Enhancement of neutralizing activity of influenza virus-specific antibodies by serum components

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Abstract

The role of serum components in enhancing virus neutralizing (VN) activity of influenza virus A/PR/8/34 hemagglutinin (HA)-specific MAbs in vitro was investigated. The degree of enhancement depended on the MAb's fine specificity and heavy chain isotype and on type of serum. Greatest enhancement (>100-fold) was seen with sera from immunodeficient mice that lacked serum immunoglobulin. At least two serum components were involved: C1q and a heat-resistant factor. C1q was mandatory for enhancement, and other components of the complement system were not required. C1q appeared to operate by improving MAb-mediated inhibition of virus attachment to host cells and was most effective with MAbs that inhibited virus attachment poorly on their own. The heat-resistant factor enhanced VN activity only in the presence of C1q and appeared to operate by enhancing VN activity at a post-attachment stage.

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Introduction

Antibodies (Abs) play important roles in protection against and recovery from influenza virus infection (reviewed in Gerhard, 2001). They may contribute to the control of the infection by reaction with (i) infectious virus particles and thereby impair their infectivity and/or (ii) infected host cells and thereby limit the yield of virus progeny. Abs may mediate protective activities autonomously or in conjunction with other host components such as complement, Fc-receptor (FcR)-expressing cells and lectins.

In a previous study, we compared four influenza virus HA-specific MAbs of G2a isotype for VN activity in vitro and prophylactic activity in a passive transfer system in vivo (Mozdzanowska et al., 1997). Although VN activity in vitro and Ab-mediated prophylaxis in vivo appear to measure similar Ab-mediated activities, we found that the four MAbs exhibited surprisingly large discrepancies in these activities. For instance, one MAb directed to the antigenic region Cb, which is located membrane-proximal of the sialic acid (SA) binding site of HA (Fig. 1) (Caton et al., 1982), exhibited VN activity in vitro at 2.5 μg/ml (Ab concentration at which 50% of MDCK cell cultures are protected from infection by 100 TCID50 of virus) and prophylactic activity in vivo at 8 μg/ml (serum Ab concentration at which 50% of SCID mice are protected from intranasal infection by 200 TCID50) (Mozdzanowska et al., 1997, and unpublished observations). By contrast, another MAb of the same isotype but directed to the antigenic region Sb of HA, which is located membrane-distal of the SA binding site (Fig. 1) (Caton et al., 1982), displayed 2500-fold greater VN activity in vitro (0.001 μg/ml) but only eight-fold greater prophylactic activity (1 μg/ml serum). Thus, in relation to their VN activity in vitro, these MAbs differed by ~300-fold in prophylactic activity in vivo. This suggested that components/conditions in vivo differentially affected their prophylactic activity, e.g., by inhibiting Sb-specific or enhancing Cb-specific MAbs. The latter appeared more likely in view of the finding that performance of the VN assay in vitro in the presence of 1.6% non-inactivated serum resulted in preferential enhancement of the VN activity of Cb-specific MAbs (Mozdzanowska et al., 1997). The general relation between Ab fine specificity and differential enhancement of their hemagglutination inhibition (HI) activity by serum

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components was subsequently confirmed with a large panel of HA-specific MAbs (Feng et al., 2002).

Here, we describe further studies on the enhancement of Ab-mediated VN activity by serum components. We found that at least two serum components were involved: C1q, the binding moiety of the first complement component, and a still undefined heat-resistant factor, whose enhancing activity was dependent on C1q. A high concentration of this factor in sera of mice that lacked serum immunoglobulin appeared to account for their elevated VN enhancing activity. C1q appeared to enhance VN activity at the pre-attachment stage and was effective mainly with MAbs that inhibited virus attachment poorly on their own. By contrast, the heat-resistant factor appeared to enhance VN activity at a post-attachment stage and was similarly effective with MAbs of high or low autonomous VN activity.

Results

Measurement of virus neutralization enhancement (VNE)

VNE was assessed in the MDCK cell assay by measuring Ab-mediated VN activity in the absence and presence of 0.5% non-inactivated naive mouse serum (NMS). Briefly, dilutions of MAbs were incubated in parallel for 60 min at 37 °C with 100 TCID₅₀ of PR8 virus in medium with or without 0.5% non-inactivated NMS. Replicate samples were then transferred to MDCK cell microcultures and incubated for another 60 min at 37 °C to allow initiation of infection by residual infectious virus. The inocula were then replaced by serum-free medium and the cultures tested for productive infection after 3–4 days of incubation. In the absence of MAb, 0.5% NMS had usually no measurable effect on virus infectivity as shown by titration of the virus inoculum in medium with or without NMS (Fig. 2A). Nevertheless, this control was performed in each assay to identify and exclude occasional serum batches that displayed significant VN activity on their own. By contrast, when HA-specific MAbs were tested in the presence and absence of 0.5% NMS, their VN activity (defined as Ab concentration at which 50% of microcultures were protected from infection) was often greatly enhanced in the presence of serum. The difference in VN activity in the presence versus absence of NMS was defined as VNE and is 92 in the example shown in Fig. 2B. VNE decreased with decreasing concentration of NMS and became insignificant below 0.05% of NMS (Fig. 2C).

SCID sera mediate stronger VNE than BALB/c sera

We noticed that SCID NMS mediated 10–20 times stronger VNE than BALB/c NMS against the HA(Cb)-specific MAb H35-C12. To determine whether this was a peculiarity of H35-
C12, we tested three additional MAbs that shared with H35-C12 G2a isotype and specificity for the antigenic region Cb of HA. All displayed substantially stronger VN activity in the presence of 0.5% SCID (148 ± 124, mean, SD) than BALB/c NMS (18 ± 9) (Fig. 3). The same was true for MAbs directed to the antigenic region Sb of HA, although in this case VNE was roughly 10 times smaller than seen with HA(Cb)-specific MAbs.

These findings indicated that HA(Cb)-specific MAbs, which in general display low autonomous VN activity in the absence of serum, are more susceptible for serum-mediated enhancement than HA(Sb)-specific MAbs, which typically display high autonomous VN activity. Furthermore, they suggested that VNE may comprise two distinct and possibly superimposed activities: one mediated by BALB/c NMS and effective in a specificity-dependent fashion (HA(Cb)>HA(Sb)) and the other mediated by SCID NMS and effective in a specificity-independent fashion.

VNE is dependent on the Ab’s Fc region and heavy chain isotype

The role of the Fc region in VNE was investigated with intact IgG and Fab fragment from the HA(Sb)-specific MAb H36-4. As reported previously (Mozdzanowska et al., 2003), IgG and Fab from this MAb exhibited similar VN activity in the absence of serum, but only intact IgG and not Fab were enhanced in the presence of NMS (Fig. 4A). The role of C1q isotype in VNE was investigated with five HA(Cb)-specific MAbs. As found previously in the serum-mediated enhancement of Ab-mediated HI activity (Feng et al., 2002), MAbs of G3, G2b and G2a isotypes showed higher VNE than those of G1 and M isotypes (Fig. 4B). Taken together, these findings indicated that serum mediated VNE through interaction with the Ab’s Fc portion and not by increasing, for instance, the sensitivity of the virus to become neutralized by MAb or by decreasing the susceptibility of MDCK cells for becoming infected.

VNE requires C1q but not C3

We have shown previously that C1q, the binding moiety of the first complement component, was responsible for enhancement of Ab-mediated HI activity by non-inactivated BALB/c- and B6 sera (Feng et al., 2002). To investigate the role of C1q in VNE, SCID NMS was mixed with an equal volume of goat-anti-human (hu)C1q antiserum or PBS, incubated for 1 h at room temperature and then tested for VNE at a final mouse serum concentration of 0.5%. This treatment reduced VNE activity of SCID NMS by ∼99% as did heat inactivation (Fig. 5A). The dependence of VNE on C1q was confirmed by the observation that sera from C1q−/− mice (B6 background) lacked VNE. By contrast, sera from C3−/− mice (B6) exhibited similar VNE as sera from intact B6 mice, indicating that activation of C3, the central complement component, was not required (Fig. 5B). Furthermore, activation of C4 and C2 was not required either as purified human C1q (huC1q) at 0.5 μg/ml, which corresponds roughly to the concentration of C1q in 0.5% NMS (Kerr et al., 1986), exhibited similar VNE as 0.5% B6 NMS, both when diluted in serum-free (BSA-containing) medium or in non-inactivated serum from C1q−/− mice (Fig. 5B). Increasing C1q concentration to 2.5 μg/ml resulted in increased VNE.

These findings were consistent with the notion that C1q was the sole serum component involved in VNE. If so, a difference in C1q concentration may explain the difference in VNE activity mediated by BALB/c and SCID NMS. However, since the latter was not the case (Fig. 5C), the results suggested that, apart from C1q, an additional serum component contributed to VNE.

A heat-resistant factor contributes to VNE

The difference in VNE mediated by BALB/c and SCID NMS could be due to an inhibitory factor in BALB/c NMS or an enhancing factor in SCID NMS.

The former possibility was tested with mixtures of non-inactivated sera from JHD mice, which exhibit high VNE similar to SCID (see below) and heat-inactivated (no VNE) or non-inactivated BALB/c NMS (low VNE). As shown in Fig. 6A, neither heat-inactivated nor non-inactivated BALB/c sera reduced the high VNE of JHD sera, indicating that sera from BALB/c mice did not contain a VNE inhibitory factor.

Next, we performed the reciprocal experiment and tested heat-inactivated SCID sera for ability to enhance VNE when mixed with non-inactivated BALB/c sera. Because of the substantial range in VNE exhibited by the non-inactivated BALB/c sera used in these experiments, samples that were tested in the same experiment are indicated by connecting lines (Fig. 6B), i.e., non-inactivated and inactivated SCID serum, non-inactivated...
BALB/c serum, and the mixture of heat-inactivated SCID- and non-inactivated BALB/c sera. The results showed that heat-inactivated SCID sera, while lacking VNE activity on their own, consistently enhanced VNE when added to non-inactivated BALB/c sera. The smallest enhancement was seen with the SCID serum that exhibited the smallest VNE prior to heat inactivation (Fig. 5B, triangles pointing down). A relatively small enhancement was seen also with a BALB/c serum that mediated high VNE by itself (Fig. 5C, assays marked by circles), possibly because this serum contained a sufficient concentration of the enhancing factor.

Taken together, these observations indicated that at least two serum factors contributed to VNE, the heat-labile C1q and a heat-resistant factor. The latter appeared to be present at higher concentration in SCID than BALB/c sera.

VNE by sera of mice with various types of immunodeficiencies

In view of the marked difference in VNE mediated by sera of immunocompetent (BALB/c, B6) and immunodeficient (SCID) mice, we screened sera from mice with other types of immunodeficiencies for VNE. The HA(Cb)-specific MAb H35-C12 was used in all assays. Fig. 7 shows VNE mediated mostly by sera from individual mice. BALB/c sera exhibited a wide range of VNE (1 to 284) with a geometric mean titer (GMT) of 10. Note that the serum with the very high VNE of 284 was tested in two independent assays, one indicating a VNE of 174 and the other of 395. B6 sera exhibited a slightly but not significantly lower VNE (GMT = 5). As mentioned previously, SCID sera mediated significantly higher VNE (GMT = 103) than BALB/c sera (non-parametric t test, P < 0.001). Similarly, sera from J12D mice on BALB/c background, which contain T cells but lack mature B cells and serum Ig, exhibited high VNE (GMT = 155) as did B cell deficient μMT mice on B6 background (data not shown). Sera from a membrane IgM-transgenic and J12-deleted mouse strain (Chan et al., 1999), which contains T- and transgenic IgM-expressing B cells but essentially no secreted Ig except for some IgA (data not shown), also gave high VNE (GMT = 224). In the case of nude mice of BALB/c background, we found that many sera exhibited significant VN activity on their own at a concentration of 0.5% and were excluded from the graph. However, five sera with low VNE activity exhibited low VNE (GMT = 15). As indicated by the deficiencies of the various mouse strains, high VNE was seen in mouse strains that lacked serum Ig.

Discussion

The study showed that the VN activity of HA-specific MAb H35-C12 was significantly enhanced when the assay was
performed in the presence of 0.5% non-inactivated serum from SCID mice. The degree of enhancement depended on the MAb’s fine specificity – HA(Cb)-specific MAbs being more strongly enhanced than HA(Sb)-specific MAbs and its heavy chain isotype – MAbs of G2a, G2b and G3 isotypes were more strongly enhanced than G1 and M. We provided evidence that at least two serum components were involved: C1q, the heat-labile binding moiety of the first component of complement, and a still unidentified heat-resistant factor, which appeared to be present at higher concentration in sera of mice that lacked circulating Ig than in sera of Ig competent mice (Fig. 7).

Enhancement of Ab-mediated VN activity in vitro by serum components has been reported previously for a variety of viruses (reviewed in Hirsch, 1982; Parren and Burton, 2001) including influenza A (Beebe et al., 1983). The common denominator of previous reports was the requirement for antiviral antibodies, which usually originated from the early immune response and presumably were of low avidity (Daniels et al., 1969; Hirsch, 1982; Linscott and Levinson, 1969) or displayed a unique specificity and exhibited poor VN activity on their own (Britt et al., 1988; Furebring et al., 2002). In addition, there was a common requirement for components of the classic complement pathway. The various systems differed, however, with regard to the extent of complement activation that was required for enhancement of VN activity, which ranged from activation of early components (C1, C4) (Daniels et al., 1969) to activation of the entire complement cascade including the formation of the membrane attack complex (Vasantha et al., 1988). Overall, the present observations fit well into this general picture in that (1) the Cb-specific MAbs, which showed the greatest susceptibility for enhancement, are a dominant component of the primary Ab response (Kavaler et al., 1990, 1991) and display low VN activity on their own (Fig. 3) and (2) a component of the classic complement pathway is involved. The findings described here are unique, however, in that only C1q was required for enhancement. Although we have evidence for deposition of C3 during incubation of virus-Ab complexes in vitro in the presence of 0.5% NMS (unpublished observation), the latter was not required for VN as indicated by similar levels of VN mediated by sera from intact and C3−/− mice (Fig. 5). We conclude that C4 and C2 deposition were not required either because purified huC1q, when used at a concentration corresponding to its anticipated concentration in 0.5% NMS, provided the expected level of VN seen with intact serum (Fig. 5B).

The findings of this study are unique also in that a second serum component was shown to contribute to VN. The nature of this component is presently unknown. Its sole known property is its heat stability, which allowed us to differentiate it from heat-labile C1q and reveal its presence in SCID mouse sera. We assume that a high concentration of this factor is responsible for the high VN mediated by sera from SCID mice and probably also for high VN mediated by exceptional sera from BALB/c mice (Figs. 6 and 7). Interestingly, the factor appeared to be present at increased concentration in sera from mice that lacked serum Ig. This excludes it from being an Ab with specificity for C1q or Ab Fc region, such as rheumatoid factor. Because this heat-resistant factor mediated no VN on its own, as shown by complete abrogation of VN upon treatment of sera with anti-C1q Ab (Fig. 5A) or heat inactivation (Fig. 6B), but substantially improved VN when admixed to non-inactivated BALB/c serum, it most likely operated through interaction with C1q.

Infection of a cell by influenza virus progresses through several steps, starting with the attachment of virus to cellular ligands, receptor-mediated endocytosis, maturation and
translocation of the endosome to the perinuclear region and acid-mediated fusion between viral and endosomal membranes, which ultimately results in the release of viral RNA–protein complexes into the cell’s cytoplasm and their transport into the cell’s nucleus for transcription (Chu and Whitaker, 2004; Lakadamyali et al., 2003; Sieczkowski and Whitaker, 2003). Both attachment and fusion are mediated by HA, the former by interaction of the HA’s sialic acid (SA) binding site with cellular ligands and the latter by a complex acid-induced conformational rearrangement of HA trimers (Skehel and Wiley, 2000). Most HA-specific Abs, including the HA(Sb)-specific MAb H36-4, which bind to regions that are membrane distal of the SA binding site or recognize epitopes that encompass part of the SA binding site, are thought to prevent infection by inhibition of virus attachment (Knossow et al., 2002). However, there is evidence for a minority of Abs that block infection at a post-attachment stage (for review, see Dimmock, 1993; Edwards and Dimmock, 2001; Imai et al., 1998). The HA(Cb)-specific MAb H35-C12 is an example of the latter type of Abs. Binding of this MAb to the Cb region, which is membrane-proximal of the SA binding site (Fig. 1), did not significantly inhibit virus attachment to chicken erythrocytes (Table 1, low HI activity) or to MDCK cells, even at Ab saturation (Mozdzanowska et al., 1997; observations to be published). Yet, H35-C12 exhibited a low level of VN activity (Table 1), which apparently resulted from interference with a post-attachment step of virus entry.

How did C1q and the heat-resistant factor enhance Ab-mediated VN? In the case of C1q, which can interact with a wide range of ligands, it is clear that VNE resulted from its interaction with the Ab’s Fc region rather than with virus or MDCK cells, as it had no effect at all on the VN activity of a Fab fragment (Fig. 4A). A likely explanation is that attachment of the large C1q (460 kDa) to HA-bound MAb resulted in increased steric hindrance of the attachment of virus to cellular ligands (Fig. 8). Accordingly, in the case of the HA(Cb)-specific MAb H35-C12, which was ineffective by itself in preventing virus attachment to chicken erythrocytes, deposition of C1q to virus-bound MAB resulted in a 131-fold enhancement of HI activity and a concomitant, though smaller, increase of VN activity (Table 1). The 10 times smaller enhancement seen with the HA(Cb)-specific MAb of IgG1 isotype (Fig. 4B) can be explained by C1q’s low affinity for this isotype and the low enhancement of HA(Cb)-specific IgM, to which C1q binds well, by the large size of IgM (950 kDa), which probably made already full use of inhibition by steric hindrance. In the case of the HA(Sb)-specific MAb H36-4, which strongly inhibited by itself attachment of virus to chicken erythrocytes (Table 1, high HI activity), increased bulk by deposition of C1q would not be expected to result in much further enhancement of Ab-mediated HI and VN activities, as was found (Table 1). Thus, C1q appeared to mediate VNE by improving Ab-mediated inhibition of virus attachment to cellular ligands and, not surprisingly, was particularly effective in the case of MAbs that bound to HA but inhibited virus attachment poorly on their own (Fig. 8). Analogous relations between Ab fine specificity and enhancement of its functional activity by C1q may operate in other virus systems.

The heat-resistant serum factor appeared to operate differently. First, in contrast to C1q, it affected only VN activity but not HI activity (Table 1, no increase in HI titer in presence of SCID compared to BALB/c NMS), and second, it had a similar effect on both HA(Cb)- and HA(Sb)-specific MAbs (Table 1, ~10-fold enhancement of VN activity of both MAbs by SCID compared to BALB/c NMS). These findings suggest that, unlike C1q, this factor enhanced VN at a post-attachment stage. Further studies are needed to identify the nature of this factor and the precise stage and mechanism of action. Also, our arguments are based on the assumption that HI provides an adequate assessment of virus attachment to MDCK cells, and that BALB/c and SCID NMS are adequate surrogates for C1q and the combination of C1q and heat-resistant factor, respectively. These assumptions need to be validated.

The preferential enhancement of the VN activity of HA(Cb)-specific Abs by C1q in vitro prompted us to test whether C1q contributed to the unexpectedly high prophylactic activity of these MAbs in vivo. To this end, we measured the prophylactic activity of passive HA(Cb)-specific MAb H35-C12 in C1q-competent and -deficient mice. We found (data not shown) that H35-C12 did not exhibit higher prophylactic activity in C1q-competent compared to C1q-deficient mice. Thus, C1q-mediated enhancement of this MAb’s VN activity did not underlie its unexpectedly high prophylactic activity in vivo. However, as virus-Ab complexes formed in vitro in the presence of serum exhibited significantly lower infectivity when inoculated into mice than virus-Ab complexes formed in the absence of serum (unpublished observation), the respiratory tract epithelium was susceptible to the C1q-mediated enhancement phenomenon. Therefore, the most likely reason for not seeing a C1q-dependent enhancement of prophylactic activity in vivo may be an insufficient C1q activity in lining fluid of the non-inflamed respiratory tract, for which there is experimental support (Giclas et al., 1987; Watford et al., 2001). It is conceivable, however, that C1q-mediated enhancement becomes operative at later stages of infection when an increasing inflammatory response results in increasing exudation of serum components into the respiratory tract. This possibility remains to be tested.

Table 1

<table>
<thead>
<tr>
<th>Assay conditions</th>
<th>GMT of MAb activity in μg/ml (n), [enhancement]</th>
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<tr>
<td></td>
<td>HI H35-C12 (Cb)</td>
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<tr>
<td>No serum a</td>
<td></td>
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<tr>
<td>BALB/c NMS b</td>
<td>0.39 (9) [131] 0.42 (25) [7] 0.090 (8) [2] 0.0042 (9) [1]</td>
</tr>
<tr>
<td>SCID NMS b</td>
<td>0.36 (8) [142] 0.035 (26) [89] 0.041 (5) [4] 0.0008 (2) [7]</td>
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</table>

a No serum means PBSN in the case of HI assay and ISC-CM containing 0.1% BSA in the case of VN assay.
b Non-inactivated sera were used at a final concentration of 0.5%. Entries are geometric mean titers (number of independent determinations) and [enhancement compared to no serum value].
In conclusion, the present study revealed a high level of complexity in virus neutralization in vitro and even greater complexity in vivo.

**Materials and methods**

**Virus**

Influenza A virus PR8 [A/PuertoRico/8/34(H1N1), Mt. Sinai strain] was propagated in the allantoic cavity of embryonated hen’s eggs. The titer of infectious virus was determined by limiting dilution in microcultures of Madin Darby canine kidney (MDCK) cells as described (Liang et al., 1994) and 50% tissue culture infectious dose (TCID50) determined by the method of Reed and Munch (1938). Infectious stocks typically contained \(\sim 10^9\) TCID50/ml. Aliquots were stored frozen (−70 °C). Purified virus was prepared from a large batch of allantoic fluid by differential centrifugation and banding in a sucrose gradient and was quantified by HA titration (Scherle et al., 1992) and determination of protein concentration by the BioRad protein assay (biorad.com).

**Media and solutions**

ISC-CM consists of Iscove’s Dulbecco’s medium (Life Technologies, Gaithersburg, MD) supplemented with 0.05 mM 2-mercaptoethanol, 0.005 mg/ml of transferrin (Sigma, St. Louis, MO), 2 mM glutamine (JRH Biosciences, Lenza KS), and 0.05 mg/ml of gentamicin (Mediatech, Herdon, VA). ISC-CM was further supplemented with fetal bovine serum (FCS) (HyClone Laboratories, Logan, UH), bovine serum albumine (BSA) (Sigma), trypsin (Whittaker Bioproducts Inc.), naïve mouse serum (NMS), human C1q (huC1q) (Calbiochem, La Jolla, CA) at specified concentrations. PBSN is phosphate buffered saline containing 3 mM NaN3. PBS(Ca,Mg) is PBS containing 0.9 mM CaCl2 and 0.5 mM MgCl2.

**Mice and sera**

BALB/c mice were obtained from HSD (harlan.com), C57BL/6 (B6) and mice with disrupted transmembrane \(\mu\) exon (\(\mu\)MT) on the B6 background from Jackson Laboratories (jax.org). Nude mice on BALB/c background were purchased from NxGen, Biosciences. C.B-17 mice, homozygous for the severe combined immunodeficiency mutation (SCID), were purchased from the Breeding Facility of the Wistar Institute. Two breeding pairs of mice with disrupted C3 gene on the B6 background (C3\(^{-}\)\(-\)) (Wessels et al., 1995) were provided by Mike Carroll (CBR, Harvard Medical School, Boston). Breeding pairs of C1q\(^{-}\)\(-\) mice on B6 background (Botto et al., 1998) were provided by Valery Fadok (National Jewish Research Center, Denver) with permission from M. Walport and M. Botto. Breeding pairs of JH-deficient mice (JHD) (Chen et al., 1993) on BALB/c background were provided by Mark Shlomchik (Yale). All mice were housed in micro-isolator cages and were maintained under pathogen free conditions. Sera from 10 JHD mice that contained a heavy chain V region transgene with a disrupted \(\mu\) secretion exon and polyadenylation site (Chan et al., 1999) were kindly provided by Mark Shlomchik (Yale). Normal human serum was obtained from a healthy volunteer and C1q-depleted human serum purchased from Calbiochem.

**Antibodies and reagents**

The main MAb used here, H35-C12-6.2 (H35-C12), was generated from a BALB/c mouse undergoing a primary response to PR8 (Kavaler et al., 1990) and is specific for the Cb site of the HA of PR8. It is of G2a/κ isotype. Its sequence (Kavaler et al.,
Measurement of Ab-mediated VN activity in vitro

MDCK cells (5 × 10^4 cells in 0.1 ml IS-CM 5%FCS) were seeded the day before assay into 96 well flat bottom plates (Falcon, Microtest™96). On the day of assay, serial dilutions of MAb were prepared in flat bottom microtiter plates in 160 μl of IS-CM 0.1%BSA (typically eight dilution steps in two replicate columns for generating 12 assay replicates/Ab dilution). An equal volume of PR8 virus at a concentration of 2000 TCID₅₀/ml in IS-CM 0.1%BSA without or with 1% mouse serum was added to the Ab dilution series. After incubation for 60 min at 37 °C, replicate aliquots (50 μl) of the MAb–virus mixtures were transferred to MDCK cell monolayers that had been washed twice with PBS. The plates were incubated for 60 min at 37 °C. The MAb–virus mixtures were then flicked out and the monolayers washed twice with PBS. IS-CM 0.1%BSA containing trypsin (1 μg/ml) was added to the plates (200 μl/well) and incubation at 37 °C continued for 3 to 4 days. Culture supernatants were then tested for presence of virus by HA assay as described (Scherle et al., 1992). The MAb concentration that protected 50% of the cultures from infection was determined by VN activity under the given virus-Ab incubation condition. Each assay included a titration of the virus dose in the presence and absence of 0.5% non-inactivated NMS, or a dilution series of purified huC1q as standard, (2) goat-anti-huC1q, (3) biotinylated mouse-anti-goat-IgG, (4) ExtrAvidin-AP and (5) pNPP. The plates were read and analyzed as above. Because goat-anti-huC1q may react more strongly with huC1q in the standard than with mouse C1q in the serum samples, the assay provides only a relative measure of C1q concentration in NMS.

Statistical analyses

Data were analyzed with the statistical software of Prism, using non-parametric t test for comparison of VNE titers.

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