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Virus-Neutralizing Antibodies of Immunoglobulin G (IgG) but Not of IgM or IgA Isotypes Can Cure Influenza Virus Pneumonia in SCID Mice

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The ability of monoclonal antibodies (MAbs) to passively cure an influenza virus pneumonia in the absence of endogenous T- and B-cell responses was investigated by treating C.B-17 mice, homozygous for the severe combined immunodeficiency (SCID) mutation, with individual monoclonal antiviral antibodies 1 day after pulmonary infection with influenza virus PR8 [A/PR/8/34(H1N1)]. Less than 10% of untreated SCID mice survived the infection. By contrast, 100% of infected SCID mice that had been treated with a single intraperitoneal inoculation of at least 175 μg of a pool of virus-neutralizing (VN^+) antihemagglutinin (anti-HA) MAbs survived, even if antibody treatment was delayed up to 7 days after infection. The use of individual MAbs showed that recovery could be achieved by VN^+ anti-HA MAbs of the immunoglobulin G1 (IgG1), IgG2a, IgG2b, and IgG3 isotypes but not by VN^+ anti-HA MAbs of the IgA and IgM isotypes, even if the latter were used in a chronic treatment protocol to compensate for their shorter half-lives in vivo. Both IgA and IgM, although ineffective therapeutically, protected against infection when given prophylactically, i.e., before exposure to virus. An Fcγ-specific effector mechanism was not an absolute requirement for antibody-mediated recovery, as $F(ab')_2$ preparations of IgGs could cure the disease, although with lesser efficacy, than intact IgG. An anti-M2 MAb of the IgG1 isotype, which was VN^- but bound well to infected cells and inhibited virus growth in vitro, failed to cure. These observations are consistent with the idea that MAbs of the IgG isotype cure the disease by neutralizing all progeny virus until all productively infected host cells have died. VN^+ MAbs of the IgA and IgM isotypes may be ineffective therapeutically because they do not have sufficient access to all tissue sites in which virus is produced during influenza virus pneumonia.

The adaptive immune response of the naive host to a virus infection usually comprises strong T-cell (CD4 and CD8) and B-cell (antibody) responses. In many infections, the brisk but relatively short-lived effector T-cell responses appear to play dominant roles in the recovery from disease, while a major function of the delayed but long-lived B-cell response is a prophylactic one, i.e., to protect against reinfection or limit the spread of previously experienced or latent viruses (reviewed in reference 12). Nevertheless, antibodies do usually contribute to recovery from primary disease, as evidenced by delayed virus clearance or increased morbidity of hosts which are defective in their B-cell response (9, 19, 24, 30, 45), and there are also examples of virus-host interactions that have been reported to depend greatly on the antibody response for recovery (21, 38, 49).

In the case of influenza virus pneumonia, current evidence indicates that the disease can be cured either by a CD8 T-cell response alone (2, 12, 34, 49) or, in the absence of CD8 T cells, by a CD4 and concomitant B-cell response (4, 6, 12, 13). While it is also generally accepted that existent virus-neutralizing (VN^+) antibodies (from prior disease) can protect against infection and that the developing antibody response in the primary infection contributes to recovery (1, 10, 30, 36, 41, 52), it remains controversial whether antibodies can cure the infection on their own in the absence of a T-cell response. In this regard, Kris et al. (31) observed that antibody treatment of infected athymic (*nu/nu*) mice led to temporary reduction of disease but not to complete recovery, and Schulman et al. (43) reported that passive antibody, which resulted in serum antibody levels equivalent to those arising in the normal primary immune response to pulmonary infection, failed to reduce pulmonary virus titers in mice that had been immunosuppressed by treatment with antithymocyte antiserum. By contrast, Virelizier (51) and subsequently ourselves (41) found that antibody treatment could cure influenza virus pneumonia in immunodeficient mice. Antibody treatments have reportedly also cured other viral diseases in immunodeficient mice, such as Sindbis virus infection of the central nervous system in severe combined immunodeficiency (SCID) mice (33), Theiler's murine encephalomyelitis virus infection in athymic mice (15), and parvovirus infection in athymic newborn rats (17). In addition, treatment with a singularly effective monoclonal antibody (MAb) but not with an antiserum cured a herpes simplex virus infection of the vaginal mucous membranes in T-cell-depleted mice (14), although passive antibody often only delayed morbidity in this infection (28, 37, 47, 55).

The reason for the discrepant observations concerning the ability of antibodies to cure an influenza virus pneumonia in immunodeficient mice is not known but may be due to differences in virus strains or antibody preparations used. In this study, we verified the ability of individual VN^+ MAbs to cure an established influenza virus pneumonia in SCID mice and defined the dose and heavy-chain isotype required for this function.

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MATERIALS AND METHODS

Mice. C.B-17 mice, homozygous for the severe combined immunodeficiency (*scid*) mutation (7) and here referred to as SCID mice, were bred in the Animal Facility of the Wistar Institute. Mice were routinely checked at 4 to 5 weeks of age for serum immunoglobulin (Ig) M concentration by enzyme-linked immunosorbent assay (ELISA) and were used at 6 to 10 weeks of age. Mice were infected with influenza virus PR8 [A/PR/8/34(H1N1)] by exposure to a virus-containing aerosol in an airborne infection apparatus (TriR Instruments). The standard infection dose was 100 50% mouse infective doses (MID_{50}) per mouse. One day later, mice were injected intraperitoneally (i.p.) with antiviral antibody preparations and 3 weeks later were killed and checked for the presence of infectious virus in the lung.

Antiviral antibodies. All antibodies used here are individual MAbs or mixtures of MAbs derived from BALB/c mice. They were used in the form of crude hybridoma ascites fluids from BALB/c mice or after purification from hybridoma culture fluids or ascites fluids generated in SCID mice. In the former case, antibody concentration was determined by comparison with purified specificity- and isotype-matched antibody preparations in ELISA. Purified MAb preparations were quantitated by the Bio-Rad protein assay with bovine serum albumin (BSA) as the standard. The hybridoma secreting the M2-specific MAb 14C2 (IgG1) was kindly provided by Zebedee and Lamb (58).

Preparation of lung extract. Mice were anesthetized by intramuscular injection of ketamine (2 mg) and xylazine (0.4 mg) and exsanguinated by heart puncture. The trachea was exposed, and the thorax was opened from the cervical side. The thymus, mediastinal lymph nodes, and heart were removed. The trachea was cut below the larynx, and the trachea with attached lung was lifted out of the thoracic cavity and immediately frozen by placing the organ on a tissue culture plate kept on dry ice. Lung extracts were made by brief homogenization of the frozen lung in a mortar containing a pinch of sterile carborundum powder and 2 ml of TE buffer (10 mM Tris HCl, 1 mM EDTA [pH 8]). The extract was transferred to a centrifuge tube, the carborundum and cell debris were pelleted (5 min at 400 × *g*), and the supernatant was tested for the concentration of infectious virus.

Determination of infectious virus titer. Dilutions of lung extracts were tested for virus infectivity in the MDCK assay as described before (41). The threshold of virus detection in the MDCK assay is $10^{2.1}$ 50% tissue culture infective doses ($TCID_{50}$) per lung. Lung extracts that were negative in the MDCK assay were further tested by inoculation of 50 μl of undiluted extract into the allantoic cavity of 10-day-old embryonated hen's eggs. The threshold of the latter assay is $10^{1.6}$ egg infectious doses (EID_{50}) per lung. Failure to detect virus in eggs was taken as evidence of clearance of the virus infection.

Production of $F(ab')_2$ fragment. Purified MAb (1 mg/ml in 0.1 M sodium citrate [pH 4.2]) was incubated with pepsin (25 μg/ml; pepsin A; EC 3.4.23.1; Sigma) for 16 h at 37°C. The digestion was stopped by increasing the pH to ~7. $F(ab')_2$ was separated from undigested MAb by passage through a protein G-Sepharose column (Pharmacia). The flowthrough was concentrated by ultrafiltration in a collodium bag (molecular weight exclusion, 25,000; Schleicher and Schuell) against phosphate-buffered saline.

ELISA. Twenty hemagglutinating units of purified virus (~150 ng of viral protein) in 25 μl of 0.02 M NaCl was used to coat the wells of polyvinyl 96-well round-bottomed plates (Costar, Cambridge, Mass.). The assay was performed as described before (27), with biotinylated C_k-specific MAb 187.1 or C_H isotype-specific MAbs (PharMingen, San Diego, Calif.) and Avidin-Alkaline Phosphatase (Sigma) as detecting reagents. Absolute antibody concentrations in ascites fluids were determined by comparison with antibody preparations of the same viral protein specificity and isotype which had been purified from hybridoma culture fluids.

HI test. The hemagglutination inhibition (HI) test was performed as described before (41).

VN in vitro. Fifty microliters of antibody dilutions in Isc-cm-0.1% BSA (Iscove's modified Dulbecco's medium [GIBCO] supplemented with 2-mercaptoethanol [0.05 mM], transferrin [0.005 mg/ml; Sigma], glutamine [2 mM], gentamicin [0.05 mg/ml], and 0.1% BSA) was added to flat-bottomed 96-well tissue culture plates (Falcon) at six to eight replicates per dilution. PR8 (100 $TCID_{50}$ in 50 μl of Isc-cm-0.1% BSA) was added to each well, and the plates were incubated for 1 h at 37°C in a CO_2 incubator. Fifty microliters of freshly trypsinized MDCK cell suspension (10^6 cells/ml of Isc-cm-0.1% BSA) was then added to each well. After overnight incubation (to permit adherence of MDCK cells), 50 μl of trypsin solution (2.5% trypsin [Whittaker Bioproducts, Inc., Walkersville, Md.], freshly diluted 1:750 in Isc-cm-0.1% BSA) was added to each well. The plates were then incubated for 2 more days, and the culture supernatant was tested for virus content.

Analysis of infected cells by flow cytometry. P1.HTR cells (54) were resuspended at 10^7 cells/ml in Isc-cm. PR8 (~10 infectious doses [ID_{50}] per cell) was added, and the suspension was incubated, with occasional mixing, for 1 h at room temperature. The cells were then pelleted, resuspended in Isc-cm-5% fetal calf serum to give ~0.5 × 10^6 cells per ml, and incubated for 8 h at 37°C in a CO_2 incubator. Cells were harvested, pelleted, resuspended in 5 ml of 40% Percoll (Pharmacia), and underlaid with 3 ml of 50% Percoll in 15-ml centrifuge tubes. The Percoll dilutions were made in phosphate-buffered saline, pH 7.2. The tubes were centrifuged for 20 min at 700 × *g* at room temperature, and the viable cells at the 40 to 50% interphase were harvested, washed, and resuspended at 2 × 10^7 cells per ml in fluorescence-activated cell sorting (FACS) diluent (phosphate-buffered saline, 0.1% NaN_3, 1% BSA, 1% rat serum). The cells were stained by primary incubation with purified biotinylated murine antiviral hybridoma antibodies (50 to 100 ng per 0.5 × 10^6 cells) followed by incubation with fluorescein isothiocyanate-avidine (Sigma). Samples were analyzed with an Ortho Cytofluorograf System 50, connected to a 2150 Data Handling System (Ortho Diagnostic Systems, Inc., Westwood, Mass.).

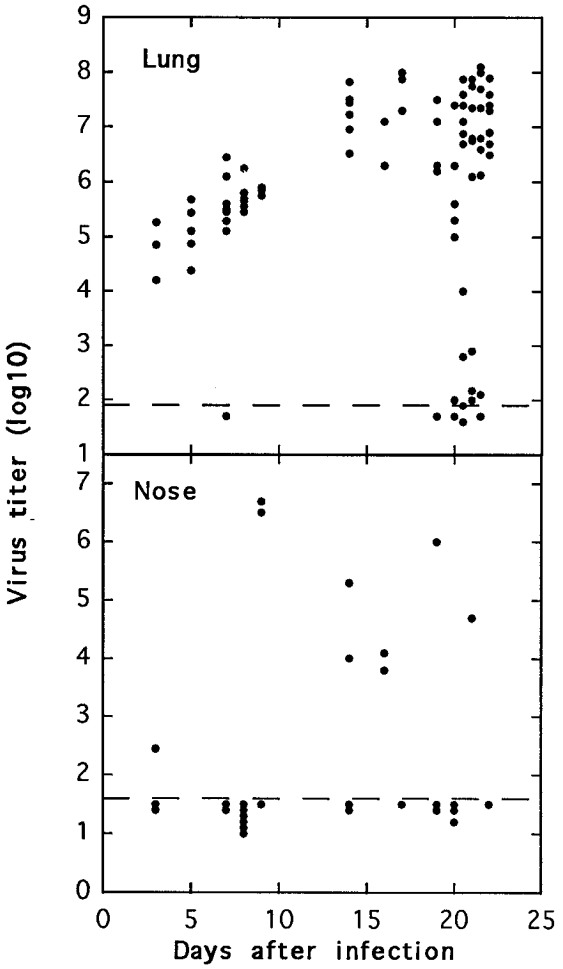

FIG. 1. Virus titers measured in extracts of lung and nose of SCID mice which had been exposed on day 0 to aerosolized PR8 (~100 MID_{50} per mouse) and treated with PBS instead of antibody (negative controls). Each dot indicates the viral infectivity titer from the tissue extract of an individual mouse. The horizontal broken lines mark the threshold of detection, i.e., no virus growth was observed after inoculation of 2.5 and 5% of total lung and nose extracts, respectively, into the allantoic cavity of embryonated hen's eggs.

RESULTS

Course of influenza virus infection in SCID mice. Exposure of mice to aerosolized PR8 virus at the dose used in this study (100 MID_{50} per mouse) leads to infection of the lower respiratory tract in essentially 100% of the mice. In immunocompetent BALB/c mice, the infection is cleared in 7 to 10 days by the antiviral immune response (41). By contrast, in similarly infected SCID mice, pulmonary virus titers increased steadily and reached a plateau by about 2 weeks after infection (Fig. 1). The lung virus titers remained at this level in most SCID mice, and deaths began to occur in the third and following weeks (mean day of death, 35 days). Exceptions to this general course of disease were rare in that only 4 (7%) of 60 mice which had

TABLE 1. Composition of the anti-HA MAb pool[a]

MAb	C_H	HA site specificity	HI titer ($-\log_{10}$)	% of pool
H3-4C4-9	IgM	Sa	4.02	17
H2-4B3-4	IgG2b	Sa	4.72	9
H37-87-1	IgG1	Sb	4.72	9
H36-4-5	IgG2a	Sb	5.80	4
H37-50-3	IgA	Sb	4.72	9
H2-4B1-14	IgG1	Ca	3.97	17
H2-4C2-2	IgG2a	Cb	3.32	35

[a] Seven hybridoma ascites fluids from BALB/c mice of the indicated heavy-chain isotypes, HA specificities, and HI titers (against PR8) were mixed in the proportion shown in the last column. This MAb cocktail contained ~5.6 mg of anti-HA antibody per ml and exhibited an HI titer versus PR8 of $10^{-4.1}$ and a VN titer versus PR8 of $10^{-5.1}$.

been used as controls in the present study and which were killed 19 to 22 days after infection scored negative for virus. The reason for the reduction and even apparent clearance of the pulmonary infection in some of the SCID mice (Fig. 1) has not been determined but is most likely related to some degree of immune responsiveness permitted by the leakiness of the *scid* mutation (7). Nevertheless, the vast majority of SCID mice exposed to aerosolized PR8 at the dose of 100 MID_{50} per mouse had either died or displayed detectable virus in their lungs at 19 to 22 days after infection. Infectious virus was only sporadically isolated from nasal extracts. This indicates that the epithelium of the nose is less susceptible than that of the lower respiratory tract to this mode of infection and displays some resistance to secondary ascending infection.

Efficiency of an HA-specific MAb pool in curing a pulmonary PR8 influenza virus infection in SCID mice. We had observed previously (41) that SCID mice could be cured from a pulmonary influenza virus infection by treatment with a single i.p. injection of a cocktail (Table 1) of VN^+ anti-HA MAbs. We wanted to define the minimum effective dose of this MAb cocktail and determine if or at which time point of the active antibody response engendered by a pulmonary influenza virus infection in immunocompetent mice the effective passive titer is reached. Table 2 shows the results from several experiments in which SCID mice that had been exposed on day 0 to 100 MID_{50} of aerosolized PR8 were injected i.p. with the indicated doses of the MAb cocktail on day 1 and then tested for infectious virus in lung extracts 22 to 65 days later. It is evident that 70 and 175 μg of the MAb cocktail cured the disease in two of three and 100%, respectively, of the recipient mice. These doses resulted in serum antibody titers in vivo (measured 1 day after injection) which were equivalent to the active anti-HA antibody response generated in immunocompetent mice during weeks 3 and 4 after infection (Fig. 2). Thus, passive anti-HA antibodies, at a concentration produced actively also in the primary antibody response of immunocompetent mice, are capable of curing a pulmonary infection in the absence of additional contributions from endogenous B or T cells. Passive antibody could also cure a further progressed pulmonary infection when given as late as 7 days after virus exposure (Table 3) to mice with a high virus titer (Fig. 1).

TABLE 2. Efficacy of the HA-specific MAb pool in clearing virus infection from the lung[a]

Dose (μg/mouse)	No. of SCID mice with infection cleared/total tested	% with infection cleared
700	6/6	100
350	6/6	100
280	2/2	100
175	4/4	100
70	4/6	66
14	0/3	0
0	1/14	7

[a] Entries show data from five independent experiments in which SCID mice were exposed on day 0 to aerosolized PR8 (corresponding to 100 MID_{50} per mouse) and treated 1 day later by i.p. injection of 250 μl of PBS containing the indicated amounts of anti-HA antibody cocktail (see Table 1). Mice were tested at 22 to 65 days after infection for the presence of infectious virus in the lung.

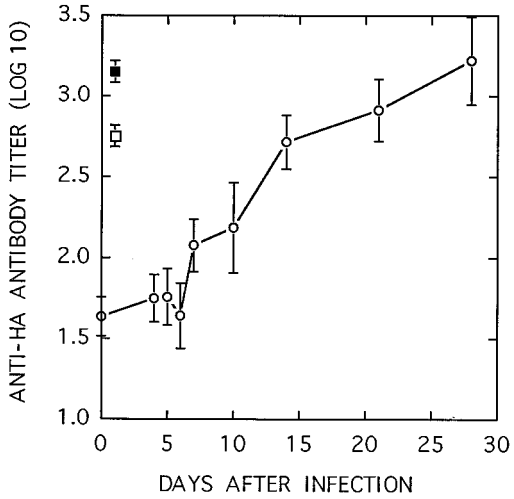

FIG. 2. Kinetics of the anti-HA antibody response in serum of immunocompetent BALB/c mice after aerosol infection with PR8. BALB/c mice were infected by exposure on day 0 to PR8 aerosol resulting in a dose of 100 MID_{50} per mouse. Anti-HA antibody titers in plasma (open circles) were determined by ELISA with purified HA as the immunoadsorbent. In parallel, anti-HA titers were also measured in the plasma of naive mice which had been injected i.p. 1 day previously with 175 μg (solid square) or 70 μg (open square) of anti-HA cocktail. Titers were expressed as the dilution giving an optical density at 405 to 750 nm of 0.500. Each datum point gives the geometric mean and standard deviation for 5 to 10 individual mice.

Influence of antibody heavy-chain isotype on virus clearance. The pool of VN^+ anti-HA antibodies used in the prior experiments included various C_H isotypes (Table 1). Since the C_H isotype is a determinant of antibody function, we next tested individual MAbs of distinct C_H isotypes for ability to cure the disease. The MAbs chosen for this comparison had in common specificity for site Sa or Sb, which lie near each other on the tip of the HA molecule (8) and are the recognition sites for antibodies with relatively high VN activity (18). Assuming that VN activity played a role in virus clearance in vivo, we

TABLE 3. Time of antibody treatment[a]

Time of treatment with MAb cocktail	No. of SCID mice with cleared infection/total tested at treatment dose:		
	700 μg	280 μg	70 μg
No treatment	0/3	0/2	0/3
Day 1	3/3	2/2	1/3
Day 3	3/3	2/2	2/3
Day 5	3/3	2/2	1/3
Day 7	3/3	2/2	0/3

[a] SCID mice were infected on day 0 with PR8 aerosol and either left untreated or injected i.p. with the indicated dose of MAb cocktail on day 1, 3, 5, or 7 postinfection. Mice were tested for the presence of virus in lung extracts between days 22 and 65 after infection.

TABLE 4. Effect of C_H isotype on virus clearance by VN-positive anti-HA MAbs[a]

MAb	C_H	Dose (log_{10} VN units)	No. of infections cleared/total	Virus titer in lung of infected mice (log_{10})	HI titer in serum on days 16–19 ($-log_{10}$)
None			0/17	6.0 ± 2.1	<1
H3-4C4	IgM	4.6	0/7	6.4 ± 2.2	<1
Y8-2C6	IgM	5.5	0/3	5.0 ± 2.6	<1
H36-7	IgG3	5.5	6/6		2.5
H37-87	IgG1	5.4	6/6		2.4
H36-12	IgG2b	5.2	6/6		1.6
H37-77	IgG2a	5.3	6/6		2.0
H37-50	IgA	4.5	0/7	6.8 ± 2.2	<1
H37-66	IgA	5.15	0/3	7.8 ± 0.2	<1

[a] SCID mice were infected on day 0 with aerosolized PR8 and injected i.p. on day 1 with PBS or the indicated doses of MAb (ascites fluids). Mice were killed between days 16 and 19, and virus titers (geometric mean ± standard deviation [SD]) in lung extracts and anti-HA antibody titers (geometric mean) in serum were determined. Failure to isolate virus by inoculation of 50 μl of undiluted lung extract into embryonated eggs was taken as evidence of virus clearance. The VN titers of the MAbs were determined in the MDCK assay.

dosed the various ascites fluids, whenever feasible, to provide roughly $10^{5.2}$ to $10^{5.6}$ VN units per mouse. These doses correspond to the VN activities exhibited by 70 and 175 μg, respectively, of the MAb cocktail, which cured two of three and 100% of the mice, respectively (Table 2). Antibody treatments were performed in all cases 1 day after infection, and surviving mice were killed 20 to 22 days later for determination of virus titer in the lung. The results (Table 4) show that antibodies of the IgG1, IgG2a, IgG2b, and IgG3 isotypes cured the disease, while antibodies of the IgM and IgA isotypes were totally ineffective.

A possible reason for the failure of IgM and IgA antibodies to cure the infection may be their shorter half-lives in vivo. The half-lives were determined in separate experiments by transfer of antibodies into uninfected SCID mice and found to be approximately 0.9 days for IgA and 1.5 days for IgM, as opposed to 4 to 10 days for IgGs, in agreement with the values reported by Vieira and Rajewsky (50). Therefore, to compensate for the shorter half-lives of IgM and IgA, infected SCID mice were injected i.p. every other day with these isotypes over a period of 5 to 11 days (Table 5). In the case of the IgM MAb H2-6C4, the total dose given over a 6-day period was roughly 10 times larger (in terms of VN units) than the fully effective single dose of an IgG MAb. However, chronic treatment with either IgM or IgA failed to cure the disease. Virus isolated

TABLE 5. Failure of IgM and IgA MAbs to clear a pulmonary virus infection cannot be overcome by repetitive inoculation of MAb[a]

MAb	C_H	Days of treatment	Total VN units transferred	No. of infections cleared/total	Virus titer in lung of infected mice (log_{10})
None				0/7	6.0 ± 2.0
H2-6C4-13	IgM	1, 3, 5, 7	$10^{6.35}$	0/7	5.85 ± 0.5
H3-4C4-9	IgM	1, 3, 5, 7, 9, 11	$10^{5.4}$	0/3	4.3 ± 3.2
H37-50-3	IgA	1, 3, 5	$10^{5.0}$	0/3	7.3 ± 0.5
H37-50-3	IgA	1, 3, 5, 7, 9, 11	$10^{5.45}$	0/3	6.6 ± 0.3

[a] The data are the composite of three independent experiments. SCID mice were infected on day 0 with PR8 aerosol and then treated with the indicated MAb ascites fluids as indicated. Mice were killed on days 17 to 21 after infection, and the virus titer in lung and HI titer in serum were determined.

TABLE 6. Treatment with IgM and IgA antibody prior to virus exposure prevents infection[a]

Treatment	Dose (VN units)	Time before infection	No. of mice infected/total	Mean virus titer ± SD (log_{10})
PBS		1 day	7/8	5.9 ± 0.4
H2-6C4-13 (IgM)	$10^{5.75}$	1 day	2/4	5.1 ± 0.2
	$10^{5.75}$	4 h	0/5[b]	
H37-66-1 (IgA)	$10^{5.25}$	1 day	0/5[b]	

[a] SCID mice were treated by i.p. injection with antibody at the indicated time points prior to exposure to PR8 aerosol. Virus titers in the lung were determined 7 to 8 days after infection.

[b] Significantly different at the 95% level, determined by chi-square analysis.

from the lungs of these chronically treated mice was still effectively neutralized by the treatment antibodies in vitro (data not shown). Therefore, selection of escape variants during sustained treatment is not the reason for the failure of these MAbs to clear the virus infection in vivo.

It was also conceivable that injected IgM and IgA MAbs failed to reach the lung in sufficient concentration. To test this, mice were treated with MAbs shortly prior to infection. As shown in Table 6, both isotypes prevented an infection under these conditions, which proves that i.p. inoculation of both IgM and IgA resulted in their presence in bronchial secretions. Therefore, these isotypes may be ineffective when given after infection for one of the following reasons: (i) an IgG-specific Fc-dependent function may be required for cure; (ii) IgM and IgA fail to reach all sites of the pulmonary tissue in which virus replication occurs during an established infection; or (iii) the disadvantage of their short half-lives could not be overcome by the chronic treatment.

Efficacy of $F(ab')_2$. The possible requirement for an IgG-specific Fc-dependent effector function was analyzed by means of $F(ab')_2$ fragments prepared from the clearance-promoting MAbs H37-77 (IgG2a) and H37-65 (IgG1). On a molar basis, both $F(ab')_2$ preparations were as active as the intact molecules in the HI test in vitro and in an ELISA and contained less than 1% intact IgG (data not shown). Although $F(ab')_2$ has a very short half-life in vivo, determined for one of the preparations to be 0.6 days, treatment with $F(ab')_2$ nevertheless cured

TABLE 7. Efficacy of $F(ab')_2$ in vivo[a]

Antibody	Form	Dose (μg) and days of treatment	No. of mice		
			Dead	Infected (mean log_{10} virus titer ± SD)	Cured
None (PBS control)			3	8 (7.66 ± 0.42)	0
H37-77 (IgG2a)	Intact	50 (1)	0	0	4[b]
	Intact	1 (1–3)	1	3 (7.24 ± 0.65)	0
	$F(ab')_2$	60 (1)	0	1 (6.1)	2[b]
	$F(ab')_2$	60 (1–3)	0	4 (3.98 ± 2.51)	0
H37-65 (IgG1)	Intact	100 (1)	0	1 (6.69)	3[b]
	Intact	1 (1–3)	0	4 (7.05 ± 0.64)	0
	$F(ab')_2$	100 (1)	0	4 (4.38 ± 1.68)	0
	$F(ab')_2$	100 (1–3)	0	2 (6.90 ± 0.98)	2[b]

[a] SCID mice were infected on day 0 with aerosolized PR8 and then injected i.p. on the indicated days postinfection with PBS or the indicated dose of antibody preparations [e.g., "1 (1–3) indicates that 1 μg was administered on each of days 1 to 3 postinfection, for a total of 3 doses). All surviving mice were killed 20 to 22 days after infection, and the virus titer (mean ± SD) in lung extracts was determined by the MDCK assay and inoculation into eggs. The number of mice in which no virus could be detected is listed under Cured.

[b] The difference between the control and treatment groups is significant at the 95% level, as assessed by chi-square analysis.

the disease in some animals (Table 7). To exclude that the partial recovery achieved with $F(ab')_2$ preparations was not due to residual intact Ig present in these preparations, some mice were treated with amounts of intact IgG that were equivalent to the maximum present as contaminants in the $F(ab')_2$ preparations. These low doses of intact IgG failed to cure the disease. Therefore, cure can be achieved by Fc-independent mechanisms. However, whether intact IgG can use additional Fc-dependent mechanisms for enhanced recovery or whether the reduced activity of $F(ab')_2$ compared with intact IgG is merely the result of its short half-life in vivo remains undetermined.

Infection is not cured by treatment with a VN-negative antibody which reacts with infected cells. We have shown above that VN^+ anti-HA antibodies of IgG isotypes are effective in curing the pulmonary infection. Since HA is expressed abundantly on the surfaces of infected cells and mature virions, these antibodies may operate in vivo both by directing effector mechanisms (antibody-dependent cell-mediated cytotoxicity and complement-mediated cytotoxicity) against infected cells (46), by suppressing intracellular virus replication (16, 33), and/or by binding to mature progeny virus and preventing it from infecting new target cells. To assess the effectiveness of anticellular mechanisms, we used the anti-M2 MAb 14C2 (IgG1). This MAb recognizes a viral transmembrane protein that is expressed at high density in the membrane of infected cells and at very low density (and in poorly or not accessible locations) in the membrane of mature virions (25, 32, 58). This MAb gives good, although slightly less intensive, staining than an anti-HA MAb when tested by flow cytometry for reaction with infected P1.HTR cells (Fig. 3). An MAb specific for the intracellular and intraviral M1 protein gave background staining (data not shown), indicating that both anti-HA and anti-M2 MAbs reacted solely with viral determinants expressed on the external surface of the plasma membrane. The MAb lacks VN activity (58; unpublished observation) but, when present during virus replication in MDCK cell cultures, reduced the virus yield (Fig. 4), as has been reported by Zebedee and Lamb (58) for most type A strains, although not the PR8 strain that they tested. Infected SCID mice which had been treated on day one with 250 μg of this MAb showed lung virus titers (eight animals; average titer, $10^{6.85 \pm 0.87}$) that were not different from those observed in control mice. Thus, the anticellular mechanisms directed by this MAb were not effective in curing the disease.

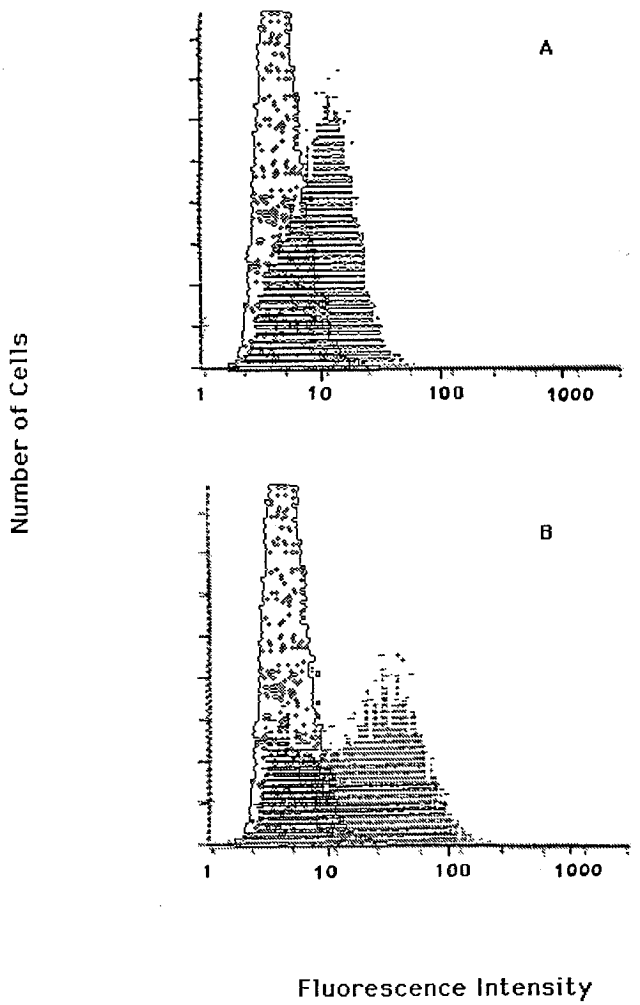

FIG. 3. Staining of PR8-infected P1.HTR cells by anti-M2 and anti-HA. P1.HTR cells were infected with 10 $TCID_{50}$ of PR8 per cell and incubated for 8 h at 37°C. At this point, >95% of the cells were viable (trypan blue exclusion), and 80% scored positive for infection (by the hemadsorption test). The cells were stained for flow cytometry by (i) incubation with biotinylated 14.4.4 (anti-class II, control), M2-1C6 (anti-M1, a viral protein which is not expressed on the cell surface), 14C2 (anti-M2), or H37-77 (anti-HA) and (ii) with fluorescein isothiocyanate-avidine. Five thousand events were analyzed. Staining with control MAb 14.4.4 is shown as a light histogram. The dark histogram shows staining by anti-M2 (A) and by anti-HA (B). Staining by anti-M1 was not different from that by MAb 14.4.4 and is not shown.

DISCUSSION

Exposure of mice to aerosolized PR8 at the dose used in this study (100 MID_{50}) leads to productive infection of the lower (but not upper) respiratory tract in essentially 100% of the mice. In immunocompetent mice, the antiviral immune response clears the infection within 7 to 10 days. By contrast, the pulmonary infection persists in most SCID mice until their death. Here we showed that infected SCID mice could be cured by treatment with VN^+ anti-HA antibodies of IgG isotypes, even when treatment was started at an advanced stage (7 days after infection) of the disease. The recovery appeared to be complete, in that no recurrent infections were observed in mice maintained until the passive antibody titer had become undetectable (41; unpublished observations). The completeness of the cure was further supported by PCR, which failed to reveal residual viral (HA) RNA (unpublished observation). By contrast, VN^+ anti-HA antibodies of the IgA and IgM isotypes were incapable of curing the disease.

The mechanisms by which the IgG antibodies cured the disease are not completely defined. Since HA is abundantly expressed on the surface of infected cells and mature virions, anti-HA antibodies could conceivably interfere with virus replication by directing antiviral effector mechanisms against infected cells and/or by neutralizing released progeny virus. The failure of anti-M2 antibody 14C2 (which recognizes a viral determinant that is abundantly expressed on infected cells but fails to neutralize virus) to cure the disease suggests that antibody-directed anticellular effector mechanisms may be incapable of curing the infection on their own, although they may contribute to its resolution, as has been concluded from other experimental systems (5, 23, 42). Residual uncertainties regarding this point relate, first, to the fact that 14C2 is of the IgG1 isotype, while IgG2a has been reported to be the most effective in directing anticellular effector mechanisms (11, 26, 29) and in exhibiting therapeutic activity in some viral systems (3, 42) and, second, the possibility that HA may be a more

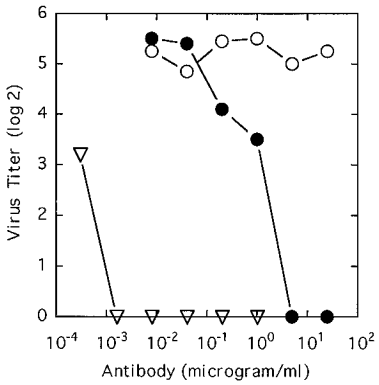

FIG. 4. Anti-M2 MAb 14C2 inhibits growth of PR8 in MDCK cells in vitro. MDCK cell monolayers in 96-well tissue culture plates were infected for 1 h with 100 $TCID_{50}$ of PR8. The medium was then replaced with fresh medium (open circles) or medium containing the indicated concentrations of anti-M2 MAb (solid circles) or anti-HA MAb H37-65 (open triangles). The cultures were incubated for 2 days at 37°C in air-CO_2, after which virus titers in culture supernatants were measured. Each datum point is the mean virus titer of two replicate plates.

suitable viral target protein than M2 for antibody-directed anticellular effects (we have not identified thus far an anti-HA MAb which binds well to HA present on infected cells but fails to neutralize virus). At present, the data are consistent with the notion that cure of the disease depends on antibody-mediated VN activity. Because influenza virus does not establish latent infections and ultimately kills the infected host cell (22, 48), it appears logical that neutralization of all progeny virus over a certain period of time would dry up the infection and cure the disease. By contrast, antibodies are often incapable of curing virus infections with a propensity for persistence and latency in immunodeficient hosts (3, 28, 37, 44, 47, 55, 57), although there are exceptions (14), most notably infections of the central nervous system (15, 21, 33).

The failure of VN^+ IgM and IgA antibodies to cure the pulmonary infection remains puzzling. This does not appear to be due to their relatively short half-lives in vivo, because chronic treatment with MAb failed to improve their efficacies, while $F(ab')_2$, which has a half-life that is three to four times shorter than that of IgM, displayed a higher curing efficacy. Furthermore, because $F(ab')_2$ showed some effectiveness, it is clear that an Fcγ-specific effector mechanism cannot be an absolute requirement for clearance. Importantly, both IgA and IgM were effective in preventing the initiation of infection when given shortly before exposure of mice to aerosolized virus. Therefore, both isotypes reached the airway secretions, in part probably by active transcytosis (35, 40), and chronic antibody treatment should have maintained an effective concentration resulting in continued neutralization of progeny virus released into the airway secretions over the period of antibody treatment. Therefore, their failure to stop a 1-day-old infection suggests that, at this stage, virus replication is no longer confined to the epithelial cell layer lining the airways and that these isotypes may not reach all tissue locations in which virus replication takes place during an ongoing pulmonary infection. The latter is also consistent with our observation that virus isolated from the lungs of IgM- and IgA-treated mice remained fully reactive in vitro with the treatment antibodies. This indicates that the virus replicated in a tissue location which contained little or no antibody and therefore was not under the influence of selective pressure for outgrowth of viral escape mutants, which arise at high frequency during influenza virus replication (56).

Other studies also have found passive IgM to be less effective than IgG in protection against, e.g., Sindbis virus (20) or rabies virus (21) infection, in the latter case even when endogenously produced (38). The failure of passive IgA to cure the influenza virus pneumonia is not in contradiction to the view that IgA plays an important role in the antiviral defense in the respiratory tract (1, 10, 35, 36, 40, 52), since this role of IgA has typically been based on its ability to protect against (as also shown here) rather than to cure an infection.

The aerosol virus exposure used here resulted in a pulmonary infection in essentially 100% of the mice but only very rarely in a nasal infection, apparently because of a higher natural resistance of the nasal epithelium to infection by this strain of influenza virus. Since a higher efficacy of passive antibody in protection against pulmonary as opposed to nasal infection is well documented (30, 39, 53), the ability of antibodies to cure a nasal influenza virus infection will have to be examined in further studies that use a different infection protocol.

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