The strong positive correlation between effective affinity and infectivity neutralization of highly cross-reactive monoclonal antibody IIB4, which recognizes antigenic site B on influenza A virus haemagglutinin

F. Kostolansky,1 E. Varečková,1 T. Betáková,1 V. Mucha,1 G. Russ1 and S. A. Wharton2

1 Institute of Virology, Slovak Academy of Sciences, Dúbravská cesta 9, 842 46 Bratislava, Slovak Republic
2 The National Institute for Medical Research, Virology Division, The Ridgeway, Mill Hill, London NW7 1AA, UK

Introduction

Neutralization by antibody is the most important mechanism an organism may use to defend itself against virus infection. The efficiency of different antibodies to neutralize virus greatly varies, even for antibodies specific for one virus antigen. One reason for this lies in the very complex antigenic structure of viral antigens involved in the interactions with neutralizing antibody. An excellent illustration is influenza virus haemagglutinin (HA), which has five dominant antigenic sites for binding antibodies, each with a differing capacity to neutralize virus infectivity (Wiley et al., 1981; Gerhard et al., 1981; Caton et al., 1982; Daniels et al., 1983). The importance of epitope localization with respect to the distance from the receptor binding site of influenza virus HA has recently been described by Fleury et al. (1999). The antibody binding sites on influenza virus HA that are essential for virus neutralization (VN) are extremely variable. Therefore antibodies with high VN activity cross-react only with closely related influenza virus strains isolated usually within a period of one influenza epidemic, and VN antibodies are not able to protect infected individuals after the appearance of new strains. Few highly cross-reactive VN antibodies against influenza virus have been reported (Okuno et al., 1993, 1994; Vanlandschoot et al., 1995). The study of such antibodies is of particular interest because it provides information about highly cross-reactive VN epitopes, which may in the future be used for the construction of vaccines with a broader protective capacity.

We described monoclonal antibody (MAb) IIB4 (Russ et al., 1987), which displays a rare combination of VN activity and broad cross-reactivity of different intensity with influenza virus...
A strains of the H3 subtype isolated in a period from 1973 to 1988. Recently we showed that the HA epitope recognized by MAb IIB4 is localized in antigenic site B, situated near the receptor binding site of HA (Wiley et al., 1981), and includes amino acids 198, 199 and 201 (Betáková et al., 1998). Here we show that amino acids 155, 159, 188, 189 and 193 also contribute to the MAb IIB4 epitope.

Because highly cross-reactive antibodies bind to HA of various influenza virus strains with different affinity they represent an ideal reagent to readdress the question of how important antibody affinity is for neutralization of virus infectivity. Using MAb IIB4 and a single epitope in antigenic site B in the HA of different influenza viruses we found a strong positive correlation between effective affinity and VN activity of MAb IIB4. Our results are compared with those of other authors. Finally we hypothesize how the affinity influences the proportion of spikes which must be occupied by antibody in neutralized virus preparation.

**Methods**

- **Viruses.** The following influenza viruses (all H3N2 subtype) were used: A/Hong Kong/1/68, A/England/42/72, A/Dunedin/4/73, A/Victoria/3/75, A/Bangkok/1/79, A/Belgium/2/81, A/Philippines/2/82, A/Praha/2/83 ‘HI+‘, A/Praha/2/83 ‘HI–‘ (the latter two strains were derived as genetically stable lines from a single isolate (Styk et al., 1986) and renamed according to their ability to cause haemagglutination in the presence of MAb IIB4). A/Caen/1/84, A/Mississippi/1/85 (all the above from the Institute of Virology, Bratislava), A/England/427/88, A/Beijing/352/89, A/Hong Kong/23/92, A/Shangdong/9/93, A/Bangkok/122/94 (these from NIMR, London). The conditions for infection of embryonated hen eggs and the mode of purification of the viruses have been described (Russ et al., 1974).

- **Selection of MAb escape variants of viruses.** Serial dilutions of infectious allantoic fluid (10⁻³–10⁻⁵) were incubated with 10-fold dilutions of MAb IIB4 acetic fluid (10⁻¹, 10⁻²) for 2 h at room temperature and inoculated (200 µl) into embryonated hen eggs. After 48 h, allantoic fluid samples with haemagglutination activity were tested for MAb IIB4 binding. Those which lost antibody binding were propagated, after limiting dilutions, in embryonated hen eggs.

- **Influenza virus HA gene sequencing.** HA genes of virus strain isolates from 1968 to 1985 were sequenced by the dideoxynucleotide chain termination method as described previously (Daniels et al., 1985; Grambas et al., 1992) using primers described by Betáková et al. (1998). Partial HA sequences (amino acids 151–250) of A/Victoria/3/75, A/Caen/1/84 and A/Mississippi/1/85 strains revealed complete agreement with information available in the GenBank database.

- **Haemagglutination-inhibition (HI) test.** The HI activity of MAb IIB4 with particular strains was tested by standard procedure, using chick erythrocytes at 0.5% concentration.

- **Virus neutralization (VN) test.** Amounts of individual influenza virus strains (dilutions of infectious allantoic fluids) used in the VN test were standardized according the quantity of viral nucleoprotein (NP) detected after 18 h of replication in MDCK cells by measuring the binding of iodinated anti-NP MAb 107-L (Varečková et al., 1995). These concentrations represented approximately 2 haemagglutination units (HU) of virus. The virus was incubated with fourfold dilutions of purified MAb IIB4 (from 10000 ng to 2.5 ng) for 90 min at room temperature. After washing with PBS pH 7.2, MDCK cells on 96-well plates were infected with virus/antibody mixtures for 1 h at room temperature. Cell monolayers were then washed with PBS and cultivation medium containing 0.2% FCS was added. Infected cells were incubated for 17 h at 37 °C, washed with PBS, fixed with 0.05% glutaraldehyde in PBS at 4 °C and permeabilized with 0.1% Triton X-100 for 10 min. Virus replication was evaluated using 125I-labelled MAb 107-L (for iodination procedure see Russ et al., 1978) and VN titre (VNₜᵣₑₜ) was established as the concentration of MAb IIB4 causing a 50% decrease in viral NP.

- **MAb IIB4 binding to viruses.** MAb IIB4 binding to viruses of the H3 subtype was determined by ELISA. Purified viruses were adsorbed onto wells of microtitre plates overnight at twofold dilutions (from 300 ng to 10 ng of virus per 100 µl of PBS pH 7.2). After washing (PBS with 0.05% Tween-20) and saturation of wells (with PBS pH 7.2 containing 1% non-fat dry milk), 100 ng of MAb IIB4 was added for 90 min at room temperature. The reaction was detected using swine anti-mouse immunoglobulin conjugated to horseradish peroxidase by measuring the enzyme activity in the presence of activated substrate 1,2-phenylenediamine dihydrochloride (1 mg/ml) with 0.03% H₂O₂ at 492 nm.

- **Homologous competitive radioimmunoassay (RIA).** The principle of the RIA was homologous competition of 125I-labelled MAb IIB4 with increasing amounts of unlabelled antibody for binding to virus adsorbed to the solid phase. In these experiments, approx. 3 ng total input of 125I-labelled IIB4 was added in each sample. The amount of purified virus was such that it had less than 50% of maximal binding of 125I-labelled IIB4. Unlabelled MAb IIB4 competitor (2 to 2000 ng) was present in the reaction mixture (100 µl). Incubation was carried out at room temperature for 3 h. After washing, bound radioactivity was measured in a gamma counter.

- **Effective affinity estimation of MAb IIB4 and epitope occupancy determination.** The effective affinity of MAb IIB4 binding was measured for various H3 strains. However, only those strains whose binding activity with MAb IIB4 was sufficiently high to allow precise measurement could be included in affinity measuring. For effective affinity estimation the data obtained from homologous competitive RIA were used (Rodbard & Feldman, 1975; Mucha, 1993; Varečková et al., 1993). Linear regression was applied to the experimental points [log₁₀(VNₜᵣₑₜ) vs log₁₀(effective affinity of binding)] representing individual virus strains, and the regression coefficient was estimated.

Epitope occupancy rate at equilibrium in virus/MAb preincubation (1 h at room temperature) in the VN test was determined on the basis of the mass action law from the formula:

\[ X/(1 - X) = K_q - K_p \times X \]  

where \( K \) is effective affinity determined as stated above, \( q \) and \( p \) are concentrations of antibody combining site and epitope, respectively, and \( X \) is epitope occupancy to be calculated. The epitope concentration was estimated on the basis of the virus concentration used in the VN experiment, taking into account a 20% proportion of HA in the virion. Protein concentration in purified virus preparations was estimated by the method of Lowry et al. (1951) using BSA as a standard.

**Results**

**Characterization of MAb IIB4**

MAb IIB4, specific for influenza virus HA, is secreted from a hybridoma obtained after immunization of BALB/c mice

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with native virus A/Bangkok/1/79 (H3N2) (Russ et al., 1987). An unusual property of MAb IIB4 is its broad cross-reactivity with different virus strains of the H3 subtype (Fig. 1). Significant binding was seen with strains isolated between 1973 and 1988. MAb IIB4 did not react with Hong Kong/68, England/72, Beijing/89, Hong Kong/92, Shangdong/93 or Bangkok/94 (the latter two strains are not shown in Fig. 1).

**Characterization of the IIB4 epitope**

**Escape mutants.** Recently we showed that the HA epitope recognized by MAb IIB4 is localized in antigenic site B and includes amino acids 198, 199 and 201 localized on the tip of the HA spikes (Fig. 2) (Betáková et al., 1998). Amino acid positions 198, 199 and 201 are highly conserved in all influenza virus HA reactive (and some non-reactive) with MAb IIB4. To characterize the IIB4 epitope in more detail, we prepared escape mutants from various strains, including the parental virus Bangkok/79. All escape mutants including those described before (Betáková et al., 1998) are summarized in Table 1. Sequencing of whole HA genes of particular strains showed that the MAb IIB4 escape mutants had substitutions at four amino acid positions, 198, 199, 201 and 248. It is noteworthy that two escape mutants, one prepared from Bangkok/79 and a second prepared from Belgium/81, had the same escape substitution A (alanine) to E (glutamic acid) at position 198.

A new escape mutant obtained from Philippines/82 had a substitution 248 I (isoleucine) to T (threonine), causing the appearance of a new glycosylation site (Fig. 2; positions 246–248). Because there were no other significant differences in the HA amino acid composition compared with the parental Philippines/82, we can conclude that oligosaccharide 246 influences the binding of MAb IIB4. The distance between positions 248 and 198 is 1.8 nm.

**Amino acid sequences of HA.** To determine other amino acids possibly involved in the binding of MAb IIB4 we compared the reactivity pattern of antibody to various H3 strains (Fig. 1). The amino acid sequences of H3 strains are shown in Fig. 3. Two non-reactive strains, Hong Kong/68 and England/72, differed from all reactive ones in positions 188 and 193, both spatially close to already determined contact amino acids 198, 199 and 201. Therefore, we suppose that positions 188 and 193 might influence MAb IIB4 binding. However, the significance of position 193 remains questionable since the substitution of neutral asparagine (N) to basic lysine (K) present in the highly reactive strain Praha/83 HI + (Fig. 3) is remarkable.

The presence of a glycosylation site at position 246 had a deleterious effect on antibody binding in the escape mutant of Philippines/82. Additional evidence arises from the significantly lowered binding of MAb IIB4 to strains Praha/83 HI — and Caen/85 (Fig. 1), both containing an oligosaccharide at this position (Fig. 3). It may be concluded that this oligosaccharide may either reduce or even eliminate the antibody binding. We observed a loss of binding in strains Beijing/89, Hong Kong/92, Shangdong/93 and Bangkok/94. In contrast, oligosaccharide 165 does not influence MAb IIB4 binding, as determined from its presence in all tested virus strains (Fig. 3).

Besides oligosaccharide 246, a second case of indirect influence on the IIB4 epitope is probably represented by tyrosine (Y) 219 in strain Praha/83 HI —. In the HA trimer this position is close to position 201 of the adjacent monomer and might influence the binding of MAb IIB4 to the adjacent monomer (Fig. 2.a).

Comparison of the sequences of the closely related strains poorly reactive England/88 and highly reactive Mississippi/85 (both lacking a sugar at 246) revealed three additional amino acid positions possibly involved in MAb IIB4 binding: 155, 159 and 189 (Fig. 3). As shown in Fig. 2, these residues are also spatially close to positions 198–201. The spatial distance between amino acid positions potentially involved in the IIB4 epitope does not exceed 18 Å. We do not exclude the fact that other even more distant amino acids may be included in the interface area that influences antibody binding. It is obvious that the area delineated by these amino acids is smaller than the buried area (1250 Å²) in the HA–Fab interface found by Bizebard et al. (1995).

In summary, the crucial amino acid positions forming the IIB4 epitope are 198, 199 and 201. We assume that amino acids 155, 159, 188, 189 and possibly 193 are also involved in the binding of MAb IIB4.
Fig. 2. Schematic diagram indicating amino acid positions involved in the epitope defined by MAb IIB4. 198, 199, 201: amino acid positions, the substitutions of which caused a loss of MAb IIB4 binding to IIB4 escape mutants; 155, 159, 188, 189, 193: positions probably involved in antibody binding as determined by comparison of HA sequences of various influenza strains with the MAb IIB4 binding pattern; 246, 247, 248: glycosylation site for oligosaccharide which strongly restricts or eliminates the antibody binding; 219: large amino acid (tyrosine) at this position might sterically influence the accessibility of the epitope on the adjacent monomer. (a) HA trimer (top view). (b) HA monomer (side view). Part (a) and (b) are not to the same scale. The RasMol software package, version 2.6-beta-2 was used for generating diagrams.

Table 1. Positions and type of amino acid substitutions in the HA of escape mutants obtained from parental strains grown in the presence of MAb IIB4

<table>
<thead>
<tr>
<th>Parental strain</th>
<th>Escape substitution</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/Bangkok/1/79</td>
<td>198 A → E</td>
</tr>
<tr>
<td>A/Belgium/2/81</td>
<td>198 A → E</td>
</tr>
<tr>
<td>A/Bangkok/1/79</td>
<td>199 S → P</td>
</tr>
<tr>
<td>A/Mississippi/1/85</td>
<td>201 R → G</td>
</tr>
<tr>
<td>A/Philippines/2/82</td>
<td>248 I → T</td>
</tr>
</tbody>
</table>

Virus neutralization depends on the effective affinity of MAb binding

The VN activity of MAb IIB4 is related to its ability to inhibit haemagglutination with particular viruses and roughly correlates with its binding reactivity in ELISA (Table 2 and Fig. 1). To find out how important antibody affinity is for VN we also estimated the effective affinity of MAb IIB4 binding to HA of influenza virus strains included into this study (Table 2). As shown in Fig. 4, VN activity was proportional to the effective affinity of binding. The application of linear regression to the experimental points resulted in a regression coefficient greater than 3. A parabolic regression line (solid line on Fig. 4) provided, however, a better fit to the experimental points. These results thus show a strong positive correlation between effective affinity and VN activity of MAb IIB4. This raises the question of whether occupancy of HA spikes required for VN is independent of the effective affinity of MAb IIB4 binding.

Affinity of MAb IIB4 determines epitope occupancy required for virus neutralization

Generally, according to the law of mass action, antibody affinity and the concentrations of epitope and antibody determine occupancy at equilibrium (see Methods, equation 1).

To evaluate the epitope concentration we determined the amount of virus present in 1 HU of purified virus preparation. The amount of virus in allantoic fluid used in the VN test was determined from its haemagglutination activity. Presuming that there is a 20% proportion of HA in a virion, 2 HU of virus used in the VN test represented the following amounts of HA in particular virus strains: A/Dunedin/4/73, 16 ng; A/Victoria/3/75, 7 ng; A/Bangkok/1/79, 14 ng; A/Praha/2/83 HI +, 7 ng; A/Mississippi/1/85, 9 ng. For these virus strains the epitope occupancy rate required for VNso was calculated (Fig. 5).

Because of the close proximity of the IIB4 epitope to the trimeric interface (Fig. 2a), we propose that only one MAb IIB4
Antibody affinity and virus neutralization

Fig. 3. Amino acid sequence of H3N2 subtype strains isolated in the period from 1968 to 1994 showing the part of the HA which influences MAb IIB4 binding. The sequence of strain A/Bangkok/1/79 (parental immunizing virus for MAb IIB4) is taken as a reference. Positions 198, 199 and 201 were determined on the basis of sequence analysis of IIB4 escape mutants. Shaded frames depict positions which probably interfere with antibody binding to a particular strain as explained in the text. Transparent frames mark glycosylation sites.

interacts per HA trimer. Electron microscopy observation of immune complexes containing an antibody which also recognizes HA position 198 has shown that only one antibody molecule can bind per trimer (Wrigley et al., 1983). Therefore, epitope occupancy is equal to the occupancy of spikes.

Provided that the epitope occupancy required for VN is constant, a relationship between binding affinity and VN titre can be described by an equi-occupancy line determined by the law of mass action. A plot of equi-occupancy lines for a given range of effective affinities and MAb concentrations used in the VN experiments described here is shown for three occupancy levels (5%, 50% and 95%) in Fig. 4 (dashed lines). There is a great difference in slopes between theoretical equi-occupancy lines and the experimental curve showing the relationship between VN activity and effective affinity. We conclude from this that the effective affinity of MAb IIB4 strongly influences epitope occupancy required for VN.

Fig. 5 shows the influence of MAb IIB4 affinity on the occupancy rate of HA spikes needed for VN$^{50}$. For the virus strains to which MAb IIB4 binds with high effective affinity (ranging from $5\times10^6$ to $6\times10^8$ I/mol), an occupancy level from 13.5% to 27% was required for VN$^{50}$. In contrast, for the virus strain with low effective affinity to MAb IIB4 ($6\times10^7$ I/mol) as many as 98% of the spikes must be occupied to neutralize the virus. The virus strain with intermediate effective affinity of binding ($1\times10^6$ I/mol) required 64% occupation of spikes. On the basis of the observed results we suggest that for the IIB4 epitope, the
Table 2. HI and VN activity, and binding affinity of MAb IIB4 to various influenza virus A strains of the H3N3 subtype and occupancy rate of HA spikes

Purified MAb IIB4 (100 µl) was added in both HI and VN tests.

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>HI titre*</th>
<th>Dilution</th>
<th>ng</th>
<th>VN₅₀ (ng)</th>
<th>Affinity (l/mol)</th>
<th>Occupancy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/Hong Kong/1/68</td>
<td>&lt; 20</td>
<td>&gt; 5380</td>
<td>&gt; 10000</td>
<td>ND†</td>
<td>NA‡</td>
<td></td>
</tr>
<tr>
<td>A/England/42/72</td>
<td>&lt; 20</td>
<td>&gt; 5380</td>
<td>&gt; 10000</td>
<td>ND</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>A/Dunedin/4/73</td>
<td>80</td>
<td>1.345</td>
<td>5000</td>
<td>6.1 x 10⁷</td>
<td>98</td>
<td></td>
</tr>
<tr>
<td>A/Victoria/3/75</td>
<td>256</td>
<td>420</td>
<td>80</td>
<td>1.7 x 10⁸</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td>A/Bangkok/1/79</td>
<td>1024</td>
<td>105</td>
<td>5</td>
<td>5.6 x 10⁸</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>A/Belgium/2/81</td>
<td>4096</td>
<td>26</td>
<td>2.5</td>
<td>8.7 x 10⁸</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>A/Philippines/2/82</td>
<td>1024</td>
<td>105</td>
<td>5</td>
<td>5.5 x 10⁸</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>A/Praha/2/83 HI+</td>
<td>4096</td>
<td>26</td>
<td>5</td>
<td>6.9 x 10⁸</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>A/Praha/2/83 HI−</td>
<td>&lt; 20</td>
<td>&gt; 5380</td>
<td>&gt; 10000</td>
<td>5.6 x 10⁷</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>A/Caen/1/84</td>
<td>&lt; 20</td>
<td>&gt; 5380</td>
<td>&gt; 10000</td>
<td>ND</td>
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<td>4096</td>
<td>26</td>
<td>2.5</td>
<td>5.7 x 10⁸</td>
<td>13.5</td>
<td></td>
</tr>
<tr>
<td>A/England/427/88</td>
<td>&lt; 20</td>
<td>&gt; 5380</td>
<td>5000</td>
<td>7.9 x 10⁷</td>
<td>NA</td>
<td></td>
</tr>
</tbody>
</table>

* HI titres are expressed as both the highest dilution and amount of MAb IIB4 capable of inhibiting haemagglutination.
† ND, Not done.
‡ NA, Occupancy could not be evaluated where no affinity data were available or no virus concentration was estimated.

**Discussion**

In this paper we studied the affinity–VN relationship in vitro using an experimental model of influenza A virus, taking into account the importance of the naturally protective role of
a specific anti-HA antibody response (Hobson et al., 1972; Dowdle et al., 1973; Virelizier 1975, 1976; Cough & Kasel, 1983). Brown et al. (1990) found that neutralization efficiency tended to be influenced by the affinity of the binding of the antibody to influenza virus HA. However, they studied a range of antibodies binding to a limited number of virus strains. When observing the VN activity of particular MAbs with the different strains, the relationship between VN and affinity was not so clear. Schofield et al. (1997) also reported on the relationship between neutralization and affinity for influenza virus. On the basis of a panel of five neutralizing MAbs directed to three antigenic sites present on the HA they found that the rates of neutralization and antibody affinity do not increase in proportion. They suggested that the epitope identity is crucial for neutralization efficiency and that the antibody affinity is of secondary importance.

We used a single MAb binding to an epitope on the HA of several related virus strains of the H3 subtype. Therefore, experiments were influenced neither by antibody variability nor by a different localization of the binding site critical for VN. Several, but not all, amino acid positions on the HA involved in the binding of MAb IIB4 were identified. We suppose that MAb IIB4 binds to the topologically identical epitope on each reactive virus strain, which does not necessarily mean that the interface in the HA–MAb IIB4 complex and the number of intermolecular hydrogen bonds are conserved throughout these strains. Possible amino acid changes within the IIB4 epitope could induce changes in its surface structure, therefore it may have variable affinity of binding to the paratope on the MAb IIB4. Moreover, mutations spatially close to the epitope could cause small differences in the surface structure of the IIB4 epitope, hence the affinity of MAb IIB4 binding to the HA may differ for different virus strains even if their IIB4 epitope consists of the same amino acids. Thus, observed variable binding affinity of MAb IIB4 to the topologically identical epitope may be accepted. Our experiments showed a strong positive correlation between the effective affinity of MAb IIB4 and its VN activity; a 10-fold increase in effective affinity corresponded to about a 2000-fold increase in VN titre. Application of linear regression to the experimental points resulted in a regression coefficient greater than 3.

Similar results were described by Nakamura et al. (1993) using the human immunodeficiency virus (HIV) model and a panel of seven MAbs directed to the C4 domain of gp120. These authors concluded that ‘small changes in antibody affinity result in large differences in the inhibitory concentration (IC_{50})’. Their affinity–VN relationship, replotted into ($\log_{10}$ affinity vs $\log_{10}$ VN) co-ordinates, which are used in our work to present results, gave not only a regression coefficient close to 3, but also a deviation from linearity similar to our results. Langedijk et al. (1991) used MAbs against an 18 amino acid long gp120-derived peptide which contains a principal neutralization domain of HIV. They found a linear relationship between $\log_{10}$ VN and $\log_{10}$ affinity with a regression coefficient approx. 5/3 (i.e. the slope was less steep than in our influenza model, but still greater than 1). Bachmann et al. (1997), using the vesicular stomatitis virus model and a set of MAbs, described a close correlation between neutralizing capacity and affinity in vitro. The regression coefficient of the relationship seems to be just above 1. On the other hand, West et al. (1994) have not found a clear correlation between neutralizing activity and binding affinities of MAbs specific to respiratory syncytial virus. Particularly, the two MAbs with the best VN activity (binding to the same site) possessed low binding affinity to F protein.

As follows from data mentioned above, the VN vs affinity relationship expressed by regression coefficient differs considerably. However, experiments leading to these conclusions were done on different viral models including different sets of epitopes and corresponding antibodies.

According to the law of mass action, the affinity value determines epitope occupancy by antibody at equilibrium (see Methods, equation 1). This enabled us to determine the occupancy level of HA spikes required for VN_{50} of examined virus strains. The regression line on Fig. 5 determines the effective affinity vs occupancy relationship: occupancy of HA spikes required for VN_{50} for particular strains was not constant but varied considerably from 13.5% to 98%, inversely proportional to the effective binding affinity. The occupancy of spikes approaching 10% probably represents the minimal occupancy warranting VN. This is feasible when one assumes a random distribution of MAb IIB4 binding to HA spikes and the localization of the IIB4 epitope on the membrane distal end of the HA molecule. Under such conditions, assuming that one antibody molecule bound to the epitope blocks the whole trimeric HA spike because of spatial proximity of the three epitope sites on the trimer, the occupation of every tenth HA spike may be enough to complete inhibition of binding of virus to cells, most probably due to steric interference, though virus aggregation cannot be excluded particularly in a certain MAb concentration range. This observation is in accordance with Taylor et al. (1987) who found that for 63% neutralization of influenza virus infectivity about 70 molecules of monoclonal immunoglobulin G per virus particle are needed (i.e. approx. 10% of HA spikes).

The very broad range of HA occupation needed for a given VN effect may be explained as follows. Apparently the minimum number of HA spikes which must be occupied by antibody (e.g. 10%) is the same for all influenza virus strains neutralized with MAb IIB4. We estimated the initial occupancy of HA in equilibrium before the virus–antibody preparations were allowed to interact with MDCK cells. However, following the exposure of the virus–MAb mixture to MDCK cells the equilibrium conditions are changed and the initial occupancy of HA spikes, due to dissociation indirectly proportional to the affinity, decreases. Provided that the effective affinity of MAb IIB4 to the particular virus strain is
sufficiently high, such a loss of bound antibody can be discounted and the initial occupation (e.g. 13.5%) of HA spikes is enough for VN. In contrast, for an interaction characterized by low effective affinity, the occupancy during incubation with MDCK cells may drop quickly from over 90% to about 10%. Therefore the initial occupation by an antibody with low effective binding affinity must be much higher than the minimal occupancy required for VN.

Additional in vivo protective experiments using mouse-adapted influenza virus strains interacting with MAbs IIB4 with various affinities could support our in vitro observations concerning the strong effect of antibody affinity on its neutralizing activity.

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References


subpopulation of native influenza virus haemagglutinin molecules. *Archives of Virology* 130, 45–56.


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