage of virus replication is yet available. It is possible that antibodies HC2, HC10 and HC61 bring about the postulated defect in tertiary uncoating in different ways, or act with differing degrees of efficiency. There are no data on the mechanism of action of HC3W and HC58.

Thus data in this report suggest for the first time that epitopes of the HA of influenza A virus differ in the efficiency with they mediate neutralization, and that neutralization is not determined solely by the affinity of the antibody concerned. Our data are consistent with those of Brown *et al.* (1990) who noted the absence of correlation between neutralization of influenza A virus and antibody affinity. The situation is less clear in regard to other viruses. While neutralization by HIV-1 MAbs specific for various antigenic sites on gp120 correlated with the rate of association (Sattentau & Moore, 1995), others have found that there was a correlation with the  $K_{\text{dissoc.}}$ . Specifically, these data were obtained with MAbs directed against the C4 region of gp120 using an ELISA format and rgp120 (Nakamura *et al.*, 1993), and with V3-specific MAbs using SPR (VanCott *et al.*, 1994). Neutralization by the V3 MAbs correlated with the rate of dissociation ( $k_d$ ) from rgp120 but as the rate of association ( $k_a$ ) was relatively invariant, this correlation is also reflected in the equilibrium value ( $K_{\text{dissoc.}}$ ) as this is calculated from  $k_d/k_a$ . However values for non-neutralizing antibodies ( $\sim 6 \times 10^{-9}$  M) were very similar to that ( $4.1 \times 10^{-9}$  M) of a good neutralizing MAb, 2F5 (Conley *et al.*, 1994), suggesting that other factors are important. Neutralization by Fab fragments showed no or little correlation with affinity (Roben *et al.*, 1994; Ditzel *et al.*, 1995). However, engineered amino acid changes in the heavy chain CDR1 improved the dissociation rate of a Fab and gave better neutralization (Barbas *et al.*, 1994). It seems that neutralization mediated through some, though not necessarily all, antigenic sites/epitopes can be improved by decreasing the  $K_{\text{dissoc.}}$  of

the antibodies concerned, but that improvements in affinity are not sufficient to compensate for the intrinsic neutralization inefficiency of an epitope.

Although our data relate to neutralization *in vitro*, they are likely to be relevant also to protection mediated by antibody *in vivo*. This realization is a step forward in the rational approach to protective immunity, since it means that vaccines should now aim to stimulate primarily antibodies to those epitopes which allow the most efficient neutralization. This may be especially relevant to those vaccines which are not particularly effective, and it will be interesting to determine if the problem with the latter is that they stimulate a preponderance of antibodies to the less efficient neutralizing epitopes. It appears that in the future, the best vaccines will stimulate high affinity antibodies directed against the most efficient neutralizing epitopes.

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