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A Pulmonary Influenza Virus Infection in SCID Mice Can Be Cured by Treatment with Hemagglutinin-Specific Antibodies That Display Very Low Virus-Neutralizing Activity In Vitro

KRYSTYNA MOZDZANOWSKA, MICHELLE FURCHNER, GEORGE WASHKO, JACEK MOZDZANOWSKI, AND WALTER GERHARD*

The Wistar Institute, Philadelphia, Pennsylvania 19104-4268

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We have previously shown that a pulmonary influenza virus infection in SCID mice can be cured by treatment with monoclonal antibodies (MAbs) specific for the viral transmembrane protein hemagglutinin (HA) but not for matrix 2. Since both types of MAbs react with infected cells but only the former neutralizes the virus, it appeared that passive MAbs cured by neutralization of progeny virus rather than reaction with infected host cells. To prove this, we selected a set of four HA-specific MAbs, all of the immunoglobulin G2a isotype, which reacted well with native HA expressed on infected cells yet differed greatly (>10,000-fold) in virus neutralization (VN) activity in vitro, apparently because of differences in antibody avidity and accessibility of the respective determinants on the HA of mature virions. Since the VN activities of these MAbs in vitro were differentially enhanced by serum components, we determined their prophylactic activities in vivo and used them as measures of their actual VN activities in vivo. The comparison of therapeutic and prophylactic activities indicated that these MAbs cured the infection to a greater extent by VN activity (which was greatly enhanced in vivo) and to a lesser extent by reaction with infected host cells. Neither complement- nor NK cell-dependent mechanisms were involved in the MAb-mediated virus clearance.

We have shown previously (31) that C.B-17 mice with severe combined immunodeficiency (SCID) could be cured of an otherwise lethal pulmonary influenza virus infection by treatment, 1 day after infection, with hemagglutinin (HA)-specific but not matrix 2 (M2)-specific monoclonal antibodies (MAbs). Since both of these MAbs react with the corresponding viral proteins expressed in the plasma membrane of infected cells but only HA-specific MAbs neutralized virus infectivity in vitro, the data appeared consistent with the idea that passive HA-specific MAbs cured the infection by neutralizing progeny virus until all infected host cells had succumbed to the cytopathic effect of the infection. However, we could not exclude the possibility that another antibody-mediated function, which was available to HA-specific but not M2-specific MAbs, underlies the antibody-mediated virus clearance in vivo. To test the latter possibility, we identified a set of HA-specific MAbs that reacted with determinants of the native HA yet displayed highly disparate virus neutralization (VN) activities in vitro and compared them for the ability to cure a pulmonary infection in vivo. The analysis indicates that VN-low HA-specific MAbs cure the infection both by VN activity, which is found to be greatly enhanced in vivo, and by reaction with infected host cells.

MATERIALS AND METHODS

Mice. C.B-17 mice, homozygous for the SCID mutation and referred to here as SCID mice, were bred in the Animal Facility of the Wistar Institute. At 6 weeks of age, the mice were tested for immunoglobulin M (IgM) concentrations in serum by enzyme-linked immunosorbent assay (ELISA). SCID mice with IgM levels in serum of <0.02 μg/ml were used for these experiments, usually at the age of 8 to 12 weeks.

General experimental protocol. SCID mice of either sex were infected with influenza virus PR8 [A/PR/8/34 (H1N1), originally obtained from Mount Sinai Hospital, New York, N.Y.] by exposure to a virus-containing aerosol in an Airborne Infection Apparatus (TriR Instruments, Rockville Center, N.Y.). In some experiments, as noted, the mice were infected under anesthesia by intranasal (i.n.) inoculation of 20 to 40 μl of PR8 in phosphate-buffered saline (PBS). In both methods, 10 to 100 50% mouse infectious doses were applied per mouse. Anesthesia was induced by inhalation of metofane or, in more recent experiments, by intraperitoneal (i.p.) injection of 0.2 ml of ketamine (10 mg/ml)-xylazine (2 mg/ml). At 6 to 24 h later, the mice were given i.p. injections of 0.2 ml of PBS containing the indicated amounts of purified antibody. On days 14 to 16, the mice were anesthetized with ketamine-xylazine and exsanguinated by heart puncture. The thorax was opened, and the lungs were excised and immediately frozen by being placed into a tissue culture dish on dry ice. For determination of virus titer, the lungs were homogenized and debris-free supernatant was subjected to titer determination in indicator cultures of Madin-Darby canine kidney (MDCK) cells as described previously (36). Lung extracts that scored negative in the MDCK assay (threshold of virus detection = $10^{2.1}$ 50% tissue culture infectious doses [$TCID_{50}$]) were tested by inoculation of undiluted extract into the allantoic cavity of two 10-day-old embryonated hen eggs (50 μl/egg, threshold = $10^{1.3}$ 50% egg infectious doses [EID_{50}] per lung).

Media and solutions. Iscove's-modified Dulbecco's medium (ISC-CM; GIBCO, Grand Island, N.Y.), supplemented with 0.05 mM 2-mercaptoethanol, 0.005 mg of transferrin (Sigma Chemical Co., St. Louis, Mo.) per ml, 2 mM glutamine (JRH Biosciences), and 0.05 mg of gentamicin sulfate (Whittaker Bioproducts Inc., Walkersville, Md.) per ml. ISC-CM was further supplemented, as indicated, with fetal calf serum or bovine serum albumin (BSA). PBS containing 0.04% NaN_3 (PBSN) was supplemented, as indicated, with BSA. PBS containing 0.1% Triton X-100 (PBST) was also used.

Virus preparations. PR8 virus was grown in the allantoic cavity of 10-day-old embryonated hen eggs. Aliquots of allantoic fluid containing infectious virus ($10^{8.8}$ $TCID_{50}$ per ml) were stored frozen at −70°C and used as infectious stock. Virus was purified from allantoic fluid by removal of cell debris by low-speed centrifugation (45 min at 250 × *g*), pelleting the virus (2 h at 18,000 rpm in a type 19 rotor), resuspending the virus in PBSN, banding in a linear (15 to 45%) sucrose gradient (45 min at 18,000 rpm in an SW28 rotor), repelleting the virus band (45 min at 25,000 rpm in a T50.2 rotor), and resuspending the pellet in PBSN. Purified virus was quantitated by HA titer determination (see below) and determination of the protein concentration (Bio-Rad Laboratories, Hercules, Calif.) with BSA as standard. One hemagglutinating unit (HAU) of virus corresponds to ~7 ng of viral protein.

Glycoprotein rosettes were prepared by mixing equal volumes of purified virus (at 5 mg/ml) with octylglucoside (150 mg/ml in 0.1 M Tris-HCl [pH 8]–1 mM EDTA). The mixture was incubated for 15 min at room temperature. The cores were pelleted (30 min at 25,000 rpm in an SW50.1 rotor), and the supernatant was dialyzed against PBSN and concentrated in collodion bags (75,000-molecular-weight exclusion; Schleicher & Schuell, Keene, N.H.).

* Corresponding author. Mailing address: The Wistar Institute, 3601 Spruce St., Philadelphia, PA 19104. Phone: (215) 898-3840. Fax: (215) 898-3868. E-mail: GERHARD@wista.wistar.upenn.edu.

Antibodies. H2-4C2 [anti-HA(Cb), IgG2a/k, Cb14 in reference 17] and H36-4-5.2 [anti-HA(Sb), IgG2a/k (38)] were generated by fusion of spleen cells 3 days after secondary immunization with PR8. H35-C12-6.2 and L2-10C1 [both anti-HA(Cb), IgG2a/k (23)] were generated by fusion of spleen cells 5 days after primary immunization. All MAbs were purified from tissue culture fluid or from ascites fluid of SCID mice by adsorption and elution from protein A columns. They were dialyzed against PBS and sterilized by filtration through a 0.45-μm-pore-size filter membrane (Millipore, Bedford, Mass.). The protein concentration was determined by the Bio-Rad protein assay with BSA as a standard and by UV light absorption. The latter was done with GeneQuant (Pharmacia, Piscataway, N.J.), and the protein concentration was calculated from the formula

$$\text{Protein (milligrams per milliliter)} = 1.55 \times (A_{280} - A_{320}) - 0.76 \times (A_{260} - A_{320}).$$

Both determinations were then averaged. The purity of each MAb preparation was verified by minigel electrophoresis (PhastSystem apparatus; Pharmacia).

VN in vitro. The VN activity of MAbs was measured as follows. MAb dilutions (50 μl) in ISC-CM–0.1% BSA, eight replicates per dilution, were dispensed into 96-well flat-bottom tissue culture plates. PR8 (50 μl) in ISC-CM–0.1% BSA (~100 $TCID_{50}$) were added to each well, and the plates were incubated for 1 h at 37°C. MDCK cells were then added to each well (25 μl ISC-CM–0.1% BSA containing 2×10^6 cells/ml), and the plates were incubated for 8 to 14 h to permit MDCK cells to adhere. The medium was then flicked out and replaced with 200 μl of antibody-free ISC-CM–0.1% BSA supplemented with trypsin (2.5% trypsin [Whittaker Bioproducts Inc.]) at a final dilution of 1/3,000 (~8 μg/ml). After another 2.5 days of incubation, culture supernatants were tested for the presence of virus by HA titer determination. The MAb concentration at which 50% of the cultures were protected from infection was computed by interpolation and taken as the MAb VN activity. Note that low concentrations indicate high VN activity.

HA titer determination. Test samples (25 μl) (or 1/2 dilution series thereof in PBSN) in round-bottom polystyrene microtiter plates were mixed with 25 μl of chicken erythrocytes (RBC) (1% in PBSN), and the pattern of RBC sedimentation was recorded after letting the plates sit for 35 min at room temperature. Partial agglutination (ringlike sedimentation pattern) was taken as the end point. The HAU per milliliter was computed by multiplying the inverse of the sample dilution giving an agglutination end point by 4.

HI. The HA inhibition (HI) test was performed in microtiter plates as described previously (35).

Yield reduction. Freshly trypsinized MDCK cells, at 5×10^6 cells/ml of ISC-CM–0.1% BSA, were infected by incubation with 10^3 $TCID_{50}$ of PR8/10^6 cells for 1 h at 37°C, with occasional resuspension. The cell suspension was then diluted to 5×10^5 cells/ml, and 100-μl aliquots were dispensed into wells of flat-bottom microtiter plates which contained 50 μl of antibody dilutions, four to six replicates per dilution. After overnight incubation, 50 μl of medium containing trypsin (32 μg/ml) was added to each well, and the cultures were incubated for an additional 2.5 days. The virus titers of culture supernatants were then determined by HA titer determination. The yield reduction (YR) activity was computed as the MAb concentration at which the virus yield was reduced by 75% compared to antibody-free control cultures.

Determination of antibody concentration by ELISA. The ELISA was performed as described previously (23) with, as the solid-phase immunoadsorbent, 20 HAU of purified PR8 (in 25 μl of 0.02 M NaCl) dried into wells of polyvinyl plates (overnight at room temperature in a fume hood). The assay comprised a 90-min incubation with 25 μl of antibody test sample, three washes, a 90-min incubation with 25 μl of biotinylated MAb 187 (rat anti-mouse C_κ, 0.5 μg/ml of PBSN–1% BSA), three washes, a 90-min incubation with 25 μl of Avidine-AP in PBSN–1% BSA (diluted as recommended by the manufacturer [Sigma]), three washes, and a 30-min incubation with 50 μl of substrate (pNPP at 1 mg/ml in substrate buffer, both from Sigma). The plates were read in an ELISA reader (Emax; Molecular Devices, Sunnyvale, Calif.) at $A_{405-750}$.

Test for escape mutants by ELISA. Virus present in lung extracts was grown up in the allantoic cavity of embryonated hen eggs and was quantitated by HA titer determination. Two sets of solid-phase immunoadsorbents, each containing virus at 20, 5, 1.25, and 0.3 HAU per 25-μl dose, were prepared from each isolate. One set was then tested with the MAb (1 μg/ml) used for treatment of the donor mouse, and the other set was tested with a MAb specific for the HA2 polypeptide and used as positive control for the presence of viral HA. Virus isolates which failed to react with the selecting MAb but reacted well with the HA2-specific control MAb were considered to consist mainly of escape mutants.

Measurement of antibody avidity with the IASYS Bio Sensor. Purified HA (5 μg/50 μl) was coupled (10 min at room temperature) to a carboxymethyl dextran-covered cuvette (Fison, Cambridge, United Kingdom) by means of *N*-hydroxysuccinimide and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide as recommended by the manufacturer. Unreacted sites were inactivated by washing with Tris buffer (150 mM; pH 8), and the cuvette was equilibrated by incubation with PBST–2% BSA. For binding studies, MAb in 200 μl of PBST–2% BSA was added to the cuvette, and data were collected with the Bio Sensor during MAb association for approximately 10 min. The MAb solution in the cuvette was then replaced with PBST–2% BSA, and MAb dissociation data were collected for approximately 5 min. The cuvette was then washed with PBST, and bound MAb was eluted by incubation with 10 mM HCl, equilibrated with PBST–2% BSA, and used for another measurement. Each MAb was tested at several concentrations ranging from 1.25 to 125 nM. Rates of association [k(on)] and dissociation [k(off)] were calculated by means of the IASYS/FASTFIT software provided with the Bio Sensor. Because of very slow dissociation kinetics observed with all but one of the MAbs, values obtained from the first phase of dual-phase association were used for the calculation of the association and dissociation constants (K_A and K_D). The good agreement of the K values determined independently from the association and dissociation phases of one MAb confirmed the validity of the analysis.

FCM. P1.HTR cells (45) were maintained in ISC-CM–5% FCS containing bromodeoxyuridine (0.1 mg/ml). For infection, they were washed with ISC-CM–0.1% BSA and suspended in this medium at 2×10^6 cells/ml. PR8-containing allantoic fluid (~$10^{6.4}$ $TCID_{50}$/10^6 cells) was added, and the cells were incubated for 1 h at 37°C with occasional rocking. The culture was then diluted fivefold with ISC-CM–5% FCS and incubated overnight (~16 h) at 32°C. Aliquots (8 ml) of the culture were then transferred to 15-ml centrifuge tubes. Percoll (100% in PBS [Pharmacia]) was added to give a final concentration of 33%, and each suspension was underlaid with ~2 ml of Percoll (70% in PBS) and centrifuged for 10 min at $600 \times g$ at room temperature. The cells at the 33%/70% interface were harvested, pelleted, and resuspended in cold flow cytometry (FCM)-diluent (PBSN–1% BSA–1% FCS) to give 20×10^6 viable cells/ml. The FCM staining procedure was as follows. Cells (0.5×10^6 in 25 μl) plus 25 μl of MAb (10 to 500 ng) in FCM diluent were incubated in microcentrifuge tubes on ice for 45 min, washed once, resuspended in 50 μl of an optimal dilution of fluorescein isothiocyanate-labeled donkey anti-mouse Ig (Jackson Laboratory, Bar Harbor, Maine), incubated on ice for 45 min, washed once, and resuspended in 0.5 ml of PBSN–1% BSA. Samples were analyzed with an Ortho Cytofluorograf System 50 connected to a 2150 Data Handling System (Ortho Diagnostic System, Inc., Mass.). The gating was set by forward and right-angle scatter for viable cells, and 5,000 events were analyzed.

Natural killer (NK) cell depletion experiments. SCID mice were given i.p. injections with 0.2 ml of PBS containing rabbit anti-asialo GM1 antiserum (Wako Pure Chemical Industries, Osaka, Japan) at 1/8 dilution or normal rabbit serum at 1/8 dilution. These injections were given on day -2 (2 days before infection), day 1, and day 8. The degree of NK cell depletion was tested in uninfected mice by i.p. injection of 100 μg of poly(I-C) (to activate NK cells) on day 14 and measurement of NK cell activity in spleen cell suspensions on day 15, with YAC cells as targets in a 5-h ^{51}Cr release assay as described previously (35).

C3 depletion experiments. SCID mice were given i.p. injections of cobra venom factor (CVF; from *Naja naja kaouthia* [Sigma]), 4 U (28 μg) on day 0 and 2 U on day 1 and day 2. Complement activity was determined on day 3 by measuring the lysis of opsonized human RBC in the presence of plasma dilutions from the experimental mice. The RBC (H reagent RBC; Gamma Biological, Houston, Tex.) were opsonized with the mouse anti-H IgM hybridoma PRD8 (kindly provided by M. Thurin, The Wistar Institute). The degree of lysis was determined after a 1-h incubation at 37°C by measurement of the optical density ($OD_{405-750}$) of the supernatant. Specific lysis was computed by subtracting from the total OD at each plasma dilution the spontaneous lysis (opsonized RBC without serum) and the OD of the plasma alone at the given dilution.

RESULTS

Structural properties and in vitro functional activities of the antibodies. The main objective of this study was to determine whether anti-HA MAbs owed their ability to cure a pulmonary virus infection in SCID mice to their VN activity. We thought that this question could be answered by means of anti-HA MAbs that differed greatly in VN activity yet reacted equivalently with native HA. To identify such MAbs, we screened a panel of 191 HA-specific IgG hybridoma culture fluids for HI titer and antibody concentration, the latter by standard ELISA (HI activity correlates well, in general, with VN activity but is less cumbersome to measure than VN activity). Of the hybridoma culture fluids, 80% exhibited more than 1 HI unit/μg of MAb/ml of culture fluid (average of 21) and 20% exhibited less than 1 HI unit per μg of MAb/ml (average of ≤0.2). Since some of the latter MAbs could conceivably be HI negative because they reacted with denatured HA, we screened them further for reaction with viable infected cells by FCM analysis. Based on the results of these analyses, we selected four MAbs of the IgG2a isotype, one from the HI-high group (H36-4) and three from the HI-low/negative group (H2-4C2, H35-C12, and L2-10C1). The HI-high MAb is specific for antigenic site Sb, which is located on the very tip of the globular head of HA (9) and corresponds to site B of the H3 subtype (43, 44). The three HI-low/negative MAbs are specific for the antigenic site Cb,

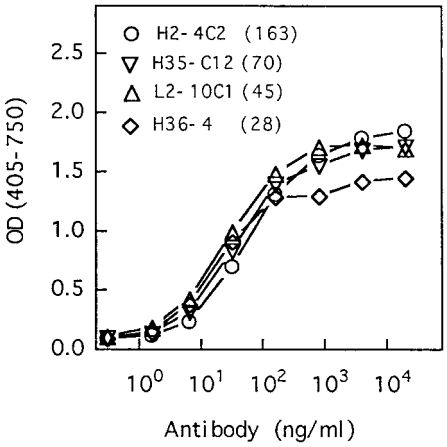

FIG. 1. Binding of MAbs to purified virus in ELISA. Dilutions of purified MAbs were tested in parallel for binding to purified PR8 solid-phase immunoadsorbent. Mean binding values (of triplicate determinations) measured in a representative assay are shown. The mean antibody concentration (in nanograms per milliliter) giving 50% maximum binding is shown in parentheses after each MAb name; it was computed from four independent assays.

which is located in the middle region of the globular head of HA, membrane-proximal to the receptor binding site (9), and corresponds to site E of the H3 model (43, 44). The MAbs were then purified, either from ascites fluid of SCID mice or from hybridoma tissue culture fluid, and compared for various functional activities in vitro.

The binding of the MAbs to purified PR8 virus in ELISA is shown in Fig. 1. The MAbs displayed slightly different binding activities, as shown by the distinct antibody concentrations required for half-maximum binding (Fig. 1). On average, H36-4 was 1.6 (45/28), 2.5 (70/28), and 5.8 (163/28) times more active in this assay than were L2-10C1, H35-C12 and H2-4C2, respectively. Figure 2 shows the reaction of these MAbs with PR8-infected cells measured by FCM. When tested at a subsaturating concentration (the data in Fig. 2 were obtained with 200 ng/ml), H36-4 gave 2.3 (48.8/21.1), 2.6 (48.8/18.4), and 4.5 (48.8/10.8) times more intensive staining than did L2-10C1, H35-C12, and H2-4C2, respectively. Thus, the MAbs displayed similar relative binding activities in ELISA and FCM. Two additional conclusions can be drawn from the FCM analysis shown in Fig. 2. First, MAb CM1-4, which reacts with denatured but not native HA, failed to stain the infected cells. Accordingly, the above four MAbs must all recognize determinants present on native HA. Second, the absence of staining by the M1-specific MAb, which is directed to a viral protein expressed solely intracellularly, shows that only antigens expressed on the cell surface are being detected.

In spite of similar reactions (within a fivefold range) of the MAbs with native HA, the MAbs showed large differences (up to 11,000-fold) in VN activity when tested in the standard VN assay in the absence of serum (Table 1). The VN activities were differentially enhanced (2- to 70-fold) when the assay was performed in the presence of 1.65% non-inactivated SCID mouse serum, but the differences between the values for the MAbs remained large (>1,000-fold). The effects of higher serum concentrations (3.3%) on the VN activity were difficult to interpret because serum on its own significantly inhibited virus infection at this concentration (data not shown).

Additional experiments were performed to identify the reason(s) for the large differences in VN activity among these MAbs. Antibody avidities (in their reaction with surface-bound

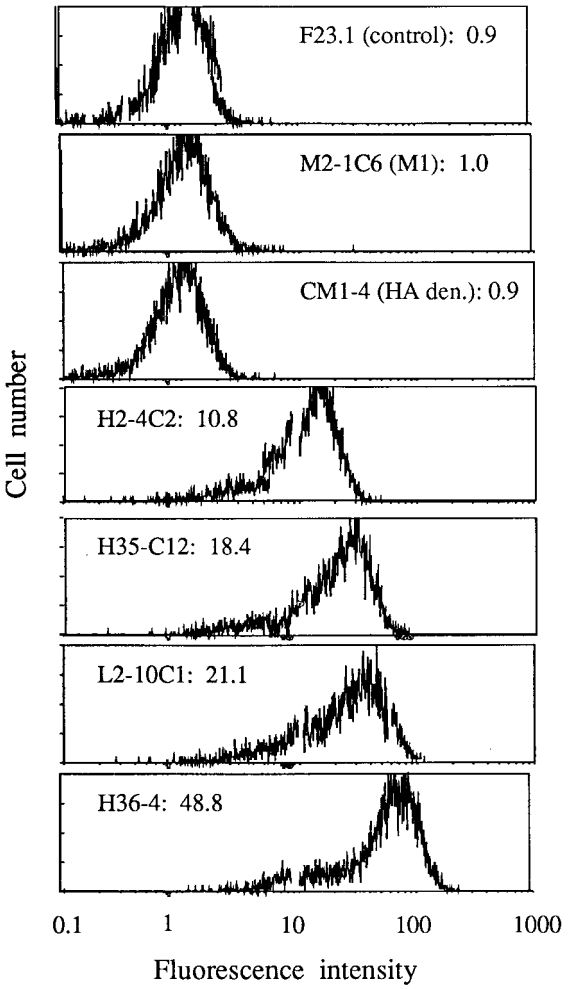

FIG. 2. Binding of MAbs to infected cells. The FCM analysis shows the reaction of MAbs at 0.2 μg/ml with PR8-infected P1.HTR cells. The MAbs used are indicated within each panel. Nonspecific background staining was determined with the nonreactive T-cell receptor Vb-specific MAb F23 (IgG2a). Cell-bound MAbs were detected with fluorescein isothiocyanate-labeled donkey anti-mouse Ig. Numbers show the mean fluorescent intensity. Live cells were gated by forward and right-angle scatter. Five thousand events were analyzed.

purified HA) were measured by plasmon resonance and found to differ within a 20-fold range (Table 1). They correlated inversely with the VN activities, except for H35-C12. Interestingly, a better inverse correlation existed between avidity and the degree of enhancement of the VN activity by serum. This supports the contention that serum preferentially enhances the activity of antibodies of low avidity (2). Differences in antibody specificity also played a role in determining the VN activity. This was indicated by the finding that the HA(Cb)-specific MAbs exhibited greatly increased HI activity against glycoprotein rosettes compared to that against intact virus (Table 2). A straightforward explanation for this observation is that the wider angle between HA spikes in rosettes than in virions improves the accessibility of the HA(Cb) site to MAbs. The HI activity against rosettes shows that these MAbs, once bound to the HA(Cb) site, can perfectly well inhibit hemagglutination and suggests that their inability to inhibit hemagglutination by intact virus is due to their ineffective binding (for steric reasons) to HA of intact virus in solution. The same argument would also explain their low VN activity. Consistent with this

TABLE 1. In vitro functional activities of antibodies

Antibody			K_A (M^{-1})	Concn (μg/ml) at which 1 U of activity is displayed			
Name	Ig class	Protein		VN[a] (no serum)	VN[a] (1.65% serum)	Fold enhancement by serum	YR[a] (no serum)
M2-1C6	IgG1	M1	ND[b]	>10	>5		>5
H2-4C2	IgG2a	HA(Cb)	2×10^7	~10	0.7 ± 0.5	14	0.87 ± 0.18
H35-C12	IgG2a	HA(Cb)	0.8×10^7	2.5 ± 0.6	0.034 ± 0.004	70	0.35 ± 0.18
L2-10C1	IgG2a	HA(Cb)	8×10^7	0.29 ± 0.10	0.023 ± 0.005	12	0.036 ± 0.021
H36-4	IgG2a	HA(Sb)	2×10^8	0.0009 ± 0.0003	0.0004 ± 0.0003	2	0.0025 ± 0.0005

[a] VN and YR activities are the mean ± standard error of the mean in three to five independent assays. For definition of activities, see Materials and Methods.
[b] ND, not done.

interpretation is the finding that the HA(Sb)-specific MAb, which binds to a region on the exposed tip of HA, showed only a threefold-enhanced HI activity against rosettes compared to that against virus. Other investigators have made similar observations (1, 24). In conclusion, differences in both avidity and accessibility of the respective determinants on HA of intact virus appear to underlie the large differences in VN activities displayed by these MAbs.

Curing efficacy of MAbs in vivo. MAbs were administered i.p. 1 day after infection of SCID mice with aerosolized PR8, and the status of the infection was assessed by determination of the virus titer in lungs 14 to 16 days later. Failure to detect infectious virus by inoculation of lung extracts into embryonated hen eggs ($<10^{1.3}$ EID_{50}/lung) was taken as evidence for cure. If virus was present, it was expanded by one passage in hen eggs and then analyzed by ELISA for recognition by the treatment MAb. If the treatment MAb failed to show significant reaction with the virus isolate, we concluded that the passive MAb had cured the wild-type virus infection and that the residual infection was now due to viral escape mutants which could no longer be controlled by the given MAb. This happened in 11 of 89 MAb-treated mice with residual infection. In addition, the antibody concentration in serum was measured at the termination of experiments to exclude the possibility that a failure to cure was due to an inadequate injection (into the bladder or intestine) of the treatment MAb.

Treatment of infected SCID mice with 200 μg of anti-M1 MAb had no detectable effect on the course of the infection compared to the nontreated control animals (Table 3). By contrast, three anti-HA MAbs cured 100% of the mice at a dose of 200 μg and one (H2-4C2) cured 38% of the mice. The first three MAbs were further tested at decreasing doses to obtain a measure of their curing activities. From these experiments, we estimated the 50% curing doses per mouse to be >200 μg for H2-4C2, ~100 μg for L2-10C1, ~50 μg for H35-C12, and ~35 μg for H36-4. Thus, the MAbs differed from each other surprisingly little in their curing activities, in marked contrast to the large differences in their VN activities. Clearly, VN activity measured in vitro, even in the presence of serum, gave a poor prediction of the curing activity of the MAb in vivo. Analogous observations have been made in many other virus systems in which the ability of MAbs to reduce mortality were tested, although these experiments were usually performed in immunocompetent mice and thus were in conjunction with effectors of the endogenous immune response (5, 6, 8, 22, 25, 28, 32, 34, 37, 41, 42).

TABLE 2. HI activity against intact virus and glycoprotein rosettes

Antibody		HI activity (μg/ml)[a] versus:	
Name	Protein	Intact virus	Rosette (enhancement)
M2-1C6	M1	>10	>5
H2-4C2	HA(Cb)	>10	0.44 (>22)
H35-C12	HA(Cb)	>10	0.020 (>500)
L2-10C1	HA(Cb)	4	0.025 (160)
H36-4	HA(Sb)	0.025	0.008 (3)

[a] The values are the mean of two independent assays in which the purified MAbs were tested in parallel for HI titer against four agglutinating doses of intact virus or glycoprotein rosettes. HI activity is expressed as the MAb concentration at which 1 U activity is produced. Enhancement, shown in parentheses, is the ratio of HI activities against intact virus and rosettes.

Comparison of prophylactic and therapeutic activities in vivo. The above observation did not exclude VN activity from playing a major role in the antibody-mediated cure because we could not dismiss the possibility that the VN activities of these MAbs in bronchial secretions paralleled their curing activities, particularly since we had shown that serum components differentially affected their VN activities. To address this issue, we compared the activities of MAbs in preventing disease when given before infection. The premise was that MAbs given prophylactically were likely to protect by neutralizing the input virus inoculum. For these studies, SCID mice were given i.p. injections of graded doses of MAb 1 day before or 6 h after infection and the virus titer in the lung was determined 6 days later. The therapeutic activity was redetermined in these experiments because a different infection protocol was used which consisted of inhalation, under anesthesia, of 30 μl of virus inoculum rather than the previously used inhalation of aerosolized virus (the aerosolizer apparatus had broken down irreparably).

These experiments (Fig. 3) indicated that 50% prophylactic doses of ~60 μg for L2-10C1, ~20 μg for H35-C12, and ~3 μg for H36-4. H2-4C2 protected fewer than 50% of the mice at a treatment dose of 200 μg (4 of 11 mice were protected [37%]). If prophylactic activity is the in vivo expression of VN activity, the large differences in VN activity in vitro between these MAbs shrank considerably in vivo but still amounted to ~7, ~20, and >70 when comparing H36-4 to H35-C12, L2-10C1, and H2-4C2, respectively. It is also noteworthy that H35-C12 displayed higher prophylactic activity in vivo but lower VN activity in vitro than L2-10C1. This appears to continue a trend indicated by the VN assays performed in the absence and presence of serum (Table 1), i.e., that the VN activity of H35-C12 was more prone to enhancement by serum components than was the VN activity of L2-10C1. Thus, 1.65% serum enhanced the VN activity of H35-C12 and L2-10C1 70- and 12-fold, respectively, and, by extrapolation, a further increase of serum-like effects by bronchial secretions could conceivably increase the VN activity of H35-C12 above that of L2-10C1. This behavior gives credence to the assumption that the pro-

TABLE 3. Curing activity of MAbs in vivo[a]

Treatment		Lung infection			Residual MAb (μg/ml of serum) (mean ± SD)
Antibody	Dose (μg)	No. (%) cured	No. infected	Log_{10} titer (mean ± SD)	
None	0	1 (4)	24	6.27 ± 0.67	
M2-1C6 (M1)	200	0 (0)	8	6.46 ± 0.59	30.7 ± 7.3
H2-4C2 (HA)	200	3 (38)[b]	5	4.45 ± 1.00	23.8 ± 8.3
L2-10C1(HA)	200	4 (100)	0		17.4 ± 4.4
	50	2 (29)[b]	5	2.82 ± 1.35	5.8 ± 2.5
	10	0 (0)	4	3.98 ± 1.21	0.4 ± 0.2
H35-C12(HA)	200	4 (100)	0		19.0 ± 12.2
	50	3 (50)	3	3.31 ± 1.82	5.9 ± 3.0
H36-4 (HA)	200	4 (100)	0		18.3 ± 5.1
	50	7 (87)[b]	1	2.75	4.0 ± 1.7
	10	0 (0)	8	5.51 ± 0.63	0.3 ± 0.1

[a] SCID mice were given i.p. injections of the indicated doses of MAb 1 day after infection with aerosolized PR8, and virus titers in lung extracts and passive MAb titers in serum were measured 14 to 16 days later. Failure to detect wild-type virus by inoculation of lung extract into the allantoic cavity of embryonated hen eggs was taken as evidence of cure of the infection. The lung virus titer (mean ± standard deviation [SD]) is shown only for mice with residual wild-type virus infection. The data are a compilation of several experiments, each comprising an untreated control group and several treatment groups of three to four mice each.

[b] One mouse within the group infected with escape mutants.

phylactic activity indeed reflects VN activity of these MAbs in situ.

The therapeutic activities measured in these experiments were similar to those measured in the previous experiments (Table 3). In all cases, cure required a larger MAb dose than did prophylaxis.

The relationship between the prophylactic and therapeutic activities of individual MAbs is shown in Fig. 4. For this plot, the data shown in Table 3 and Fig. 3 were combined and recalculated for 37% cure and prophylaxis, respectively, so that MAb H2-4C2 could also be included. The plot revealed a fairly linear relationship among these activities. The segment of the curve connecting H35-C12, L2-10C1, and H2-4C2 was a straight line with a slope of 0.8. This plot suggested that VN, or, more generally, the activities used in prophylaxis, also played a significant role in therapy. In addition, the fact that the curve did not extrapolate to the origin but intersected the *x* axis indicated that part of the passive MAb used in therapy was consumed for an activity other than VN. This most probably reflected the reaction of the MAbs with infected host cells. Such cell-bound MAb may contribute to cure by targeting infected host cells for complement-dependent effector mechanisms and/or FcR- and CR-expressing effector cells. This was tested in the following experiments.

Depletion of C3 or NK cells does not diminish the therapeutic activity of MAbs. SCID mice were depleted of C3 by daily i.p. injections with CVF. The mice were infected with PR8 at the time of the first CVF injection and were treated 6 h later with 200 μg of MAb L2-10C1. On day 3, a blood sample was obtained for subsequent measurement of complement activity, and the mice were then killed for determination of virus titers in the lungs. Although CVF treatment decreased the complement activity in plasma to <2% of that in the control mice (Fig. 5, inset), it did not detectably reduce the curing activity of MAb L2-10C1.

NK cells were depleted by treatment of SCID mice with rabbit anti-asialo-GM_1 antiserum. Control mice received an analogous treatment with normal rabbit serum. All mice were infected on day 0 by i.n. inoculation of PR8. On day 1, some of the mice in each group were treated with 200 μg of H35-C12. The mice were killed on day 16, and the virus titer in the lungs was determined. In addition, several mice, which underwent the antibody treatments but were not infected, were given i.p. injections of 100 μg of poly(I-C) on day 14 (to induce NK cell activity) and spleen cells were tested the next day for the presence of NK cell activity. As shown in the inset in Fig. 6, the anti-asialo-GM_1 treatment resulted in a strong reduction of the NK-cell activity in these mice. However, the presence or absence of NK cells had no detectable effect on the therapeutic activity of the MAb. The virus challenge dose used in both experiments in Fig. 6 was two to three times larger than the standard dose used in the experiments in Fig. 3 and 5. This may explain the more virulent course of the infection (most of the untreated mice had died by day 16) and the finding that most MAb-treated mice showed residual infections by escape mutants. The parental virus infection, however, had apparently been cured in all mice tested. Depletion of NK cells also had no detectable effect on the course of the infection in the absence of MAb treatment.

These findings thus indicate that complement and NK cells do not play an indispensable role in the cure mediated by these VN-low MAbs.

Activity of MAbs when present in vitro during the entire virus culture period. It is well established that antibodies can also interfere with virus replication and maturation merely by binding to infected cells, independent of complement- or FcR-expressing effector cells. To test this activity, MAbs were added to cultures that had been infected at low multiplicity, and they remained present throughout the subsequent culture period (by contrast, MAb was present only during the initial infection period in the VN assay). Virus yield was then measured, and the antibody activity was expressed as the concentration required for 75% virus YR. MAbs may act in this assay both by inhibiting the release of virus from infected cells and by neutralizing released progeny virus. Mean YR activities are listed in Table 1. It is evident that the YR activity is not a simple reflection of the VN activity of the MAb. Instead, it is increased relative to the VN activity in the case of the HA(Cb)-specific MAbs and decreased in the case of the HA(sb)-specific antibody. This supports the notion that the activity measured in this assay does not result merely from neutralization of progeny virus but also from inhibition of maturation and/or release of virus from infected cells. Overall, the differences among MAbs in the YR assay were smaller than in the VN assay performed in the absence of serum. However, they still greatly exceeded those observed in cure in vivo. The YR assay could not be performed in the presence of non-inactivated serum, because serum interfered with the measurement of virus yield.

DISCUSSION

To confirm our previous proposition that passive MAbs cure a pulmonary influenza virus infection in SCID mice by neutralizing progeny virus and thus preventing the spread of the infection until all infected host cells have succumbed to the viral cytopathic effect, we selected a group of four HA-specific MAbs with the IgG2a isotype that differed greatly in VN activity when tested in vitro and compared them for curing effi-

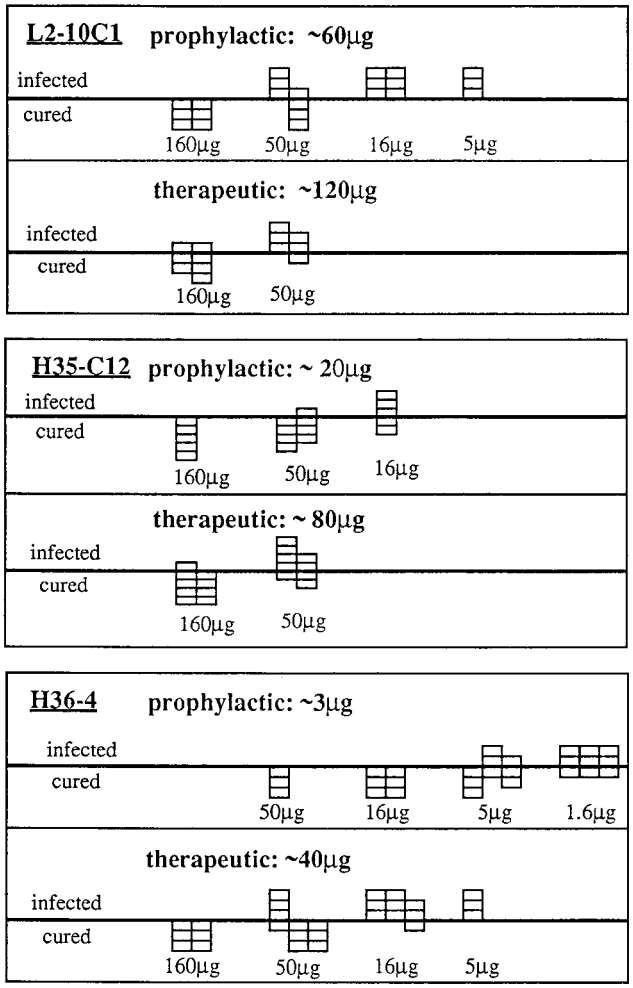

FIG. 3. Comparison between prophylactic and curing activities. SCID mice were given i.p. injections of the indicated doses of MAb 1 day before (prophylactic) or 5 to 6 h after (therapeutic) infection. The infection was initiated by i.n. inoculation under anesthesia of 30 μl of PR8 (100 $TCID_{50}$). The status of the infection in the lungs was tested 6 days later. The lung infection was considered cured if no wild-type virus could be recovered by inoculation of lung extract into the allantoic cavity of embryonated hen eggs ($<10^{1.3}$ EID_{50} per lung). The data are from several experiments. Each box within a column indicates an individual mouse, and replicate columns at a given MAb dose show data from independent experiments. Prophylactic and curing activities were computed by interpolation and indicate the micrograms of MAb required per mouse for 50% protection or cure, respectively.

cacy in vivo and various addition activities in vitro and in vivo. The data in these analyses are summarized in Table 4, in which all activities are expressed relative to MAb H36-4, which displayed the highest activity in each assay. The analysis revealed that VN activity measured in vitro predicted the curing activity of the MAb only poorly and even ranked the MAbs incorrectly. However, VN activity in vitro may not be an adequate measure of VN activity in vivo (13, 29) because the latter may be influenced by additional factors such as host cell types which behave differently from the cell lines used in vitro, the presence of substances that inhibit viral infectivity (3, 19, 20) or enhance antibody activity (2), and the presence of FcR- and CR-expressing cell types which may take up and degrade opsonized virus (5, 36). Indeed, we found that serum components differentially enhanced the VN activity of these MAbs in vitro (Table 4). Thus, to take all these factors into account, we measured the activity of the MAbs in preventing an infection when given before virus challenge. The assumption was that this prophylactic activity would provide the most realistic measure of VN activity in vivo. Indeed, prophylactic activity paralleled therapeutic activity better than did VN activity measured in

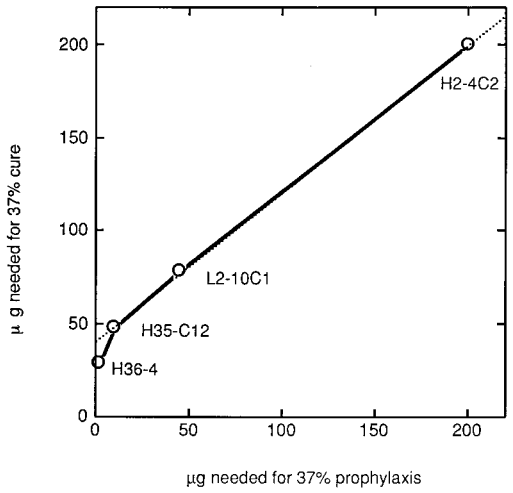

FIG. 4. Comparison between prophylactic and therapeutic activities. The data shown in Table 3 and Fig. 3 were combined and recalculated for provision of 37% cure and prophylaxis. This was done so that data for H2-4C2 could be incorporated.

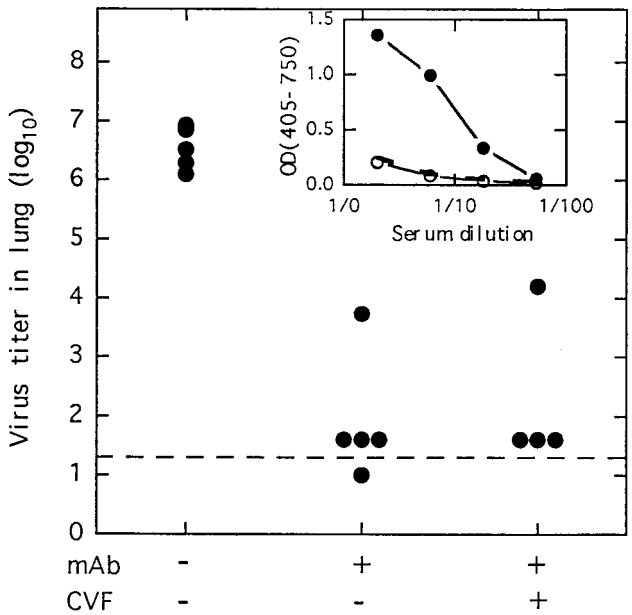

FIG. 5. MAb curing activity is not decreased in decomplemented SCID mice. SCID mice were depleted of complement component C3 by daily treatment with CVF, and MAb L2-10C1 (200 μg i.p.) was then tested for the capacity to cure a pulmonary infection in these C3-depleted mice. SCID mice were infected by i.n. inoculation of 30 μl of PR8 (100 $TCID_{50}$) and treated 6 h later by i.p. inoculation of MAb, and 3 days later, the status of the lung infection and complement activity in plasma was determined. Each dot shows the virus titer in the lung of an individual mouse. The dashed horizontal line indicates the threshold of virus detection, i.e., $10^{1.3}$ EID_{50} per lung. The inset shows the complement activity of mouse plasma, as determined by the lysis of human RBC opsonized with blood group O-specific mouse IgM antibody. Open symbols, mean for two CVF-treated mice; solid symbols, mean for two control mice. The dashed line in the inset shows the background OD of the pooled plasma.

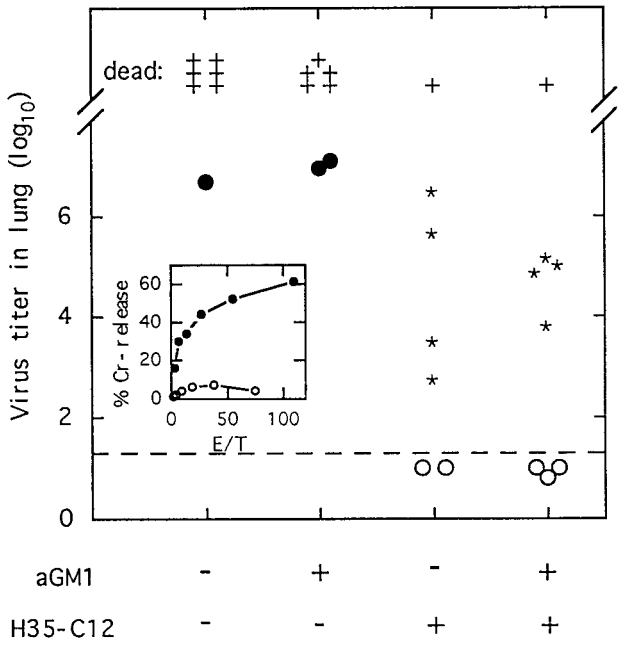

FIG. 6. MAb curing activity is not diminished in NK-cell-depleted SCID mice. SCID mice were treated with anti-asialo GM_1 serum or with normal rabbit serum on days -2, 1, and 8, infected with 300 $TCID_{50}$ of PR8 on day 0, and treated with 200 μg of H35-C12 on day 1, as indicated. Virus titers in the lungs of surviving mice were determined 16 days later. The data from two independent experiments are shown. Plus signs indicate individual mice which did not survive 16 days. Solid circles show virus titers in the lungs of individual mice that were infected with wild-type PR8 virus. Asterisks show virus titers in the lungs of individual mice that were infected with mutant viruses with which H35-C12 failed to react. Open circles indicate mice in which no residual infectious virus could be detected. The inset shows NK activity detected in the spleens of mice which were similarly treated with anti-asialo GM_1 (open symbols) or normal rabbit serum (solid symbols) but were not infected. These mice were given injections of poly(I-C) on day 14, and NK activity was measured in spleens (two mice per group) 1 day later by a ^{51}Cr-release assay with YAC cells as targets. Mean specific releases at various effector-to-target ratios are shown.

vitro, and the MAbs were ranked in the same order in both prophylaxis and cure. The relationship between these activities, which is shown in Fig. 4, supports the following propositions. (i) The slope of the curve (0.8 in the segment defined by H35-C12, L2-10C1, and H2-4C2) indicates that VN (or, more generally, the antibody activities that provide prophylaxis) also makes a major contribution to the cure provided by these MAbs. (ii) The fact that the curve does not extrapolate to the origin but intersects the *y* axis indicates that, in therapy, a fraction of the passive MAb is used for an activity other than VN, presumably for reaction with HA expressed in the plasma membrane of virus-infected host cells. Thus, these MAbs appear to operate during therapy both by neutralizing progeny virus and by binding to infected host cells.

Neutralization of newly released progeny virus appears to make an obvious contribution to virus clearance by preventing the spread of the infection to uninfected host cells. By contrast, binding of MAb to infected host cells does not necessarily mean that this contributes to virus clearance. Effective targeting of cells for complement-mediated effector mechanisms and FcR- and CR-expressing effector cells may require a higher density of cell-bound antibody than can be achieved here by giving passive MAb by an extrapulmonary route. It is difficult to test this in vitro because we do not know the actual passive MAb concentration in native bronchial secretions. Transudation of IgG from serum into the extravascular compartment of the lungs and from there through basement membranes and epithelial cell layers into the airways, where it finally can act against the infection, appears to be quite inefficient. By comparing the efficacy of respiratory syncytial virus (RSV)-specific human IgG in RSV-infected rats after topical (i.n.) and parenteral (i.p.) application, Prince et al. (33) estimated the parenteral application to be 160-fold less effective than the topical application. Perhaps because there is no significant inflammatory reaction 1 day after low-dose infection of SCID mice, an even less effective movement of passive MAb from serum into the airway secretions is indicated by the present finding that 3 μg of H36-4 given i.p. (1 to 1.5 μg of MAb/ml of serum) protected 50% of the mice against a virus inoculum of $\sim 10^2$ $TCID_{50}$ while a roughly 3,000-fold-lower concentration (0.0004 μg/ml) protected 50% of the MDCK cell cultures in vitro against infection by a similar dose of virus. Nevertheless, we believe that cell-bound MAb contributed to virus clearance for the following reasons. (i) The 0.8 slope of the curve in Fig. 4 suggests that MAb is slightly more effective therapeutically than prophylactically; equal effectiveness would be indicated if the slope were 1. This may be so because cell-bound MAb contributes to cure. (ii) We have found (unpublished observation) that a subcuring dose of VN-positive MAb can promote cure if given together with a noncuring dose of an MAb that targets only infected cells. Thus, it appears that both VN and cell targeting are involved in the MAb-mediated virus clearance in this system.

While all four MAbs appeared to use both VN and cell targeting for cure, it is interesting to consider whether both activities are actually required for effective cure or whether a single activity would suffice. Intuitively, one would believe that VN activity alone should be capable of curing an infection provided that the virus (i) is cytopathic and will ultimately result in the death of the virus-producing host cells and (ii) does not spread through intercellular contacts. These conditions apply for influenza virus but not for many other viruses. Most relevant in this context is the study of Crowe et al. (11), which showed that an ongoing infection with RSV could be stopped and cured by three daily i.n. treatments with RSV-specified Fab fragment (the daily treatment was necessitated because of the short half-life of Fab in vivo). However, since these experiments were performed in immunocompetent mice, it is not clear whether the ultimate cure indeed resulted solely from the continuous neutralization of progeny virus (until all infected cells had succumbed to the viral cytopathic effects) or whether effectors of the endogenous immune response were involved in the ultimate cure. On the other hand, MAb-dependent cell-targeting mechanisms may often not be effective enough to

TABLE 4. Relation between relative curing activity, VN, and HA binding[a]

MAb	Cure (50%)	VN with:			HI with:		HA binding (ELISA)[b]
		No serum	1.65% serum	50% prophylaxis	Virus	Rosettes	
H2-4C2	>6	17,000	1,750	>70	>400	55	6
L2-10C1	3	430	60	20	>400	3	2
H35-C12	2	3,500	85	7	160	3	3
H36-4	1	1	1	1	1	1	1

[a] MAb activities from Tables 1 and 2 and Fig. 1 and 8 have been normalized with respect to H36-4, which was the most effective MAb in each assay. The curing activity is the mean curing activity computed from the data in Table 3 and Fig. 8. The values provide a relative measure of the MAb dose needed for the indicated unit of activity.

[b] ELISA binding activities are computed from the half-maximum binding values.

clear a virus infection on their own. This is supported by the finding that treatment of infected SCID mice with a VN-negative MAb specific for the viral transmembrane protein M2 reduced the virus titer in the lungs but failed to cure the infection (reference 31 and our unpublished observations). Also, infections by noncytopathic viruses such as lymphocytic choriomeningitis virus persist in spite of high antibody levels in serum (7, 30). Furthermore, with the notable exception of VN-negative IgA MAbs that cured a mucosal rotavirus infection apparently in the course of their transcytosis (8), VN-negative MAbs have generally been found incapable of curing infections in immunoincompetent mice (for the reasons given above, we do not consider the MAbs used here to be VN negative in vivo). Antibodies appear more proficient in attacking cells present in blood and lymphoid tissues, as shown by the effective elimination of lymphocyte subpopulations by MAb treatment in vivo (10).

We do not know at present by which mechanism(s) cell-bound MAb contributes to virus clearance. Complement-dependent mechanisms do not appear to play a role, since the curing activity of a VN-low MAb was not diminished by severe depletion of C3. Although we verified C3 depletion only in serum, it is likely that C3 was similarly depleted in bronchial secretions, because CVF is a small protein (62 amino acids) which would be expected to transudate readily into extravascular compartments of the lungs. Similarly, NK cells, which are an important constituent of the FcR-expressing effector cell system and could conceivably contribute to recovery through antibody-dependent cytotoxic activity and local release of gamma interferon, also do not appear to play an indispensable role because NK-cell depletion failed to diminish the curing activity of a VN-low MAb. The possibility that NK cells, while effectively depleted in the spleen (as verified here), were not depleted in the lungs is not likely, because other investigators have shown (39) that NK depletion in the lungs parallels the depletion observed in the spleen after i.p. treatment with anti-asialo GM_1. Although neither complement nor NK cells appear to be required for MAb-mediated virus clearance, it is still possible that these effector mechanisms are involved but can substitute for each other. Also, we cannot exclude a role for other FcR-expressing effector cells such as macrophages and polymorphonuclear leukocytes. Macrophages, in particular, are present in substantial numbers in the airways of the lower respiratory tract of healthy mice (4), and both pulmonary macrophages and polymorphonuclear leukocytes play a role in the early defense against influenza virus infection (16). Our previous finding (31) that $F(ab')_2$ of a VN-high MAb displayed reduced curing activity does not clarify this issue because we could not exclude the possibility that the reduced therapeutic activity of $F(ab')_2$ reflected its short half-life in vivo rather than an involvement of Fc-dependent mechanisms. It should also be noted that while complement-dependent effector mechanisms (21) and NK cells (40) have been reported to contribute to recovery from influenza virus infection, those studies used large virus challenge doses and thus may have tested mainly the ability of complement and NK cells to rapidly convert an overwhelming lethal challenge into a nonlethal challenge which subsequently could be handled by the host immune response. Lastly, antibody may inhibit virus release from infected cells through its mere binding to viral HA expressed in the plasma membrane. This activity is usually evidenced in vitro as a reduction in the plaque size, when antibody is incorporated into the agar overlay, or reduction in the virus yield, as in the present study. It can readily be differentiated from neutralization of progeny virus if the antibodies under investigation lack VN activity at the concentration used, as is the case here. Cell-bound MAb may inhibit virus yield by suppressing virus replication at intracellular stages, as reported for other viruses (12, 15, 26, 27), and/or by cross-linking fully budded virions to HA present in the cell membrane and thus inhibiting their release from infected host cells. The contribution of direct effects of MAb on infected host cells and indirect effects mediated by FcR-expressing effector cells, other than NK cells, remains to be determined.

The present demonstration that passive anti-HA MAbs can cure a pulmonary influenza virus infection in the absence of T cells does not mean that an active endogenous anti-HA antibody response in immunocompetent mice makes the same decisive contribution to recovery from infection. In the latter situation, T- and B-cell-dependent mechanisms contribute to recovery and no single effector mechanism appears to be indispensable for recovery (14, 18, 35).

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