A haemagglutinin (HA1)-specific FAb neutralizes influenza A virus by inhibiting fusion activity

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H9-D3-4R2 (referred to as H9), a murine monoclonal HA1-specific IgG3, recognizes an epitope within antigenic site Cb of influenza virus A/PR/8/34 (H1N1). At 50% neutralization, inhibition of virus-mediated fusion was responsible for the majority of neutralization but, at higher antibody concentrations, the attachment of virus to target cells was also inhibited and may have contributed to neutralization. H9 FAb was also neutralizing, although the concentration needed was two orders of magnitude greater than for the IgG. Functional affinity of the IgG and affinity of the FAb were almost identical, and it is not clear why the neutralization efficiency of the FAb was so low. Unlike its IgG, H9 FAb had no detectable effect on virus attachment but inhibited virus fusion activity. It thus appears that monovalent binding by this antibody is sufficient to inhibit fusion activity and that this was directly responsible for neutralization of infectivity.

Introduction

Influenza A virus has three surface proteins. The haemagglutinin (HA) is responsible for attachment to target cells and the low pH-mediated fusion–entry process, the neuraminidase allows progeny virions to avoid binding local receptors by removing terminal N-acetyl neuraminic acid residues from the cell and other virions, and M2 channels protons into the virion and permits proper secondary uncoating of the virion core. The HA is a homotrimer, with each monomer consisting of an outer HA1 subunit that is linked covalently to a membrane-anchoring HA2 subunit. The HA elicits protective immunity in man, which results largely from the antibody response (Ghendon, 1990), and is also the major neutralization antigen. Neutralizing antibodies are produced largely to HA1, and these are confined to recognizing specific clusters of overlapping epitopes (antigenic sites) on the surface of the globular head.

Neutralization is defined as an in vitro process in which virus binds antibody and loses infectivity, and it correlates strongly, although not exclusively, with protection from infection in vivo (Dimmock, 1993). Which functions of the virion are inhibited during neutralization, which are directly responsible for loss of infectivity and exactly how the antibody brings this about are poorly understood. However, neutralization is known to be a complex, three-way process that is dependent upon the interaction of the virus, the antibody and the target cell. In addition, the efficiency of neutralization and its mechanism have been shown to be affected by intrinsic properties of the epitope, the affinity, isotype and concentration of the applied antibody and the valency with which it binds (Dimmock, 1993, 1995; Edwards & Dimmock, 2000; Schofield & Dimmock, 1996; Schofield et al., 1997b). While it is never possible to show unequivocally how a virus is neutralized, loss of infectivity can, with care, be correlated with the loss of a particular virus function or functions that is known to be essential in the initial stages of infection. However, to establish a putative causal relationship, loss of a particular virus function must be able to account quantitatively for the loss of infectivity, as represented by the antibody dose–neutralization curve or kinetics of neutralization.

FAb fragments bind to the same epitope on virions as the IgG from which they were derived, and can be neutralizing. The FAb is monovalent and has a simpler structure than IgG, and its neutralization activity may thus prove more amenable to analysis. Not much is known about FAb-mediated neutralization of influenza A virus (or viruses in general), but a recent study compared neutralization by IgGs and their FAbs specific to sites Ca2 and Sb on the distal surface of the globular head of the HA of an H1N1 influenza A virus (Edwards &...
Dimmock, 2000). It was found that these IgGs exerted a complex, concentration-dependent spectrum of neutralization activity. Inhibition of virus–cell fusion was the predominant mechanism at low antibody concentration, while inhibition of attachment of virus to target cells predominated at high concentration. The two mechanisms occurred simultaneously at intermediate concentrations. In contrast, neutralization by their FAb was simple and, at all concentrations, correlated closely with inhibition of attachment to the target cell. However, this required a much higher molar concentration for the same loss of infectivity compared with IgG neutralization. The loss of neutralization activity might be expected, as the FAb is smaller in size than the IgG, but the change in neutralization mechanism is counterintuitive, as one might have expected the FAb to neutralize in the same way as the IgG, particularly as one of the MAbs had a functional affinity very similar to that of its FAb and would thus be expected to bind monovalently.

In this report, we have studied H9-D3-4R2 (referred to as H9), an HA1-specific murine monoclonal IgG3 that recognizes antigenic site Cb on the under-surface of the globular head. H9 IgG neutralized primarily by inhibiting virus–cell fusion in two different cell types, although virus attachment to target cells was also inhibited at higher IgG concentrations. The H9 FAb was also neutralizing but, within the concentration range tested, neutralized entirely by inhibiting virus fusion. However, a given amount of neutralization required a higher molar concentration of FAb than of IgG. How a monovalent ligand to the HA1 such as the FAb inhibited the virus fusion process is also discussed.

Methods

**Virus.** Influenza virus A/Puerto Rico/8/34 (H1N1) (PR8) was grown in the allantoic cavity of 10-day-old embryonated chicken eggs (Poynden Egg Farm, Goss Oaks, Herts, UK) for 48 h at 33 °C. Virus was pelleted, resuspended and centrifuged for 90 min on a 10–45% (w/v) sucrose velocity gradient and then overnight on a 20–70% (w/v) sucrose density gradient. The virus band was pelleted and stored at −70 °C.

**Cells.** Madin–Darby canine kidney (MDCK) cells (a gift from W. Barclay, University of Reading, UK) were maintained in DMEM (Life Technologies) supplemented with 4 mM glutamine (Life Technologies), 5% (v/v) heat-inactivated foetal calf serum (HIFCS; Life Technologies) and 50 µg/ml gentamicin (Life Technologies) and BHK cells (ECACC, Porton Down, UK) were cultured in GMEM (Life Technologies) supplemented with 4 mM glutamine, 10% HIFCS and 50 µg/ml gentamicin. The mouse hybridomas (see below) were grown in RPMI 1640 (Life Technologies) supplemented with 10% HIFCS, 2 mM glutamine and 50 µg/ml gentamicin.

**Antibodies.** Here, we studied the murine MAbs H9-D3-4R2, which is specific for the HA1 of PR8 (H1N1). This is an IgG3 that maps to antigenic site Cb (site E of H3) and was kindly donated by Walter Gerhard (Caton et al., 1982; Staudt & Gerhard, 1983). RT–PCR and sequencing of neutralization-escape mutants showed that there was an amino acid residue change (S79 → P) in the expected antigenic site (A. C. Marriott, C. Parry and N. J. Dimmock, unpublished data). We also used the HA1-specific MAbs H37-45-5R3 (IgG3, site Ca2) and H37-66-1 (IgA, site Sb) (also from W. Gerhard) and MAb HB67, specific for the nucleoprotein (NP) of influenza virus A/WSN (IgG1; from Robert Webster, St Jude’s Children’s Research Hospital, Memphis, TN, USA). When required, IgG MAbs were purified from TCF by affinity chromatography on Protein A–Sepharose (Sigma) under low-salt conditions. IgG was eluted in 100 mM glycine (pH 3) and fractions were neutralized by the addition of 1 M Tris–HCl (pH 8). Peak protein fractions were pooled and concentrated by using an Amicon 8010 pressure concentrator with a 10000 r cut-off filter. Antibody was dialysed against PBS and quantified by measurement of A280.

**Production of FAbs by digestion of IgG with papain.** H9 IgG3 (1 mg) was mixed with 0.2 µl freshly made 1 M cysteine (Sigma), 20 µl EDTA and 1 U agarose-immobilized papain (Sigma). This was adjusted to pH 5.5 with 200 mM sodium acetate to a final volume of 1 ml and shaken for 3 h at 37 °C. The reaction was stopped by pelleting the papain, making the digest 5.5 mM with respect to iodoacetamide (Sigma) and raising the pH to neutrality with 1 M Tris–HCl (pH 8). Undigested IgG and Fc fragments were removed by repeated passage through Protein A–Sepharose. FAbs were then dialysed against PBS, concentrated and quantified spectrophotometrically. Purity of FAbs was assessed by PAGE (Edwards & Dimmock, 2000) and no IgG was detected (<50 ng/ml).

**Haemagglutination assay.** Virus was diluted with an equal volume of PBS in a 96-well round-bottom plate (Greiner) and detected by agglutination of 0.13% chicken red-blood cells (Sorotech). Fifty per cent agglutination (1 HAU) was estimated by interpolation between complete agglutination and no agglutination and the titre was expressed as the reciprocal of the dilution in HAU/ml.

**Plaque assay for virus neutralization.** Virus (400 p.f.u./ml) was mixed with an equal volume of a range of dilutions of MAb for 60 min at 37 °C and then 100 µl was inoculated onto washed MDCK or BHK monolayers in 6-well plates (Falcon) and incubated for 45 min at 20 °C. Monolayers were then overlaid with 0.9% agar (Life Technologies) in 199 medium buffered with sodium bicarbonate and containing 0.2% (w/v) BSA in PBS (Sigma), 0.01% (w/v) DEAE dextran (Sigma), 100 U/ml penicillin, 100 µg/ml streptomycin and 30 U/ml TPCK trypsin (Sigma). After incubation for 3 days at 33 °C, monolayers were fixed with formal–saline and stained with toluidine blue (BDH) for plaque counting. Virus controls had approximately 40 p.f.u. per plate. Neutralization was calculated as the titre, as a percentage of the virus control, subtracted from 100.

**Neutralization ELISA.** A neutralization ELISA (Höring et al., 1992) was used to measure infectivity loss in the same format and from the same virus–antibody mixtures as were used to measure virus attachment and virus internalization. These were carried out in 96-well plates. Virus and MAb were incubated together for 60 min at 37 °C and 100 µl was inoculated onto washed MDCK or BHK monolayers in a 96-well cell culture plate (Life Technologies) and incubated for 25 min at 4 °C. After washing, 100 µl of warm DMEM, 1% HIFCS, 4 mM glutamine and 20 µg/ml gentamicin (Life Technologies) was added and the plate was incubated overnight at 37 °C. Exogenous trypsin is required to cleave the precursor HA0 molecule into the components HA1 and HA2 that are required for infectivity. Thus, without trypsin, any viron products will be non-infectious and only one round of replication can take place. Cells were then washed, fixed with 10% formal–saline and blocked with 3% BSA in TBS (20 mM Tris–HCl, 140 mM NaCl, pH 7.6) for 90 min at 20 °C. HA expressed de novo on the cell surface was then assayed as a measure of virus replication by using a murine monoclonal anti-HA IgA (H37-66-1), an anti-IgA alkaline phosphatase conjugate (Sigma) and dinitrophenyl phosphate (DNPP). The plate was read on an
optical plate reader at 405 nm (Titretek Multiscan Plus; Life Sciences International). Non-neutralized virus controls had an $A_{485}$ of approximately 1.1. To ensure that different assays were carried out under the same conditions, the ELISA neutralization was also done occasionally in 3 cm diameter dishes. The procedure was the same as for the 96-well ELISA except that the volumes were scaled up. The coloured product was then transferred to 96-well plates for reading.

### ELISA of virus attachment to target cells and its inhibition by antibody

Virus was neutralized and monolayers in 96-well plates were inoculated as described above. After removing non-attached virus by rinsing with cold DMEM, virus was fixed and permeabilized with methanol at $-20\,^\circ C$ and then blocked overnight at $4\,^\circ C$ with 3% BSA in TBS. Attached virus was assayed by using an NP-specific MAb (HB-67, IgG1) in 1% BSA in TBS containing 0.1% Tween (Sigma), rabbit anti-mouse IgG1 (Dako), goat anti-rabbit alkaline phosphatase conjugate (Dako) and DNPP. The plate was read as described above. The virus control had an $A_{485}$ of approximately 1.3. In addition, parallel monolayers were treated before inoculation with 0.025 U of *Clostridium perfringens* neuraminidase type V (Sigma) for 10 min at $37\,^\circ C$ to determine whether neutralized virus was attaching to $N$-acyethyl neuraminic acid (NANA) receptors. Virus attachment and neutralization were determined in exactly the same 96-well system.

### ELISA of virus internalization by target cells and its inhibition by antibody

Virus was neutralized and monolayers in 96-well plates were inoculated as described above. After washing with cold DMEM, monolayers were incubated with warm DMEM at $37\,^\circ C$ for 30 min to allow the attached virus to be internalized. Non-internalized virus was removed by washing and treatment twice with 0.025 U *Clostridium perfringens* neuraminidase for 10 min at $37\,^\circ C$. Monolayers were then permeabilized by freeze–thawing three times and fixed in methanol containing 0.15 M NaCl for 30 min at $-20\,^\circ C$. After washing in TBS–Tween, monolayers were blocked overnight at $4\,^\circ C$ with 3% BSA in TBS. Virus was detected as virion NP antigen, as described above. In order to show that virus was being internalized by receptor-mediated endocytosis, we used conditions that are known to inhibit this process: either incubation at $4\,^\circ C$ (Matlin et al., 1981; Richman et al., 1986) or incubation of cells in hypertonic medium (0.25 M sucrose) for 30 min at $20\,^\circ C$ before inoculation of virus. This latter treatment prevents formation of the clathrin lattice required for receptor-mediated endocytosis (Daukas & Zigmond, 1985; Heuser & Anderson, 1989). The amounts of virus internalization and neutralization were determined in exactly the same 96-well system. In addition, virus-attachment assays were carried out in parallel. This enabled the internalization data to be corrected for any inhibition of virus attachment by antibody (% internalization + % inhibition of attachment). The virus control had an $A_{485}$ of approximately 0.8.

### Assay for virus–cell fusion, using virus labelled with octadecyl rhodamine B chloride (R18), and its inhibition by antibody

Freshly prepared purified virus (1.25 x 10^7 HAU in 500 µl) was labelled by incubation with 5 µl R18 (final concentration 85.5 µM; Molecular Probes) for 60 min at $20\,^\circ C$ in the dark. After centrifuging to remove any precipitate, free R18 was removed by pelleting virus through 20% sucrose at 11000 x $g$ for 90 min. Virus was resuspended in 100 µl PBS and stored at $-70\,^\circ C$. When solubilized in 1% Triton X-100 (BDH), R18-labelled virus gave a 150-fold increase in fluorescence, indicating that the R18 was self-quenched and incorporated into the virus lipid bilayer. For assay of fusion, 200 HAU non-neutralized or neutralized R18-labelled virus in 100 µl was inoculated onto a 3 cm monolayer of MDCK or BHK cells for 25 min at $4\,^\circ C$. Unbound virus was removed by washing with cold DMEM and 200 µl of warm DMEM was then added and incubated for 30 min at $37\,^\circ C$. Cells were then washed with cold DMEM, harvested by incubating with cold versene for 5 min, pelleted and fixed by resuspending in cold 2% paraformaldehyde in the dark. Fluorescence was determined with a Luminescence Spectrophotometer LS-5 (Perkin-Elmer), exciting at 560 nm and emitting at 590 nm, with a slit length of 10 nm. The virus control had fluorescence of approximately 300 units. To demonstrate that the increase in fluorescence signal was due to virus–cell fusion, either cells inoculated with R18-labelled virus were kept at $4\,^\circ C$ to inhibit fusion (Matlin et al., 1981; Richman et al., 1986) or cells were treated prior to infection with 500 nM bafilomycin A1 from *Streptomyces griseus* (Calbiochem) for 90 min at $37\,^\circ C$. Bafilomycin is a V-ATPase inhibitor that prevents acidification of endosomal vesicles in the cell and hence inhibits HA-mediated fusion (Guinea & Carrasco, 1995; Ochiai et al., 1995; Palokangas et al., 1994). Data were corrected for inhibition of attachment of virus to target cells that occurred with increasing antibody concentration, as described above. In order to keep the assays comparable, neutralization was determined by ELISA with the same virus–antibody mixtures and in the same 3 cm diameter dish system as was used for the fusion assays. Neutralization was carried out exactly as in the 96-well ELISA except that the volumes were scaled up. The coloured product was then transferred to 96-well plates for reading.

### Assay of the functional affinity of H9 MAbs and affinity of its FAb for virions using surface plasmon resonance

Functional affinity/affinity was determined with whole virions (Schofield & Dimmock, 1996). The HA-specific monoclonal H37-45-SR3 IgG was bound covalently to a CM5 biosensor chip of a BIAcore 2000 (Biacore AB) by amine coupling and any MAbs bound non-specifically was removed with 0.05 M triethylamine (Sigma). Purified virus [320 HAU in 40 µl of 100 mg/ml CM5 dextran in HBS (Biacore)] was then allowed to bind to the MA at a flow rate of 20 µl/min. The signal from the blank control, lane 1, is subtracted from the signal in lane 2, so that bulk changes in refractive index are minimized and the sensorgram shows actual binding events. Approximately 700 resonance units (RU) of virus were bound and used for kinetic analysis of MAbs and FAbs. Kinetic measurements for MAbs and FAbs were made simultaneously at $25\,^\circ C$ on the same chip surface to minimize the effect of surface differences on kinetics and over four different concentrations. Antibody in HBS buffer containing 10 ng/ml CM5 dextran was injected over lanes 1 and 2 at a flow rate of 20 µl/min and a dissociation time of 600 s. After dissociation, the chip surface was regenerated to the covalently coupled MAb H37 baseline by eluting bound virus with 0.05 M triethylamine. Kinetics of the binding of the MAbs and FAbs were analysed by using the BLAevaluation software package (version 2.2). Data were analysed by fitting each concentration of antibody to a Langmuir 1-to-1 binding model, with the association and dissociation rates being calculated separately. Initially, the off-rate constant ($k_d$) was calculated by selecting 300 s of dissociation data (kept constant to compare MAbs and FAbs) and this rate was used together with the molar analyte concentration to determine the on-rate constant ($k_a$). The equilibrium dissociation constant ($K_{\text{dissoc}}$) is the ratio $k_d / k_a$.

### Data analysis

Dose–response curves were calculated by non-linear regression analysis using Prism Graphpad software. An unpaired $t$-test was applied to compare curves and $P < 0.05$ was considered significant.

### Results

Neutralization efficiencies of H9 IgG and FAb

MAb H9 (IgG3) binds an epitope in antigenic site Cb of the HA1 subunit (equivalent to site E of the H3 HA) that is situated...
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**Fig. 1.** Location of the epitope of MAb H9 (site Cb/E). The locations of the epitopes of MAbs H36 (site Sb/B) and H37 (site Ca2/A) are shown for comparison (adapted from Wiley et al., 1981 and Caton et al., 1982).

**Fig. 2.** Dose–response relationship of the percentage infectivity of PR8 remaining after incubation with increasing concentrations of H9 IgG (■) and FAb (▼). Virus and antibody were incubated together for 1 h at 37 °C. Data are from plaque assays in MDCK cells and are the means of five experiments, each with three replicates. Virus controls had approximately 40 p.f.u. per plate. Bars represent the SEM. Curves (r > 0.98) were generated by non-linear regression analysis using Prism Graphpad software.

Table 1. Comparison of the neutralizing activities of H9 IgG and FAb in MDCK cells

Data were taken from Fig. 2. The ratios given indicate the excess of FAb over IgG required to achieve the same degree of neutralization.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Concentration (mM) required to give neutralization of</th>
<th>FAb:IgG ratio at neutralization of</th>
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<tbody>
<tr>
<td></td>
<td>50%</td>
<td>90%</td>
</tr>
<tr>
<td>IgG</td>
<td>0.04</td>
<td>0.12</td>
</tr>
<tr>
<td>FAb</td>
<td>15.0</td>
<td>25.0</td>
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</table>

FAb occurred over a similarly narrow concentration range, but the N_{50} and N_{90} required concentrations over two orders of magnitude higher (Table 1). Similar data were obtained when neutralization assays were conducted in BHK cells (data not shown).

**H9 IgG neutralized more infectivity than could be accounted for by inhibition of virus attachment to the target cell, while H9 FAb neutralized without affecting the attachment process**

Data for neutralization and inhibition of virus attachment to MDCK and BHK cells are shown for a range of IgG concentrations. To permit accurate comparisons, we used the same experimental system, the same sized monolayers, the same virus–antibody mixture and the same inoculum volume for both assays. Neuraminidase pre-treatment of the target cell monolayer reduced the level of virus attachment by > 82% and this decreased with increasing antibody concentration, showing that both non-neutralized and neutralized virus bound to NANA receptors and that binding to Fc receptors was not involved (Fig. 3). The proportion of virus that attached decreased with increasing neutralization, but the correlation with IgG concentration in both MDCK and BHK cells was poor (Fig. 3a, b), and curves were significantly different by an unpaired t-test (for P values see Fig. 3). The N_{50} values in MDCK and BHK cells were accompanied by 14 and 22% inhibition of attachment and N_{90} by 52 and 54% inhibition of attachment. Thus, neutralization was complex. The majority of virus was neutralized by a mechanism other than inhibition of attachment. The remainder may have been neutralized by inhibition of attachment and the proportion of attachment inhibition certainly increased with increasing MAb concentration. However, except at very high concentrations of antibody, this did not account for the neutralization observed. Equally, we cannot be certain that IgG is inhibiting attachment of infectious virus, and we may be observing inhibition of attachment of virus that has already been neutralized.

towards the underside of the globular head (Caton et al., 1982; Staudt & Gerhard, 1983) (Fig. 1). Both IgG and the FAb were neutralizing, and data for MDCK cells are shown in Fig. 2. Curves were fitted by non-linear regression analysis and showed that 50 and 90% neutralization by IgG (N_{50} and N_{90}) required 0.04 and 0.12 mM, respectively. Neutralization by
Inhibition of virus fusion by a neutralizing FAb

In marked contrast to the data above, H9 FAb neutralized without any detectable inhibition of virus–cell attachment in both cell types (Fig. 3c, d) and thus inhibits a later stage of the virus–cell interaction.

**H9 IgG- and FAb-neutralized virus that attached to target cells was internalized**

After attaching to the target cell, influenza virus undergoes receptor-mediated endocytosis. The specificity of this reaction was confirmed by chilling cells at 4 °C or pre-treating cells with medium made hypertonic with sucrose (see Methods). Internalization of non-neutralized virus or of H9 IgG- or FAb-neutralized virus by MDCK or BHK cells was inhibited by ≥ 90% at 4 °C and by ≥ 78% by hypertonic medium (data not shown). We found that internalization of virus into MDCK cells, after correcting for inhibition of attachment, was unaffected by increasing concentrations of H9 IgG (Fig. 4a). Similar data were obtained with H9 FAb, although a higher concentration of FAb than IgG was required for a given amount of neutralization (Fig. 4b). Similar data were obtained for BHK cells for both IgG and FAb (not shown). Thus, inhibition of the virus-internalization process was not involved in neutralization by H9 IgG or FAb.

**Inhibition of virus fusion to target cells by H9 IgG and FAb was directly proportional to neutralization**

Since IgG did not inhibit virus internalization, we next investigated virus–cell fusion. Infection of cells at 37 °C with R18-labelled virus results in fluorescence dequenching consistent with fusion of virus and cell membranes. The specificity

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**Fig. 3.** Dose–response relationship between infectivity and attachment of virus to target cells after incubation with increasing concentrations of H9 IgG (a, b) and FAb (c, d). Virus and antibody were incubated together for 1 h at 37 °C. Infectivity and attachment were assayed in parallel by ELISA in the same batch of monolayers in 96-well plates with the same virus–antibody mixtures. MDCK cells (a, c) and BHK cells (b, d) were used and percentage infectivity remaining (▲), percentage attachment of virus to cells (■) and percentage attachment of virus to cells pre-treated with neuraminidase from *Clostridium perfringens* (*) are plotted. Data are the means of five experiments, each with three replicates. The neutralization and attachment assay virus controls gave A$_{405}$ readings of approximately 1–1 and 1–3, respectively. The bar represents the SEM. Curves were generated as described in Fig. 2. r > 0.98 for all infectivity and IgG attachment inhibition curves. P values indicate the significance of the difference between the infectivity and attachment curves by an unpaired t-test; a value < 0.05 is considered significant.
of this reaction was confirmed by chilling cells to 4 °C or pre-treating cells with bafilomycin. These reduced fusion by ≥ 89 and ≥ 79%, respectively (data not shown). Fig. 5 shows the relationship between neutralization and virus-fusion activity in MDCK and BHK cells. R18-labelled non-neutralized virus was lysed with Triton X-100 to give a fluorescence signal that indicated the amount of virus that had attached to the surface of the cells. This value was taken as 100% attachment and the extent of inhibition of attachment by the IgG was determined by comparing the level of fluorescence released after Triton X-100 treatment of the attached neutralized virus with the value obtained for the virus control. The percentage fusion of neutralized virus was then calculated as a percentage of that achieved by the non-neutralized virus control. Although the curves are not perfectly superimposable, they did not differ significantly from each other by an unpaired t-test (see Fig. 5 for P values). Thus, we concluded that neutralization of most of the virus infectivity by H9 IgG was caused by inhibition of virus-induced fusion.

The ability of H9 FAb to inhibit fusion is shown in Fig. 5(c, d). As with IgG, there was a close correspondence between residual infectivity and fusion activity, and the curves did not differ significantly by an unpaired t-test. However, interpretation of the mechanism of neutralization by the FAb is more clear-cut, as neutralization was not accompanied by any inhibition of virus attachment, as discussed above (Fig. 3c, d). Thus, for the FAb, there appears to be a direct causal relationship between neutralization and inhibition of virus fusion.

**Measurement of functional affinity of H9 IgG and the affinity of H9 FAb**

On- and off-rates with immobilized whole virus were measured by surface plasmon resonance using four antibody concentrations. Values for H9 IgG and FAb were very similar and equilibrium dissociation constants for IgG and FAb were calculated as 3.3 × 10⁻¹⁰ and 3.5 × 10⁻¹⁰ M, respectively (Table 2).

**Discussion**

Neutralization of PR8 by H9 and other IgGs

Fifty per cent neutralization (N₅₀) by H9 IgG (specific for antigenic site Cb/E) required 40 nM, whereas neutralization by monoclonals H37 (Ca2/A) and H36 (Sb/B) IgGs required between 0.2 and 5 nM (Edwards & Dimmock, 2000). This is not explained by their functional affinities, as H9 IgG was the most avid (0.33, 0.56 and 0.39 nM, respectively). Nor does the isotype appear relevant, as H9 and H37 are both IgG3 and H36 is IgG2a. Thus, it seems that the epitope per se is the key factor in neutralization by these antibodies, as was found earlier by Schofield et al. (1997b). This may be a positional effect, in the sense that a good neutralizing antibody binds to an epitope that allows it to interfere more efficiently with the functions of the HA than IgGs bound to other epitopes. In addition, the orientation of an IgG molecule with respect to the HA may affect its neutralization efficiency, as antibodies that compete for the same epitope might, for example, bind radially to the HA, bind perpendicularly to the long axis of the HA or bind at any intermediate angle (Schofield et al., 1997a). Differences in antibody action seem unlikely to be due to the nature of the target cell and/or receptor-bearing molecule, as similar results with H9, H36 and H37 IgGs were found using MDCK and BHK cells (Edwards & Dimmock, 2000).

Another reason why H9 neutralized less well than H36 and H37 is that access to its epitope in site Cb/E on the underside of the HA1 globular head is probably less easy than for IgGs to epitopes on the loop or tip of HA1 (Fig. 1). Indeed, while H36 and H37 attached to a BIAcore chip were able to capture virus as it flowed past, H9 was unable to do so (unpublished...
Inhibition of virus fusion by a neutralizing FAb

Fig. 5. Analysis of the relationship between PR8 infectivity and fusion of virus to target cells after incubation with increasing concentrations of H9 IgG (a, b) and FAb (c, d). Virus and antibody were incubated together for 1 h at 37 °C. Data were obtained with 3 cm monolayers of MDCK cells (a, c) and BHK cells (b, d). Percentage infectivity (▲) was determined by plaque reduction and percentage fusion activity (●) was measured by fluorescence dequenching of R18-labelled virus. The fusion assay virus controls gave readings of approximately 300 fluorescence units. Data are the means of two experiments, each with three replicates, and have been corrected for inhibition of virus attachment to target cells. Bars represent the SEM. Curves (r > 0.98) were generated as described in Fig. 2 and P values were calculated as in Fig. 3. A value < 0.05 is considered significant.

Table 2. Functional affinity of H9 IgG and affinity of its FAb for virus particles determined by surface plasmon resonance

<table>
<thead>
<tr>
<th>Antibody</th>
<th>On-rate constant (M⁻¹ s⁻¹)</th>
<th>Off-rate constant (s⁻¹)</th>
<th>Equilibrium dissociation constant (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG</td>
<td>1.08 × 10⁷</td>
<td>3.64 × 10⁻⁴</td>
<td>0.33</td>
</tr>
<tr>
<td>FAb</td>
<td>1.25 × 10⁵</td>
<td>4.47 × 10⁻⁴</td>
<td>0.35</td>
</tr>
</tbody>
</table>

data). However, in the reciprocal arrangement (solid-phase virus/flow-through antibody), H9 exhibited a high functional efficiency (0.33 nM). A similar conclusion was reached with an H7 influenza virus and an IgG that bound with high affinity in an equilibrium system, but completely failed to bind solid-phase virus in a flow system (Schofield & Dimmock, 1996; Schofield et al., 1997a). In another example, a site E MAb had lower neutralization activity than a site B MAb for an H3 virus (Fleury et al., 1999). It seems possible that, in all these cases, the site E MAb is less efficient at neutralizing because it has to interdigitate with HA trimers in order to access its epitope.

Despite differences in the efficiency of neutralization, the three PR8-specific MAbs, H9, H36 and H37, studied here and by Edwards & Dimmock (2000) have very similar mechanisms of neutralization. That is, all MAbs preferentially inhibited fusion activity. However, when antibody concentrations were increased, an increasing proportion of neutralization could be accounted for by inhibition of attachment of virus to the target cell. At intermediate concentrations, both mechanisms operated simultaneously. None of the MAbs had any inhibitory effect on the internalization of virus that attached to
cells. At present, there is little information on the role of HA1 in the virus-fusion process, except that it has to move out of the way of the low pH-triggered extension of HA2 (Doms, 1993; Gaudin et al., 1995). However, dissociation of the trimeric globular head of HA1 was not required for H3 virus fusion at 4 °C or H2 virus at 37 °C (Puri et al., 1990; Stegmann et al., 1990), and it was proposed that fusion only required rearrangement of the HA2 stem (Stegmann & Helenius, 1993; Stegmann et al., 1990). Fusion inhibition by MAbs H9, H36 and H37, which are specific for three different sites on HA1, could be mediated by steric hindrance, a global change in the HA1 structure that is effected by all the MAbs or the involvement of each site in a separate part of the fusion process.

The location of the epitopes suggests an explanation for the varying abilities of antibodies to inhibit attachment to target cells (H36 > H37 > H9) and it seems likely that IgGs that bind to the HA tip or loop would interfere relatively easily sterically with the binding of the cell receptor to the virus-attachment site. However, this would depend on the orientation of antibody with respect to the long axis of the HA trimer, as discussed above. By the same argument, it would seem likely that H9 IgG, which attaches to site Cb/E on the underside of the HA1 globular head, would interfere poorly with cell attachment regardless of its orientation. In fact, it is difficult to see how H9 interferes with virus attachment since, even if it lay radially with respect to the virion, only about half of the IgG molecule would extend beyond the tip of HA1. Alternatively, H9 IgG might cause conformational changes in HA1 that affect the functioning of the virus-attachment site adversely.

Neutralization by H9 FAb

The H9 FAb was capable of > 90% neutralization although, like the H36 and H37 FAbs (Edwards & Dimmock, 2000), it required a molar concentration that was approximately two orders of magnitude higher than that needed for the IgGs (Table 1). A similar differential between IgG and FAbs has been observed by others (Schofield et al., 1997a). This difference in neutralization efficiency of H9 IgG and FAb could not be explained by changes in on-rate or off-rate binding to virus, as these were virtually the same, and the overall equilibrium affinity constants were high (0.033 and 0.35 nM, respectively). The same was true for H36 IgG and FAb (Edwards & Dimmock, 2000). Such results suggest that these IgGs are binding monovalently. Thus, the neutralization differential between IgGs and their FAbs was maintained, regardless of the location of the cognate antigenic site, although one might have expected the smaller H9 FAb to access the Cb/E site more easily than its IgG. The functional affinity of another IgG and FAb to site E of an H3 HA were also similar (Fleury et al., 1999). It seems possible that the FAb is unable to block attachment because it is smaller in size than the IgG and, hence, offers less steric hindrance. However, the reason why the FAb neutralizes less efficiently than the IgG is not clear, as both are thought to bind monovalently and both inhibit fusion.

In contrast to H36 and H37 FAbs, which neutralize by inhibiting attachment (Edwards & Dimmock, 2000), H9 FAb neutralized without any demonstrable effect on virus-attachment (Fig. 3b, c). This is entirely expected, as any FAb binding to site Cb/E would not reach beyond the distal end of the HA. These data contrast with the neutralization activity of the H9, H36 and H37 IgGs, which inhibited a combination of virus fusion and cell attachment (Edwards & Dimmock, 2000). FAb H9 did not inhibit virus internalization, and its dose-response curve for fusion inhibition did not differ significantly from that of neutralization. Thus, it appears that FAb H9 neutralized the majority of the virus by inhibition of fusion. Evidently, H9 does not need to bind bivalently to cause neutralization or fusion inhibition.

Finally, it is interesting to consider how H9 FAb inhibits fusion, since FAbs are monovalent and are unable to cross-link HA1 monomers in the conventional sense. An antibody footprint covers around 20 amino acid residues of antigen (Wilson & Stanfield, 1993) and it may be that the H9 footprint alone is able effectively to hold together parts of the HA1 that the fusion process requires to move apart. This would be intrafootprint cross-linking. Other possibilities are that crosslinking of an HA molecule by antibody stabilizes its structure and decreases the pH required for fusion or that antibody hinders the mobility of the HA trimers in the virion and their ability to form a fusion pore. Further experimentation will be required to address these issues.

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