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Influenza A Vaccine Based on the Extracellular Domain of M2: Weak Protection Mediated via Antibody-Dependent NK Cell Activity

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Vaccination of mice with a peptide corresponding to the extracellular part of M2 protein coupled to the immunodominant domain of hepatitis B core can protect mice from a lethal challenge with influenza A virus. As the extracellular part of M2 protein is highly conserved in all known human influenza A strains, such a vaccine may protect against all human influenza A strains, which would represent a major advantage over current vaccine strategies. The present study demonstrates that protection is mediated exclusively by Abs, a very important feature of a successful preventive vaccine. However, these Abs neither bind efficiently to the free virus nor neutralize virus infection, but bind to M2 protein expressed on the surface of virus-infected cells. The presence of NK cells is important for protection, whereas complement is not, supposing that protection is mediated via Ab-dependent, cell-mediated cytotoxicity. The absence of neutralizing Abs results in much weaker protection than that achieved by vaccination with UV-inactivated influenza virus. Specifically, whereas neutralizing Abs completely eliminate signs of disease even at high viral challenge doses, M2-specific Abs cannot prevent infection, but merely reduce disease at low challenge doses. M2-specific Abs fail to protect from high challenge doses, as vaccinated mice undergo lethal infection under these conditions. In conclusion, protection mediated by M2-hepatitis B core vaccine would be insufficient during the yearly epidemics, for which full protection is desirable, and overall is clearly inferior to protection achieved by immunization with classical inactivated viral preparations. ***The Journal of Immunology,*** **2004, 172: 5598–5605.**

In mice, protection from primary infection with influenza virus is mediated by CTLs, Th cells, and Abs. In contrast, protection from reinfection is largely mediated by Abs directed against the surface glycoproteins, principally hemagglutinin (HA),[2] but also neuraminidase (NA). Also in humans little epidemiological evidence is given for the involvement of T cell-mediated immunity in protection from reinfection with influenza virus (1). Nevertheless, humans frequently have memory CTLs specific for internal influenza A proteins, which are highly conserved between different influenza subtypes (2, 3). However, despite the presence of cross-reactive CTLs, immunity to natural influenza reinfection is attributed to specific anti-HA Abs; immunity is lost if Abs fail to recognize HA (1).

Influenza A viruses constantly evolve by the mechanisms of antigenic drift, which involves point mutations in the antigenic sites of HA and NA, and antigenic shift, the sudden replacement of HA or NA of one subtype by another subtype (4). Antigenic variants of influenza A viruses emerge every year and demand an updated vaccine formulation based on ongoing international surveillance of influenza virus by the World Health Organization. Contemporary vaccines typically contain strains representative of two influenza A subtypes (A/H3N2 and A/H1N1) and one influenza B virus. The idea of developing a vaccine based on an invariant influenza A protein that would induce long-lasting immunity is very tempting. One candidate protein for such a vaccine is M2. The M2 protein is an integral membrane protein of influenza A virus that is expressed at the plasma membrane of influenza virus-infected cells, and it is also incorporated in small amounts into budding virions (5). It consists of a 23-aa N-terminal extracellular domain, a single transmembrane domain of 19 aa, and a 54-aa cytoplasmic tail (6). The extracellular domain is conserved in all known human influenza strains, except A/PR/8/34 and A/Brevig Mission/1/18, which both contain one amino acid change, and A/Fort Monmouth/1/47 which has three amino acid changes (7–9). The M2 protein is a homotetramer with proton-selective ion channel activity (10–12) and is involved in virus uncoating in the endosome (13–16) and in virus maturation in the *trans*-Golgi network (10, 17–22). The antiviral drug, amantadine (1-aminoadamantine hydrochloride), which is presumably responsible for the inhibition of replication, binds to the *trans*-membrane domain of the M2 protein (23). The mAb 14C2 directed toward the extracellular part of M2 considerably reduces the spread of different influenza virus strains, as determined by plaque size reduction; the same effect is achieved with amantadine (5). Moreover, passive administration of 14C2 reduced the level of replication of influenza A virus strain A/Udorn/307/72 (H3N2) in the lung; in contrast, replication of an influenza B virus strain was not reduced (24).

Several studies have shown that immunization with M2 can protect against influenza A infection. Mice were protected against homologous and heterologous infection with influenza A virus after vaccination with whole M2 protein (25). Vaccination with a DNA plasmid containing the M1 and M2 genes of influenza A/PR/8/34 induced protection against homologous and heterologous infection. Protection was due to CTL activity and activation of Th

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[2] Abbreviations used in this paper: HA, hemagglutinin; CDC, complement-mediated cytotoxicity; ADCC, Ab-dependent cytotoxicity; BAL, bronchoalveolar lavage; HBc, hepatitis B core; NA, neuraminidase.

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cells, whereas Abs played a minor role (26). M2-hepatitis B core (M2HBc) particles, based on the genetic fusion of the extracellular part of M2 to the N terminus of HBc protein, also induced protection against lethal homologous and heterologous influenza A challenge (27). In a passive immunization experiment it was demonstrated that Abs were sufficient for protection. However, the N terminus of hepatitis B virus core protein is buried within the particle, which probably makes the fused M2 poorly accessible to B cells. To improve the accessibility to B cells, we coupled a peptide corresponding to the extracellular part of M2 to the immunodominant region of HBc. The Ab response to M2 peptide induced by coupled M2 vs fused M2 was previously shown to be much stronger. In addition, the induction of protective immunity was improved (28). In this study we analyzed the mechanism by which mice vaccinated with M2 coupled to HBc were protected and compared M2-mediated protection with protection in mice vaccinated with inactivated influenza virus. We found that vaccination with M2 coupled to HBc induces protective Abs, whereas the contribution of T cells to protection was negligible. M2-specific Abs failed to neutralize the virus in vitro, suggesting indirect mechanisms of protection, such as complement-mediated cytotoxicity (CDC) or Ab-dependent cytotoxicity (ADCC). Additional experiments showed that C3 was not required for M2-mediated protection, whereas NK cells played a role, demonstrating ADCC to probably be the mechanism in question.

However, protection induced by vaccination with M2 coupled to HBc was weak overall and failed to prevent weight loss in vaccinated infected animals, and mice succumbed to high dose infection. Thus, although protective against many viral strains, vaccines based on M2 confer much weaker protection than vaccines based on single inactivated strains.

Materials and Methods

Mice

C57BL/6 and BALB/c mice were purchased from Harlan (Horst, The Netherlands) at the age of 6 wk. A breeding pair of $C3^{-/-}$ mice (29) was obtained from the laboratory of M. Caroll (Department of Pathology, Harvard Medical School, Boston, MA). Animals were kept under specific pathogen-free conditions.

Construction of plasmid ab1, expression and purification of HBcAg(1–149)-Lys-2cys-Mut, and coupling procedure to HBc

Construction of plasmid ab1 as well as expression and purification steps and coupling procedure were performed as previously described (28).

Immunization of mice

Six-week-old female C57BL/6 or BALB/c mice were immunized s.c. with 30 or 15 μg, respectively, of M2 coupled to HBc without adjuvant or with 15 μg of UV-inactivated influenza virus PR8 in alum.

Adoptive transfer of T cells

Sixteen BALB/c mice were immunized twice (days 0 and 14) with 30 μg of M2 coupled to HBc. On day 33, immunized mice were killed, spleen and lymph nodes were isolated, and viable lymphocytes were recovered by density separation (Lympholyte-M; Cedarlane Laboratories, Hornby, Canada) according to the manufacturer's instructions. $CD4^+$ and $CD8^+$ cells were isolated by MACS (Miltenyi Biotec, Bergisch-Gladbach, Germany) according to the manufacturer's instructions, pooled, and transferred i.v. into eight naive BALB/c mice (2.5×10^6 $CD4^+$ cells/mouse; 7.5×10^5 $CD8^+$ cells/mouse) in a volume of 500 μl/mouse before infection of the recipient mice with virus.

In vivo $CD4^+$ and $CD8^+$ T cell depletion

Mice were treated i.p. on days -3 and -1 before infection with 500 μg of anti-CD4 mAb YTS 191.6 (30) and anti-CD8 mAb YTS 169 (30). This treatment depleted $CD4^+$ and $CD8^+$ T cells below the detection level as measured by FACS analysis (not shown).

Passive immunization

Mice were immunized twice, on days 0 and 14, with 15 μg of UV light-inactivated influenza virus PR8, or 15 or 30 μg of M2 coupled to HBc. Blood samples were taken at several time points starting from day 21 until day 50. Sera from equally immunized mice were collected, heated for 30 min at 57°C to inactivate complement, and injected i.p. (500 μl/mouse) into naive mice the same day that mice were infected with live influenza virus PR8.

ELISA

UV-inactivated influenza virus (diluted in PBS) at 2.5×10^7 PFU/ml or 10 μg/ml peptide, coupled to RNase diluted in coating buffer (0.1 M NaHCO, pH 9.6), was used to coat ELISA plates (Immuno Maxisorp; Nunc, Naperville, IL). ELISAs were performed according to a standard protocol, using HRP-conjugated secondary Abs (Sigma-Aldrich, St. Louis, MO). Plates were developed with *o*-phenylenediamine substrate buffer (0.5 mg/ml *o*-phenylenediamine, 0.01% H_2O_2, 0.066 M Na_2HPO_4, and 0.038 M citric acid, pH 5.0; 100 μl/well) and were read in an ELISA reader at 450 nm. ELISA titers are given as the dilution at which half-maximal OD was reached.

Virus growth, titration, and neutralization assay

Influenza virus PR8 (A/Puerto Rico8/34, H1N1 subtype) was grown, purified, and titrated as previously described (31). Lung virus titers and neutralization titers were determined on Mardin-Darby canine kidney (MDCK) cells as previously described (31). Mice were infected intranasally twice with 50 μl of virus diluted in PBS (lethal infection, 4000 PFU/mouse of live virus; sublethal infection, 400 PFU/mouse of live virus).

Surface staining of virus-infected MDCK cells with anti-M2 or anti-PR8 polyclonal serum

MDCK cells (10^7) were infected (or left uninfected as a control) with 10 multiplicity of infection of influenza virus PR8 in a volume of 10 ml of DMEM (5% FCS, glutamine, and antibiotics) in a Falcon tissue culture flask (75 cm^2; BD Biosciences, Franklin Lakes, NJ) and incubated for 15 h at 37°C. Cells were washed once with PBS before gentle dissolution with a cell scraper (Corning Glass, Corning, NY) and resuspended in 1.2 ml of PBS. Preimmune, anti-M2, and anti-PR8 sera were preabsorbed with MDCK cells to avoid unspecific binding to MDCK cells. The pellets were separated into six parts for staining with undiluted anti-M2 serum, 1/5 diluted anti-M2 serum, undiluted anti-PR8 serum, 1/5 diluted anti-PR8 serum, undiluted preimmune serum, and 1/5 diluted preimmune serum. The samples were stained with an FITC-labeled anti-mouse IgG Ab for FACS analysis (BD PharMingen, Heidelberg, Germany).

Harvest and analysis of bronchoalveolar lavage (BAL) cells

On the days indicated, mice were sacrificed by CO_2 inhalation, a tracheal cannula was inserted, and BAL was performed with five 0.3-ml aliquots of PBS. The total number of leukocytes in 25 μl of BAL fluid was determined using a Coulter counter (Hialeah, FL). Differential cell counts were made counting 200 cells on May-Grünwald-Giemsa-stained cytospins by light microscopy using standard morphological criteria. BAL cells were pelleted by centrifugation, and the supernatant was removed for measurement of Ab levels. The pellet was separated into three parts: one part for staining cells with allophycocyanin-labeled anti-CD4, PE-labeled anti-CD8, and FITC-labeled anti-TCR$\alpha\beta$ for flow cytometric analysis of $CD4^+$ and $CD8^+$ cells; the second part for staining cells with FITC-labeled anti-MHC class II, PE-labeled anti-B220, or allophycocyanin-labeled anti-CD19 for FACS analysis of B cells; and the third part for staining cells with allophycocyanin-labeled anti-NK1.1, FITC-labeled anti-CD3, or PE-labeled anti-TCR$\alpha\beta$ for FACS analysis of NK and NKT cells. Flow cytometric analysis was performed with a FACSCalibur flow cytometer using CellQuest software (BD Biosciences, San Jose, CA).

The first staining was used to analyze the percentages of $CD4^+$ and $CD8^+$ cells, the second staining to analyze the percentage of B cells, and the third staining to analyze the percentages of NK and NKT cells in the lymphocyte cell population. From differential cell counts, the total number of lymphocytes in BAL was calculated and multiplied by the percentages of $CD4^+$, $CD8^+$, B, NK, and NKT cells to obtain the total number of each cell type in BAL.

Depletion of NK and NKT cells

Mice were treated i.v. with 30 μl of anti-asialo-GM1 (Wako Pure Chemical, Osaka, Japan) starting 3 days before infection, and treatment was

repeated every third day as previously described (32). This treatment depleted NK and NKT cells below the detection level measured by FACS analysis of blood samples on day3 after infection with PE-labeled anti-NK1.1 and FITC-labeled anti-CD3.

Statistics

Statistical analysis of differences in viral titers in the lung between immunized and nonimmunized mice were performed using Student's *t* test. The significance of differences between survival curves was analyzed by Kaplan-Meier survival analysis using the program PRISM (version 4.00). One-tailed analysis was used. A value of $p < 0.05$ was regarded as statistically significant.

Results

Protection of mice immunized with M2 coupled to HBc is mediated via Abs rather than T cells

Immunization of mice with M2 peptide in alum gave raise to IgG titers to M2 peptide (data not shown), a fact that demonstrated that the M2 peptide contained a Th epitope. A study by Okuda et al. (26) previously revealed protective immunity against influenza A virus induced after immunization with a DNA plasmid containing the influenza M gene. This protection was attributed to the induction of a CTL response (26). To study the contribution of T cells to protection of mice vaccinated with M2 peptide coupled to HBc (M2-HBc), two strategies were followed. Firstly, immunized animals were treated with anti-CD4 and anti-CD8 mAbs to deplete mice of T cells before infection with a lethal dose of influenza A virus PR8. Secondly, T cells from immunized animals were isolated and adoptively transferred into naive BALB/c mice before infection. To study the contribution of M2-specific Abs, groups of mice were passively immunized with 500 or 50 μl of serum derived from mice previously immunized with M2-HBc (Fig. 1). M2-HBc-immunized mice showed 100% protection against infection with a lethal dose of influenza A virus PR8. The same was true for mice depleted of T cells by mAbs (depletion was >95%). In addition, adoptively transferred T cells from immunized mice could not protect against a lethal challenge. Taken together these results demonstrate that T cells are not responsible for protection. In contrast, passively immunized mice showed 50 or 25% protection depending on the dose of passively transferred serum, demonstrating that M2-specific Abs account for protection and that the degree of protection is dependent on Ab titer. All negative controls succumbed to the lethal infection.

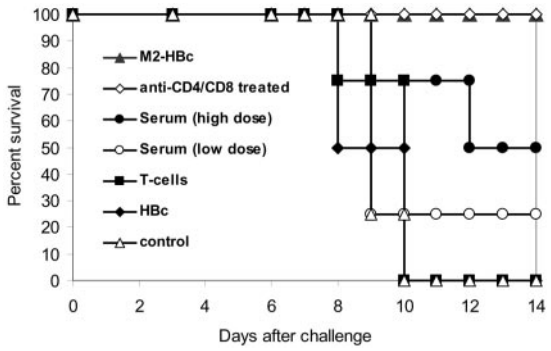

FIGURE 1. Protection of mice immunized with M2 coupled to HBc is mediated via Abs, rather than T cells. Six-week-old female BALB/c mice were immunized with M2 coupled to HBc (30 μg), boosted with the same amount of protein on day 14, and bled on day 22. Serum was collected for passive serum transfer. On day 33, 16 mice were killed, and $CD4^+$ and $CD8^+$ T cells were isolated from spleen and lymph nodes and together passively transferred into naive BALB/c mice. $CD4^+$ and $CD8^+$ T cells isolated from two mice were transferred into one mouse. Groups of four naive BALB/c mice received 500 μl of serum i.p., 50 μl of serum diluted 1/10 with PBS i.p., T cells i.v., or T cells i.v. together with 500 μl of diluted serum (data not shown). One group of four mice immunized with M2 coupled to HBc was treated on days 3 and 1 of infection i.p. with 500 μg of anti-CD4 and 500 μg of anti-CD8 mAbs to deplete T cells. On day 33, mice were infected with 4×10^3 live influenza A virus/mouse. As a positive control, one group of four mice was immunized with M2 coupled to HBc; as a negative control, groups of four mice, either nonimmunized or immunized with HBc (30 μg) alone, were also infected with the same amount of virus.

Abs induced by immunization with M2-HBc act upon binding to influenza A virus-infected cells rather than binding to virus and neutralization

Polyclonal serum from mice immunized with M2-HBc or UV-inactivated influenza virus was analyzed by ELISA to identify IgG Abs directed against M2 peptide or UV-inactivated influenza virus. In addition, the capacity of the sera to inhibit virus infection of MDCK cells was tested in a neutralization assay. Serum samples were taken 13 days after the first immunization and 20 days after the second immunization, which took place on day 14. Mice immunized with M2-HBc developed a considerable Ab response against M2 peptide (Fig. 2*A*), but Abs did not recognize influenza virus (Fig. 2*B*) and did not neutralize virus infection of cells (Fig. 2*C*). Titers of M2-specific Abs in serum samples from mice immunized with UV-inactivated virus were very low (Fig. 2*A*), but a moderate Ab response against UV-inactivated virus could be detected (Fig. 2*B*). Moreover, these Abs were able to neutralize virus infection of MDCK cells (Fig. 2*C*). In a subsequent experiment, MDCK cells were infected with influenza virus to allow expression of M2 protein on the surface of MDCK cells. The cells were incubated with preimmune serum or serum from M2-HBc immunized mice (Fig. 2*D*), and anti-M2 protein IgG Abs bound to infected cells would be detected by FACS. Only sera from immunized mice, but not preimmune sera, bound to infected cells. This allows two conclusions. First, Abs induced against M2 peptide are cross-reactive with a protein expressed on virus-infected cells, apparently native M2 protein, and second, Abs probably act in vivo by binding to the surface of infected cells. Thus, protection is probably dependent on Fc- or complement-mediated effector functions such as ADCC or CDC. As expected, serum from mice immunized with the UV-inactivated influenza virus also contained IgG Abs that stained virus-infected cells, probably by recognizing HA protein expressed on the surface of infected cells (Fig. 2*D*).

Analysis of cell populations in BAL, Ab titers and isotypes in BAL and blood, and virus titers in lung

Among the cells that can mediate ADCC are NK cells, macrophages, neutrophils, and eosinophils. To compare infiltration of cells at the site of infection, BAL of naive infected mice was compared with that of infected mice that had been previously immunized with a sublethal dose of influenza virus PR8 on days 5, 7, and 9 after infection (Fig. 3*A*). Surprisingly, there were only small differences in the number of infiltrating cells. However, virus titers in the lungs of immunized mice were slightly, but significantly, reduced on days 5 and 7 after infection (Fig. 3*D*). Nevertheless, this reduction did not result in a decreased recruitment of cells. On day 9 virus was almost cleared from lungs of immunized mice as well as from nonimmunized mice, correlating with a strong infiltration by T cells.

Next, we assessed M2 peptide-specific IgG Ab titers in blood and BAL before and after infection (Fig. 3*B*). Mice immunized with M2-HBc showed a considerable anti-M2 IgG titer before infection in the blood, but not in BAL. After infection of these mice

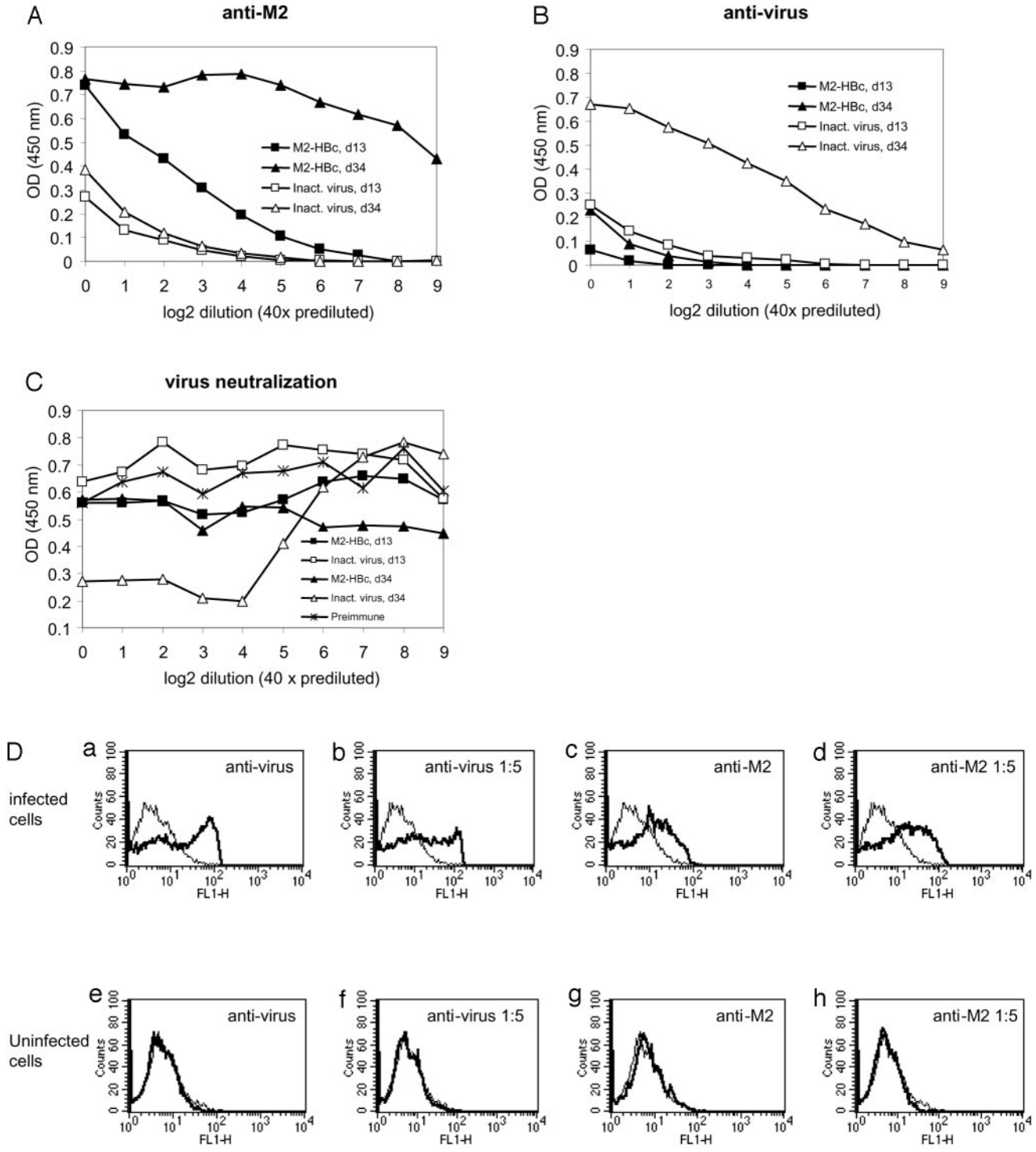

FIGURE 2. M2-specific Abs act upon binding to influenza A virus-infected cells rather than binding to virus and neutralization. *A*, ELISA against M2 peptide coupled to RNase using sera from mice immunized twice with 15 μg of M2-HBc or 15 μg of inactivated virus (day 13, after first immunization; day 34, after second immunization). *B*, ELISA against UV-inactivated influenza A virus using the same sera in *A*. At the lowest dilution, preimmune sera showed OD values <0.05 (data not shown). *C*, Virus neutralization assessed by ELISA on virus-infected cells. Sera from mice immunized as described in *A* were used in the neutralization assay on MDCK cells. Absorbance values at 340 nm were evaluated using an ELISA reader after 20 min. Preimmune serum was used as a negative control. *D*, MDCK cells were infected with 10 multiplicity of infection of influenza A virus overnight and subsequently stained with undiluted (*a*), 1/5 diluted serum from mice immunized twice with UV-inactivated influenza A virus (*b*), or undiluted (*c*) or 1/5 diluted (*d*) serum from mice immunized twice with M2 coupled to HBc. As a control, uninfected MDCK cells were stained with undiluted (*e*), 1/5 diluted serum from mice immunized twice with UV-inactivated influenza A virus (*f*), or undiluted (*g*) or 1/5 diluted (*h*) serum from mice immunized twice with M2 coupled to HBc. Control samples were stained with preimmune serum. An FITC-anti-mouse IgG secondary Ab was used, and cells were analyzed by flow cytometry (normal lines, control samples; bold lines, specifically stained samples).

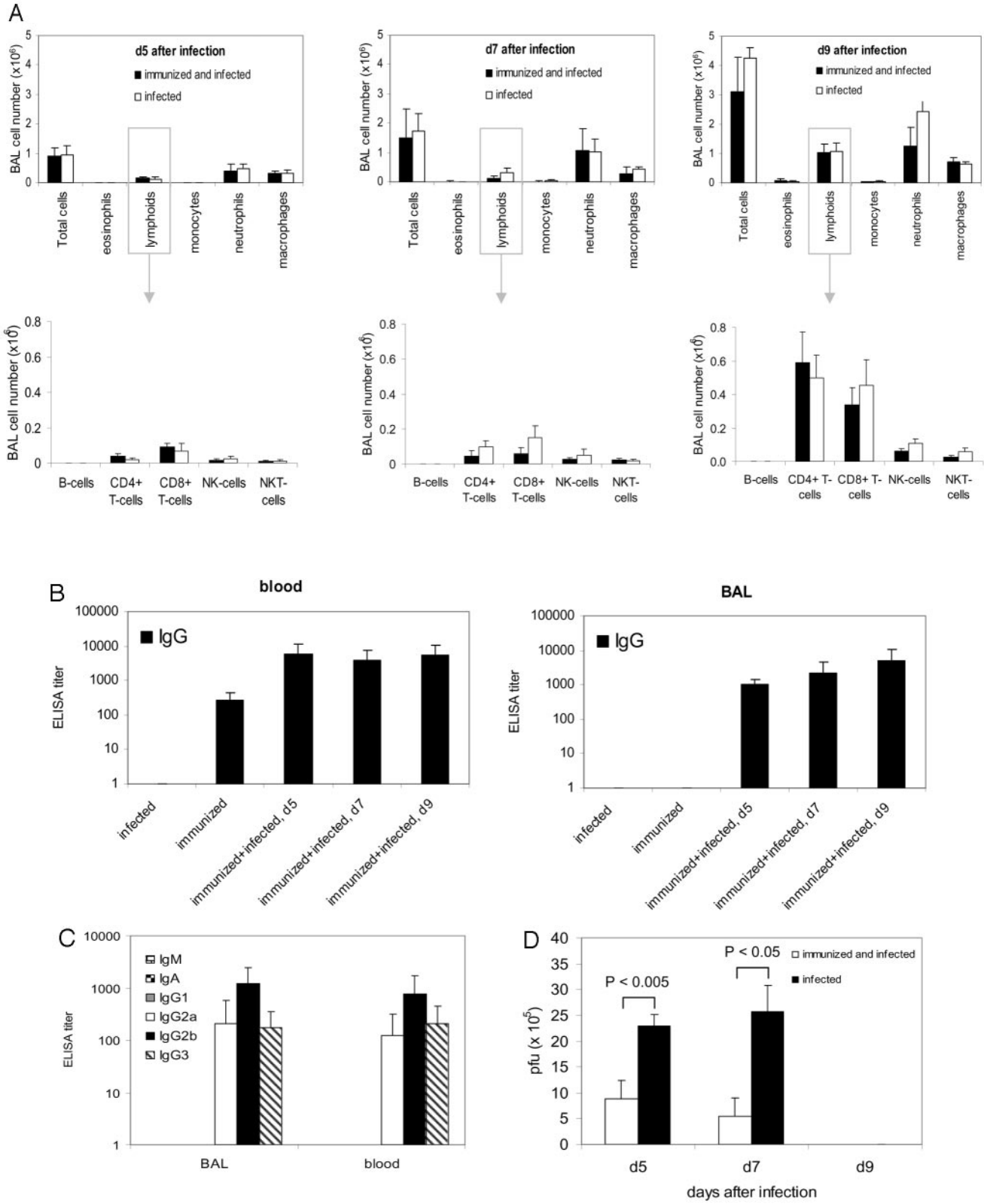

FIGURE 3. Analysis of cell populations in BAL, Ab titers and isotypes in BAL and blood, and virus titers in lung. *A*, Six-week-old female C57BL/6 mice were immunized and boosted with M2 coupled to HBc (30 μg) and infected 8 days later with 4×10^2 PFU of live influenza virus (sublethal dose). As a comparison, naive mice were infected with the same amount of virus. BAL of immunized and nonimmunized mice was taken on days 5, 7, and 9 after infection. Different cell types were visualized by May-Grünwald-Giemsa staining, and lymphoid subtypes were analyzed by FACS. *B*, IgG titers against M2 peptide in blood and BAL of mice only infected with virus or immunized with M2 coupled to HBc and infected with virus at different time points. *C*, Ab isotypes in BAL and blood of mice immunized with M2-HBc and infected with virus. *D*, Virus titers in the lungs of mice either immunized and infected or infected only at different time points. The *p* values were determined by Student's *t* test. Results are represented as the mean ± SD.

with live virus, the anti-M2 IgG titer increased in blood, and substantial Ab titers became detectable in BAL. As Ab isotypes are important in Fc-mediated effector functions, we determined the IgG isotypes in BAL and blood after infection (Fig. 3*C*). The predominant isotype was IgG2b; lower amounts of both IgG2a and IgG3 were detected, but no IgG1, IgM, or IgA was found.

Protection by M2-specific Abs in the absence of C3

Once a virus-infected cell is opsonized by IgG, it may cross-link FcRs on the cell surface of effector cells that mediate ADCC. In addition, arrays of Ab Fc regions bound to the surface of an infected cell may also activate the classical or alternative pathway of complement activation, ultimately leading to CDC. To distinguish between these two different mechanisms, we assessed M2-mediated protection in $C3^{-/-}$ mice. It has been shown that the T cell-dependent isotype switch from IgM to IgG is dramatically reduced in $C3^{-/-}$ mice (33). Therefore, active immunization of $C3^{-/-}$ mice with M2-HBc in comparison with $C3^{+/+}$ mice would not have resulted in an equal IgG-Ab response, making a proper comparison impossible. This problem could be avoided by passive immunization with 500 μl of serum from C57BL/6 mice immunized twice with M2-HBc. As a positive control, mice were passively immunized with serum from mice previously immunized with UV-inactivated influenza virus. As a negative control, mice were transfused with preimmune serum. Sixty percent of $C3^{-/-}$ (Fig. 4*A*) as well as 60% of C57BL/6 mice (Fig. 4*B*) passively immunized with serum from M2-HBc-immunized mice survived ($p > 0.05$, by Kaplan-Meyer survival analysis). These results show that complement does not play an important role in protection. In addition, $C3^{-/-}$ (Fig. 4*A*) and $C3^{+/+}$ mice (Fig. 4*B*) passively vaccinated with serum from mice immunized with UV-inactivated influenza virus were fully protected.

Impaired protection of M2-HBc-immunized mice in the absence of NK and NKT cells

The analysis of BAL fluid did not answer the question of whether ADCC was the primary mechanism involved in protection. As NK cells seem to be the most important mediators of ADCC, we depleted immunized mice of NK (and NKT) cells and compared protection with immunized mice containing intact NK and NKT compartments. To do this, C57BL/6 mice were immunized twice with M2-HBc, and half the mice were treated with anti-asialo-GM1 to deplete NK cells. FACS analysis showed that NK and NKT cells were depleted (below detection level; not shown). Mice were infected with a lethal dose of 4×10^3 PFU/mouse of PR8 influenza virus, and as a negative control, naive C57BL/6 mice were infected. Depletion of NK cells in immunized mice diminished protection compared with nondepleted immunized mice ($p < 0.03$, by Kaplan-Meyer survival analysis; Fig. 5). Thus, NK cells are a key mediator of anti-M2 Ab-dependent antiviral protection. However, depleted mice showed slightly enhanced protection compared with naive C57BL/6 mice, leading to several, not mutually exclusive, possibilities: 1) other cell types, such as macrophages, neutrophils, or eosinophils, which are also able to act via ADCC, could be involved in protection; 2) ADCC is not the only factor involved in protection; and 3) some NK cells are not depleted from the tissue.

Protection of mice after immunization with M2 coupled to HBc is less potent than protection in mice immunized with UV-inactivated influenza virus

There is some evidence that Abs are less effective against infected cells than against free virus (34). A higher concentration of Abs has been shown to be required for effective CDC and ADCC compared with the amount required for neutralization (35). Therefore, the protective potential of M2-HBc was assessed by infecting M2-HBc-immunized or UV-inactivated influenza virus-immunized mice with three different infectious doses of live influenza virus PR8 (Fig. 6*A*). The low dose corresponded to 4×10^3 PFU/mouse, the medium dose to 4×10^4 PFU/mouse, and the high dose to 4×10^5 PFU/mouse. Although mice previously immunized with UV-inactivated influenza virus showed 100% protection at all doses, this was only true for M2-HBc-immunized mice infected with a low dose of influenza virus. Only 50% of mice survived a medium virus dose, and only 33% of mice survived a challenge with a high virus dose. Mice immunized with M2-HBc and infected with low dose virus survived; however, they showed signs of disease, which become apparent by weight loss before recovery from illness (Fig. 6*B*).

Discussion

In this study we analyzed in detail the potential of an influenza A vaccine based on the conserved extracellular domain of M2. Such a vaccine might have the advantage of providing protection against most known human influenza A strains. The data presented in this study show that protection induced by the M2 vaccine is exclusively mediated by Abs, which allows the assumption that the vaccine would induce long term protection. Indeed, it has been argued

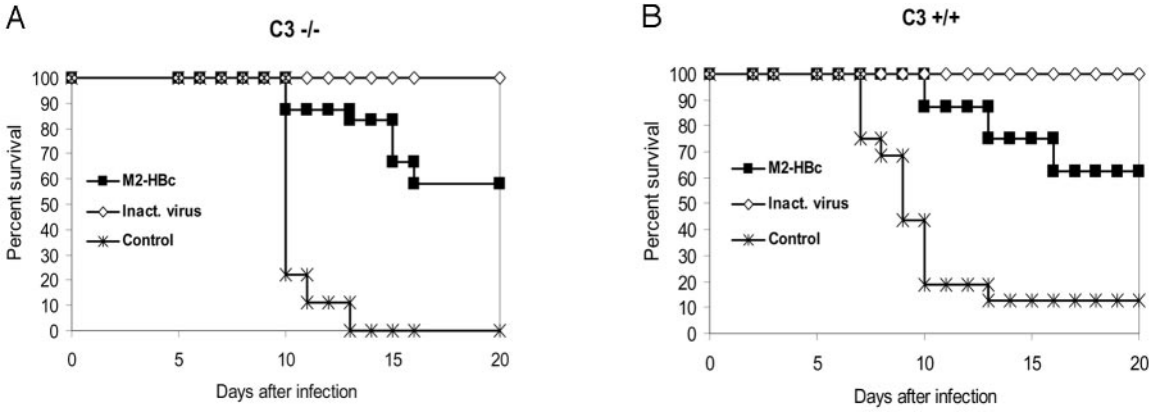

FIGURE 4. Protection of M2-specific Abs in the absence of C3. Serum was collected from mice immunized as described in Fig. 2*A* and passively transferred i.p. into groups of 6-wk-old female $C3^{-/-}$ (*A*) or C57BL/6 (*B*) mice (500 μl of serum/mouse). Mice were infected immediately after serum transfer with 4×10^3 PFU of influenza A virus/mouse (strain PR8), and the survival of the mice was followed over a period of 20 days. In each figure, data from two independent experiments are shown.

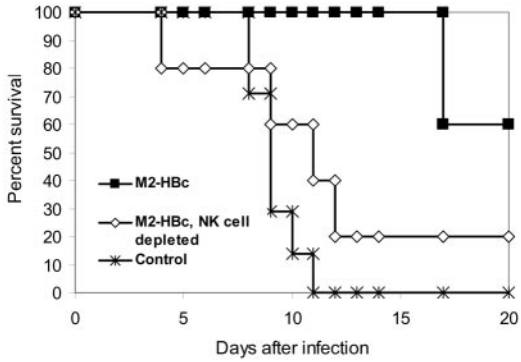

FIGURE 5. Impaired protection of M2-HBc-immunized mice in the absence of NK and NKT cells. Ten C57BL/6 mice were immunized twice (days 0 and 14) with 30 μg of M2-HBc, and half the mice were treated i.v. with 30 μl of anti-asialo-GM1 Abs starting on day 3 of infection. Treatment was repeated every third day to deplete NK cells. Mice were infected on day 21 with a lethal dose of 4×10^3 PFU/mouse of PR8 influenza virus. As a negative control, seven naive C57BL/6 mice were infected. The survival of the mice was followed until day 20. A Kaplan-Meier survival analysis was performed to compare M2-HBc-immunized mice with M2-HBc-immunized and NK cell-depleted mice, and a p value of 0.028 was obtained. In contrast, the difference between nonimmunized and NK cell-depleted, immunized mice was not significant.

that Ab-mediated immunity is more long-lived than T cell-mediated immunity, and that therefore most successful preventive antiviral vaccines are based on the induction of neutralizing Abs (36–38).

Despite antiviral protection being exclusively provided by Abs, the failure to induce neutralizing Abs may be a major drawback for an M2-based vaccine. Mice immunized with UV-inactivated influenza virus were protected against infection with much higher doses of influenza virus than mice immunized with the M2-HBc vaccine, although Ab titers were comparable. Anti-HA Abs are likely to be the most important species in mice immunized with inactivated virus, and these Abs have been shown to bind to free virus as well as to virus-infected cells. HA Abs are more effective than M2-Abs because they can 1) neutralize and prevent infection, 2) induce elimination of virus by FcR-mediated phagocytosis (39), and 3) mediate killing of infected cells via ADCC or CDC. Anti-M2 Abs only have the third capacity; therefore, although mice immunized with M2-HBc are protected, they show signs of disease, which is visible as weight loss, before recovering from illness.

Our data demonstrate that the complement component C3 is not critical for the protective capacity of the Abs; rather, protection may be mediated via NK cells, as NK cell-depleted mice showed reduced protection by vaccination with M2-HBc. We would hypothesize that NK cells recognize Ab-decorated infected cells and mediate the lysis of these cells. However, anti-M2 Abs seem to be poorly efficient in the initial phase of infection, as the infiltration of cells at the site of infection is the same in immunized mice and nonimmunized mice. There may be several different reasons for this. 1) The virus has to infect cells and induce expression of M2 on the surface before Abs can bind and become effective. 2) Anti-M2 Abs may simply not be present at the site of infection in the initial phase, as anti-M2 IgG Abs appear in BAL only after infection.

Nevertheless, anti-M2 IgG titers in the blood rise after infection in vaccinated mice. Normally, mice infected with influenza virus do not generate sizeable titers of Abs recognizing M2. The dominant M2-specific Ab isotype after infection of vaccinated mice is IgG2b with some IgG2a. These two isotypes have been shown to be the most important mediators of ADCC in mice (40). Thus, the right amount and the right isotypes of Abs seem to be present at the right site only some time after infection. Current knowledge indicates that the major Ab isotype in upper respiratory secretions is IgA, which is produced locally. In contrast, the major isotype in lower respiratory secretions is IgG derived primarily from serum (41, 42). As no anti-M2 IgA Abs could be found in BAL before and after infection, infection is probably not controlled in the upper respiratory tract by IgA Abs. This problem could be avoided by intranasal administration of the vaccine. Nevertheless, s.c. immunization with inactivated virus also did not induce IgA Abs, yet mice were fully protected. Failure to induce IgA Abs with the M2-HBc vaccine, therefore, is not the only reason for the inefficiency of the vaccine, rather it is the specificity of the Abs that seems to play the major role.

Influenza is a unique virus with a particular epidemiology. It causes epidemics and pandemics, which may result in quite different vaccination requirements. Although only those at risk are supposed to get vaccinated during an epidemic, it would be wise to vaccinate everybody against a pandemic. Because of its poor protection capacity, the M2-HBc vaccine is not suitable for use during an epidemic, when the goal is to produce full protection with no

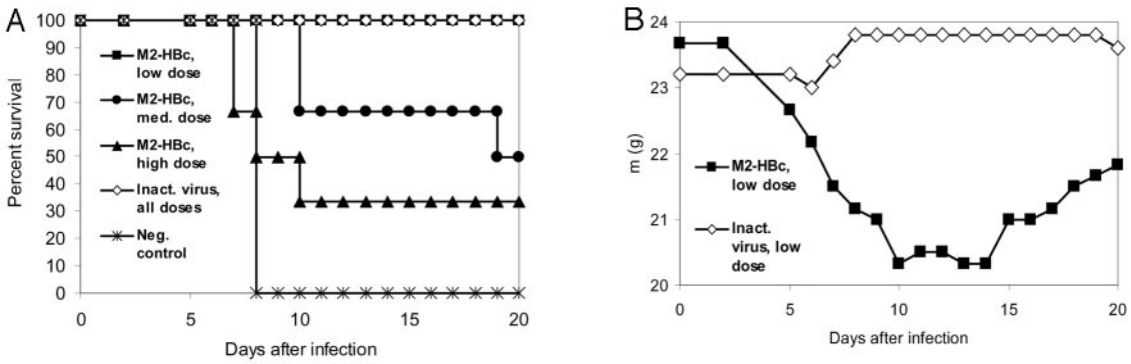

FIGURE 6. Protection of mice after immunization with M2 coupled to HBc is less potent than that of mice immunized with UV-inactivated influenza A virus. *A*, Groups of 6-wk-old female C57BL/6 mice were immunized twice s.c. with M2 coupled to HBc (15 μg) or UV-inactivated influenza A virus (strain PR8) (15 μg) and infected with a low dose (4×10^3 PFU/mouse), a medium dose (4×10^4 PFU/mouse), or a high dose (4×10^5/mouse) of live influenza A virus (strain PR8). Each treatment group consisted of three mice. As a negative control, naive mice were immunized with a low dose of virus. The survival of the mice was followed over a period of 20 days. *B*, Weight loss of mice immunized either with M2 coupled to HBc or UV-inactivated influenza A virus and infected with a low dose of virus.

signs of disease. However, immunization with the M2-HBc vaccine may be considered as a precaution to prevent or at least reduce mortality during a pandemic. Recently, the coding region of influenza A virus RNA segment 7 from the 1918 pandemic virus, consisting of the open reading frames of the two matrix genes, M1 and M2, has been sequenced (9). The extracellular domain of the M2 protein of the 1918 strain has only one amino acid change compared with the conserved sequence of other known human influenza strains. Furthermore, a recombinant influenza virus that possesses the M segment of the 1918 virus and the remaining seven genes of the amantadine/rimantadine-resistant A/WSN/33 virus was inhibited effectively both in tissue culture and in vivo by amantadine and rimantadine (43). Therefore, it can be assumed that the M2-HBc vaccine would also have been effective against the 1918 pandemic. Reemergence of a 1918 or 1918-like influenza virus, whether through natural means or as result of bioterrorism is of significant concern, as the 1918 pandemic killed 20–40 million people worldwide. From this point of view, the M2-HBc vaccine may represent a well-chosen influenza prophylaxis, especially considering the dangers of producing a 1918-like influenza virus for the purposes of vaccine production.

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