Universal influenza A vaccine: Optimization of M2-based constructs

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Abstract

M2e is the external domain of the influenza A M2-protein. It is minimally immunogenic during infection and conventional vaccination, which explains in part its striking sequence conservation across all human influenza A strains. Previous research has shown that when M2e is linked to an appropriate carrier such as hepatitis B virus core (HBc) particles, it becomes highly immunogenic, eliciting antibodies that fully protect mice against a potentially lethal virus infection. Different M2e-HBc particles and adjuvants suitable for human use were compared for induction of protective immunity. Strong immunogenicity and full protection were obtained after either intraperitoneal or intranasal administration. The most protective particle contained three consecutive M2e-copies linked to the N-terminus of HBc. Although HBc is highly immunogenic, the optimized M2e-HBc vaccine induced an anti-M2e antibody titer even higher than that of anti-HBc.
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Introduction

Influenza virus typically infects 10–20% of the total worldwide population during seasonal epidemics, resulting in three to five million cases of severe illness and 250,000 to 500,000 deaths per year (Anonymous, 2003). In the US, influenza kills an average of 20,000 people per year, while an average of 114,000 influenza-related hospitalizations result in an estimated economic cost of $12 billion per year (Poland et al., 2001). Moreover, novel influenza strains appear occasionally in the human population, causing pandemics. The death toll of the 1917–1920 Spanish Flu pandemic resulted in over 50 million deaths worldwide (Johnson and Mueller, 2002), and the danger of a new influenza pandemic is always present.

Nowadays, conventional vaccines contain antigenic determinants from three virus strains, two A-strains and one B, predicted to be the most likely to cause the next epidemic. They are dosed on the basis of their hemagglu-

tinin (HA) content, the major glycoprotein of the virus. Approximately 250 million doses of influenza vaccine are produced each year (Gerdil, 2003), with protective efficacy in children and adults ranging between 60% and 90% (Cox and Subbarao, 1999). Although these vaccines are less effective in the elderly, 80% of influenza-caused deaths in this age group are prevented (Patriarca et al., 1985). However, conventional vaccines have important drawbacks, the most important of which is the uncertainty in the choice of virus strains to be included in each year's vaccine formulation. Over a 10-year period, the match between predicted and circulating strains was correct in 88% of cases (Palache, 1992), but inaccurate predictions can greatly affect vaccine efficacy. In the 1997–1998 winter season, for example, efficacy of vaccination against serologically confirmed influenza illness was reduced to 50% (Bridges et al., 2000). During the 2003–2004 season, the A/Panama/2007/99 strain, a component of the conventional vaccines, did not satisfactorily match the related, prevalent, circulating strain A/Fujian/411/2002. An even greater threat, however, exists in the form of transmission of highly virulent avian influenza viruses from birds to humans, as occurred in Hong

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Kong in 1997 and 1999, and, more recently, in several Far East countries in 2003–2004 and the ongoing 2004–2005 winter season (Claas et al., 1998; Normile, 2004; Peiris et al., 1999; Suarez et al., 1998). 45 cases of avian H5N1 transmission to humans were identified in 2004, of which 32 were fatal (Anonymous, 2004). These rare infections in humans may, by gene reassortment, give rise to novel human influenza A strains with pandemic potential. In addition to the uncertainty associated with strain prediction, conventional vaccine production has inherent deficiencies. These vaccines are produced by growing virus in embryonated chicken eggs or tissue culture, procedures which are not without potential pitfalls and uncertainties. These shortcomings are related to, for instance, adequacy of egg supply, appropriateness of virus inoculating stocks, biological safety, residual avian proteins, killing of chicken embryos by highly virulent avian viruses, etc. (Normile, 2004; Webby et al., 2004).

In view of the inherent limitations in the approved, conventional influenza vaccines, we have taken a completely different approach, based on the highly conserved, extracellular domain of the viral M2-protein (M2e). The M2-protein is a tetrameric, type III transmembrane protein scarcely present on virus particles, but abundant on virus-infected cells (Lamb et al., 1985; Holsinger and Lamb, 1991; Pinto et al., 1992). The human influenza A M2e-sequence is only 23 amino acids long. It has remained nearly unchanged since the first human influenza strain was isolated in 1933, despite numerous epidemics and two major pandemics since then (Fiers et al., 2004; Neirynck et al., 1999). By linking M2e to a carrier, such as the hepatitis B Virus core particle (HBc), the sequence can be presented in a much more immunogenic form to the immune system. Antibodies produced after vaccination with M2e-HBc particles fully protect mice against a potentially lethal challenge by a mouse-adapted influenza strain (Neirynck et al., 1999). Procedures for expression and purification of M2e-HBc are straightforward, efficient, and reproducible. Because the M2e-HBc material is made in *Escherichia coli*, safety concerns often associated with preparations derived from eukaryotic expression systems are avoided. In view of the conserved nature of the target sequence, human influenza A M2e, we can refer to this system as a "universal vaccine" (Neirynck et al., 1999). Further experiments documenting the universality of the vaccine for all human-transmissible influenza A viruses are ongoing.

We previously published the proof of concept for this M2e-based influenza A vaccine and demonstrated that the protection elicited was due to antibodies/humoral immune responses as it was transferable by serum (Neirynck et al., 1999). Since then, we have carried out experiments whereby various parameters were evaluated, such as different molecular constructs, adjuvants, and routes of administration. These efforts have led to an improved vaccine candidate, as here described.

Results

Protection against a lethal influenza A infection can be achieved when the M2e-antigen is genetically linked to the N-terminus or inserted in the immunodominant loop of C-terminally truncated HBc particles

We previously reported protection of mice against a potentially lethal influenza A infection using a vaccine consisting of full-length HBc particles with M2e genetically fused to the N-terminus of the HBc-subunits (Neirynck et al., 1999). As no modifications are made at the N-terminus of M2e, it remains as it is in the native influenza A M2-protein. The icosahedral HBc-particle consists of 240 monomers, arranged as dimeric building blocks (Wynne et al., 1999). The particle carries 120 spikes on its surface, each of which consists of two interacting α-helical loops formed by the two partners of the dimer. These spikes are highly immunogenic and can be used for insertion of foreign B-cell epitopes (Kratz et al., 1999; Murray and Shiau, 1999; Pumpens and Grens, 2001).

The M2e-HBc particle used previously (Neirynck et al., 1999), now renamed particle 1559, contains the full-length HBc protein, except for the first 4 amino acids, which are replaced by M2e. The basic C-terminal domain of the HBc protein, which is 34 amino acids in length, contains protamine-like clusters and functions as an anchor to retain the viral DNA in the particle. When produced in *E. coli* and purified, this domain binds host RNA, which accounts for approximately 25% of the particle weight (Wingfield et al., 1995). Although these contaminants may contribute to the immunogenicity by acting as adjuvants, they negatively impact yields and complicate the rigorous chemical characterization of the vaccine preparation. Because the basic C-terminal domain (amino acids 150–183) is not needed for HBc particle assembly (Zheng et al., 1992), it was deleted from particle 1473, and a Cys residue was added (position 150) to increase stability (Birkett, 1999). M2e contains two Cys-residues at positions 17 and 19. It is conceivable that these cysteines could result in aggregation under oxidative conditions by the formation of inter-particle disulfide bonds. Therefore, the two Cys residues in M2e were replaced by Ala in particle 1473 (replacement in all later particles was by Ser, which better mimics Cys).

In our earlier experiments (Neirynck et al., 1999), vaccination with particle 1559 (previously referred to as IM2HBc), administered either intraperitoneally (i.p.) with "MPL-TDM emulsion/MPL-MDP" as adjuvant, or intranasally (i.n.) without adjuvant, fully protected mice against a potentially lethal infection with mouse-adapted (m.a.) influenza A (strain X47). Therefore, these experiments were repeated as positive controls. The results, shown in Fig. 1, confirm that these formulations, indeed, confer full protection. Moreover, vaccination with particle 1473 also resulted in complete protection. This means that the M2e-peptide does not have to start with a free
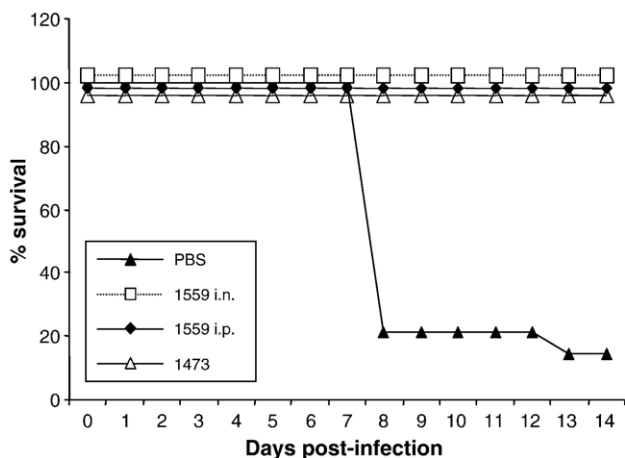
Fig. 1. Survival of vaccinated BALB/c mice after challenge with 4 LD50 of m.a. X47 influenza A virus. The experiment involved 4 groups of 14 female mice, 8 weeks old. Three immunizations with 10 μg vaccine per mouse were given at 3-week intervals. Mice were challenged 3 weeks after the last boost. Particles 1559 and 1473 (Table 1) were administered i.p. in combination with MPL-TDM emulsion (priming) followed by MPL-MDP (boosters) as adjuvant. Particle 1559 was also administered i.n. without adjuvant (cf. Materials and methods and Neirynck et al., 1999 for further details).

N-terminus in order to be effective, that the basic C-terminal domain of the HBc subunit is dispensable, and that the Cys-residues in the M2e-sequence can be replaced by residues of similar size.

Particles 1559 and 1473 confer full protection when administered i.p. with different adjuvants

The most commonly used adjuvants in approved human vaccines are aluminum salts, namely aluminum hydroxide and aluminum phosphate. Although this class of adjuvants has a long record of safety, their efficacy has been surpassed by new types of adjuvants. One of these is Montanide ISA-720, a water-in-non-mineral oil emulsion that has been studied in human Phase I and Phase II clinical trials (Aucouturier et al., 2002, and Seppic data sheet P/1755/GB/01/July 2001—Revised August 3, 2001). We evaluated the effects of particles 1559 and 1473 alone, adsorbed on Alhydrogel, or in combination with Montanide ISA-720. When formulated with adjuvants, each of these particles provided complete protection against a potentially lethal challenge infection (Fig. 2A). In the absence of adjuvant, protection was still complete for particle 1559 but only partial for 1473; the difference in survival was significant (Kaplan–Meier method, $P < 0.05$).

In all experiments, morbidity following challenge was monitored by measuring weight and rectal temperature. There was usually a weight loss after challenge, even in the fully protected groups. As discussed previously (Neirynck et al., 1999), the incomplete prevention of morbidity may be explained by the severity of the

challenge. A highly lethal dose for challenge ensures that all, or nearly all, control animals will die as a consequence, and unambiguously confirms the protective efficacy of the vaccine. But it should be stressed that this strong challenge dose results in a much more abrupt and intense illness than humans are likely to experience when naturally infected. It may be noted in this respect that a conventional, HA-based vaccine, tested in mice in a similar vaccination/lethal challenge model as used here, was protective but was also unable to alleviate morbidity as monitored by weight loss (Huber et al., 2001). The data presented in Fig. 2B reveal that immunization in the presence of adjuvant does not prevent morbidity but significantly reduces it (Tukey test, $P < 0.05$ for adjuvanted relative to unadjuvanted vaccination; data for day 8). Additionally, Fig. 2B suggests that Montanide ISA-720 may be slightly superior to Alhydrogel in reducing morbidity, but the difference between the two adjuvants was not statistically significant. Similar conclusions about morbidity could be drawn from the body temperature data (not shown).

We also monitored the anti-M2e immune response by determining the titer of the various M2e-specific IgG
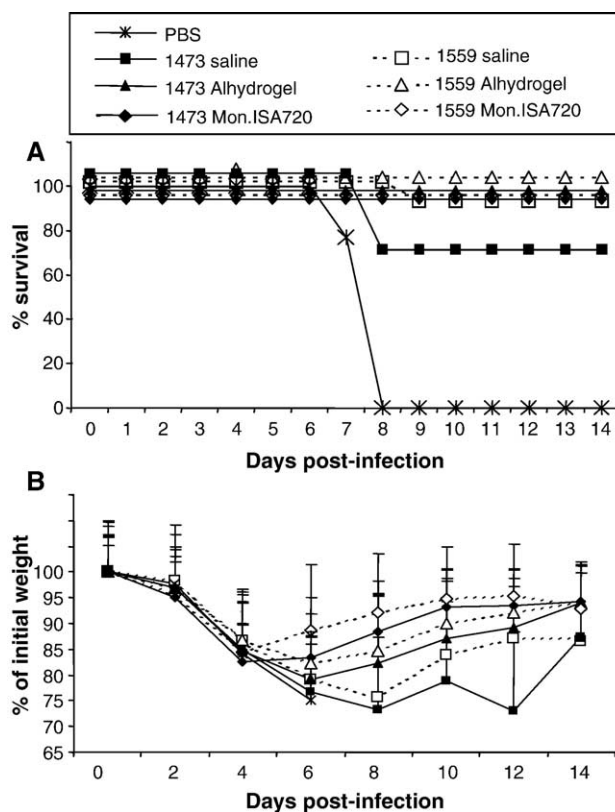

Fig. 2. Seven groups of 14 BALB/c mice were vaccinated i.p. with particle 1473 or 1559 in absence or presence of adjuvant, Alhydrogel, or Montanide ISA-720, as indicated, followed by challenge with 4 LD50 of m.a. X47 virus (cf. Materials and methods for further details). (A) Mortality after challenge. (B) Percent of initial body weight after challenge (with standard error bars).

subclasses (IgG1, IgG2a, IgG2b, and IgG3). The IgG1 titer was considerably enhanced by the inclusion of an adjuvant in the vaccine preparation, especially Montanide ISA-720 (Fig. 3A). For example, both for particles 1559 and 1473, the serum titers obtained after boost 2 for the Montanide ISA-720 and Alhydrogel-adjuvanted groups were significantly higher than the titers obtained for the unadjuvanted group (Tukey multiple comparison of means test at 95% confidence level). It may be noted that the IgG1 titer after two boosts with M2e-HBc particles formulated with Montanide ISA-720 was so high that it was not enhanced further by the challenge infection. In Fig. 3B, the IgG2a data are shown. We have previously reported that protection seems to correlate with a high IgG2a titer, which suggests that a T-helper cell type 1 response is needed for efficient protection (Fiers et al., 2004). Therefore, it is of interest to note that particle 1473, which provided only partial protection in the absence of adjuvant (Fig. 2A), had induced only a fairly weak IgG2a response. Conversely, the IgG1 titer after the second boost of particle 1473 in saline was higher than that of 1559 in saline (Fig. 3A; difference not statistically significant). To conclude, the analysis of humoral immune responses correlates with the morbidity data. For both particle 1473 and 1559, IgG1 and IgG2a titers after the second boost were higher in combination with Montanide ISA-720 than with Alhydrogel.
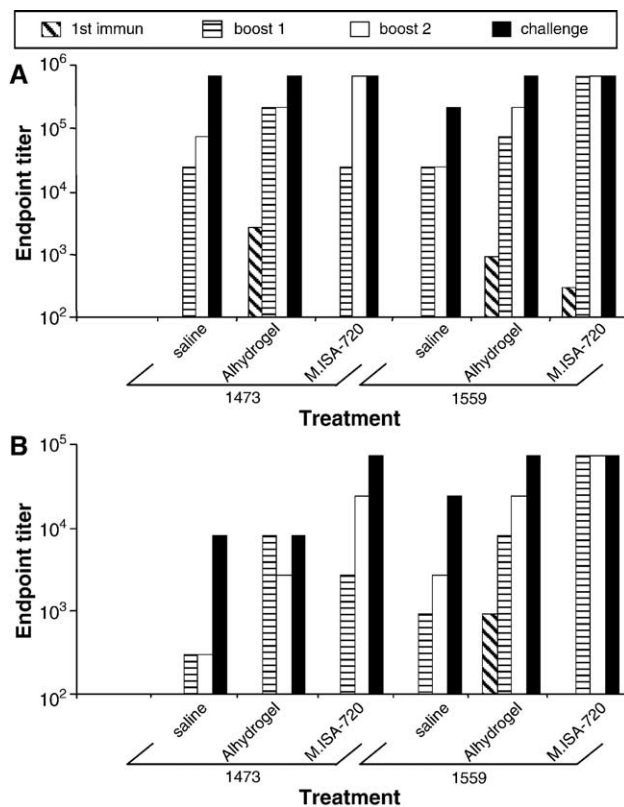

Fig. 3. M2e-specific IgG subclasses in pooled sera from mice used in the experiment shown in Fig. 2. (A) IgG1. (B) IgG2a (similar titers were obtained for IgG2b).

In combination with adjuvants appropriate for human use, particles 1604 and 1569 provide complete protection against a potentially lethal influenza challenge

Particle 1604, like particle 1559, contains the M2e sequence fused to the N-terminus of the HBc subunit, but the C-terminal basic domain (34 amino acids) was replaced by a single Cys residue. Particle 1569 is identical to the aforementioned 1473, except that the two M2e cysteines were replaced by serines, rather than alanines. Particles 1604 and 1569 were formulated on Alhydrogel, Alhydrogel + RC-529-AF, or "MPL-TDM emulsion/MPL-MDP" used previously (Neirynck et al., 1999). RC-529-AF is a well-defined, synthetic compound based on natural, detoxified lipopolysaccharide A (Evans et al., 2003).

The results of the vaccination/challenge experiment, shown in Fig. 4A, reveal that both vaccine particles, in combination with either adjuvant, fully protected mice against a potentially lethal infection. The few control mice that survived experienced severe weight loss and drop of body temperature before recovering. The survival of these mice may be due to activation of the innate immune system by MPL that can induce nonspecific resistance against viral pathogens like influenza (Persing et al., 2002).

The M2e-specific IgG1 immune responses induced by the two types of particles were similar (Fig. 4B), meaning that positioning M2e at the N-terminus (1604) or internally in the immunodominant loop (1569) of the HBc subunit does not appreciably affect the immunogenicity of the M2e peptide. Addition of RC-529 enhanced the adjuvant effect of Alhydrogel such that it reached the same level as observed using MPL-TDM emulsion (priming) and MPL-MDP (boosters) in previous studies (Neirynck et al., 1999). In groups treated with particles adsorbed on Alhydrogel, no further increase in titer was observed when a second boost was administered. It is interesting to note that the M2e-antibody titer in the few surviving, non-vaccinated mice was barely detectable after challenge, highlighting the low immunogenicity of natural M2-protein.

M2e-HBc vaccine also provides complete protection when administered intranasally, and efficacy is considerably enhanced by using detoxified enterotoxin adjuvant

Nasal administration of vaccines targeting respiratory infections has several important advantages. Many people have a fear of injection needles, and nasal administration can be delivered safely and with minimal effort to a large group of subjects. Moreover, as influenza is a respiratory disease, it is likely that i.n. vaccination preferentially stimulates the lymphoid system associated with the upper respiratory tract and enhances local immunity (Liang et al., 2001; Tamura et al., 1998).

We have shown previously that an M2e-HBc vaccine can protect efficiently when administered i.n. (Neirynck et al., 1999, and Fig. 1). Here we report that the induced immune
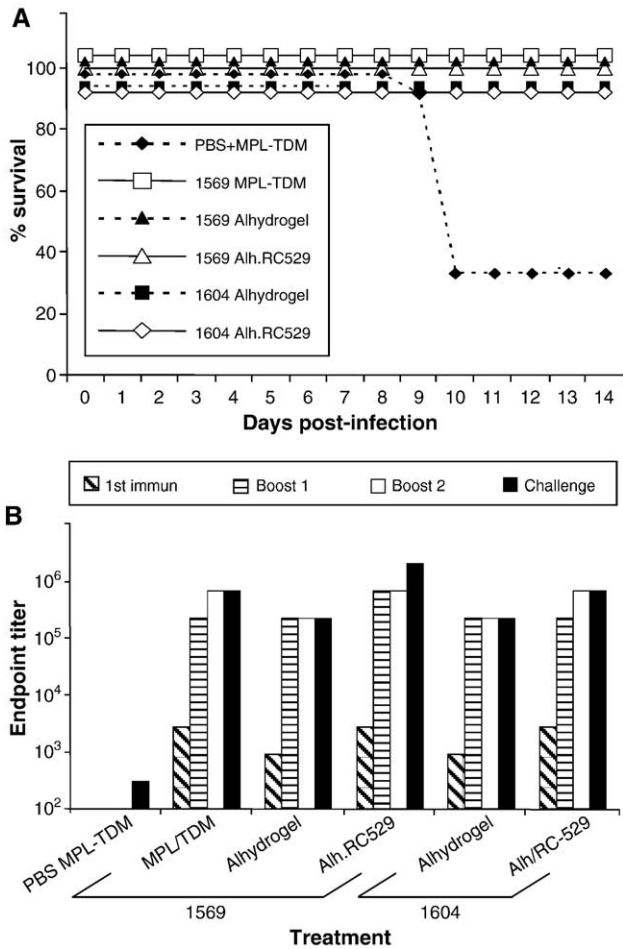
Fig. 4. Six groups of 15 BALB/c mice were vaccinated i.p. with no protein, particle 1604 or 1569 in combination with adjuvant (MPL-TDM emulsion/MPL-MDP, Alhydrogel, or Alhydrogel plus RC-529-AF); controls received an injection of buffer. Mice were challenged with 4 LD50 of m.a. X47 virus (cf. Materials and methods for further details). (A) Post-challenge survival of control mice and M2e-HBc vaccinees after challenge. (B) M2e-specific IgG1 titers in sera of BALB/c mice after vaccination with particle 1569 or 1604 in combination with different adjuvants.

response can be considerably enhanced by addition of an adjuvant suitable for i.n. delivery. J. Clements has characterized an *E. coli* heat labile enterotoxin mutant, LTR192G, that has lost nearly all toxicity but has retained its potent adjuvancy (Dickinson and Clements, 1995; Freytag and Clements, 1999). Whereas the unadjuvanted M2e-HBc vaccine (particle 1559) provided almost complete protection (Fig. 5), addition of LTR192G increased survival to 100% (the difference was not significant, according to the Kaplan–Meier method), and dramatically elevated the M2e-specific antibody titers in circulation (Table 2). Indeed, the IgG1, IgG2a, and IgG2b titers were all significantly increased by addition of LTR192G (two-sample *t* test, $P <$ 0.005). Moreover, morbidity was almost completely abolished in the adjuvanted group (Fig. 5B; $P < 0.05$ on days 6 and 8, two-sample *t* test). Similar results were obtained using particles 1569 and 1604 (data not shown). We observed consistently that i.n. immunization resulted in an

evident IgA response in circulation, which did not occur after i.p. immunizations.

Constructs with multiple, consecutive M2e-sequences induce an enhanced immune response that leads to strong protection

Jegerlehner et al. (2002) have described an M2e-HBc particle, referred to as M2-VLP, made by chemical linkage of the M2e-peptide to the immunodominant loop as an alternative to genetic fusion to the N-terminus. This required prior modification of the HBc particle by insertion of an acceptor lysine residue in the subunit loop, as previously described (Birkett, 1999). Jegerlehner et al. (2002) reported that this M2-VLP particle provided better protection than an 1559-like particle constructed according to our earlier description (Neirynck et al., 1999). Because insertion of the M2e-peptide in the immunodominant loop did not significantly enhance immunogenicity (particles 1473 and 1569, discussed above), we produced particles with two (1817) and three (1818) M2e-copies fused to the N-terminus of HBc subunits. These new constructs were compared to particle HBc/K77-M2e, which, like M2-VLP (Jegerlehner et al., 2002), was made by chemical fusion of an M2e-sequence to a lysine residue introduced into the immunodominant loop of HBc particle subunits.

The results of a vaccination/challenge experiment are shown in Fig. 6. In this experiment, we used buffer (PBS),
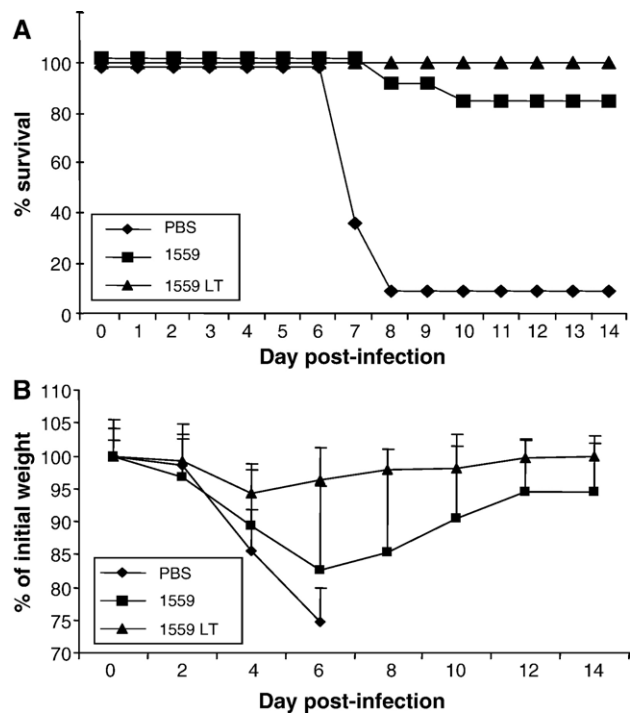

Fig. 5. Vaccination by i.n. administration and effect of detoxified mutant enterotoxin as adjuvant. Mice were immunized 3 times i.n. with either PBS (control), unadjuvanted 1559, or 1559 adjuvanted with 5 μg LTR192G. (A) Survival after challenge with 4 LD50 of m.a. X47. (B) Percent of initial body weight after challenge (with standard error bars).
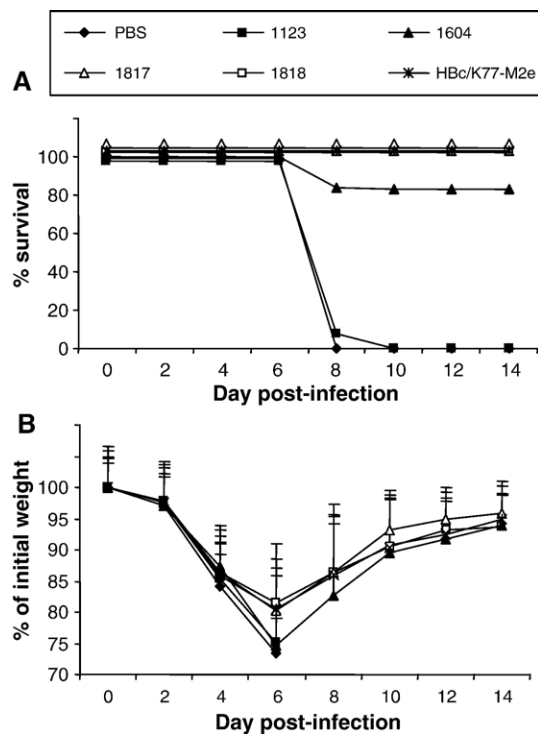
Fig. 6. Survival and morbidity of control mice and M2e-vaccinees after challenge with 4 LD50 of m.a. X47. All mice received three i.p. injections; particles 1123 (HBc control), 1604, 1817, 1818, and HBc/K77-M2e were formulated with Alhydrogel plus RC-529-AF as adjuvants. (A) Survival. (B) percent of initial body weight after challenge (with standard error bars).
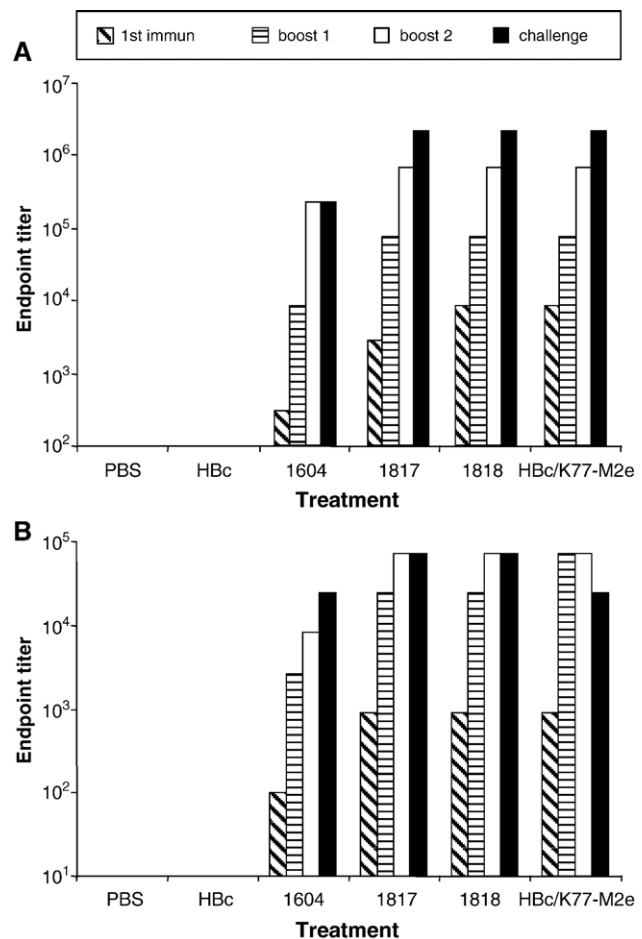

Fig. 7. M2e-specific IgG1 and IgG2a titers in sera of mice after immunization (mice from same experiment referred to in Fig. 6). (A) IgG1. (B) IgG2a.

as well as particle 1123 (HBc, Table 1), as controls. Protection after vaccination with 1817, 1818, and HBc/ K77-M2e was complete while 2 of 12 mice in the 1604 group died (the lower rate of survival of this group was not significantly different from the 1817, 1818, and HBc/K77-M2e groups; Kaplan–Meier method) (Fig. 6A). Morbidity was reduced (Fig. 6B), although not statistically significantly. The IgG1 titers were remarkably high and almost identical in sera of animals immunized with particles 1817, 1818, and HBc/K77-M2e (Fig. 7A). These titers were significantly higher than those obtained for group 1604 (Tukey test, $P < 0.05$). IgG2a titers were also almost indistinguishable for the former three particles and again significantly higher than those of the 1604 group (Fig. 7B; Tukey test, $P < 0.05$). As 10 μg M2e-vaccine was

administered per mouse per injection, it follows that mice treated with particle 1817 received about twice the molar concentration of M2e-peptide compared to those treated with 1604, whereas mice treated with 1818 received about three times the molar concentration per injection. We have shown previously that there is only a limited dose/response correlation in the range 5–50 μg vaccine/injection/mouse (Neirynck et al., 1999). Therefore, the higher immune response induced by particles 1817 and 1818 when compared to particle 1604 (e.g., 10-fold higher IgG2a after

Table 1
Designation of the different vaccine particles used in the present study[a]

	N-terminal fusion	HBc NH$_2$-terminus start	Immunodominant loop fusion	C-terminus end	Results shown in
1123	None	1	None	183	Figs. 6 and 7
1559	M2(1–24)	5	None	183	Figs. 1, 2, 3, and 5
1473	None	1	M2(1–24/2C > 2A)	150	Figs. 1, 2, and 3
1569	None	1	M2(2–24/2C > 2S)	150	Fig. 4
1604	M2(1–24)	5	None	150	Figs. 4, 6, 7, and 8
1817	M2(1–24/2C > 2S)-M2(2–24)	5	None	150	Figs. 6, 7, and 8
1818	M2(1–24/2C > 2S)-M2(2–24/2C > 2S)-M2(2–24)	5	None	150	Figs. 6, 7, and 8

[a] The position of the M2e-copy in the HBc carrier, the nature of endogenous M2e cysteine replacements, the position of the C-terminus of the carrier, and the use of each particle in experiments shown in the figures are indicated.

boost 2) cannot simply be due to the dose of M2e-peptide, but rather reflect enhanced immunogenicity of these particles. In nearly all previous experiments, the IgG2b titers (not shown) were approximately the same as the IgG2a titers, but in the present experiment the IgG2b titers elicited by particles 1817 and 1818 exceeded the IgG2a titers. In the control groups (buffer or HBc plus adjuvant), neither protection nor M2e-specific immunoreactivity could be detected, supporting the specificity of the assays used.

In most experiments in this study, anti-HBc immune responses were also measured in all groups of mice. HBc is a potent immunogen by itself, which is the reason for its frequent use as a carrier for foreign epitopes (Kratz et al., 1999; Murray and Shiau, 1999; Pumpens and Grens, 2001). The immune response is mainly directed against the spikes (Salfeld et al., 1989; Wynne et al., 1999), which allow efficient interaction with B-cell receptors by virtue of their position and spacing. Indeed, we observed a strong immune response to HBc in nearly all experiments. As expected, particles containing an insertion in the immunodominant loop (1473 and 1569) had about a 10-fold lower anti-HBc response, indicative of disruption of major B-cell epitopes. We measured not only total HBc-specific antibodies, but also IgG1, such that the immune response could be directly compared to the anti-M2e data. For the most immunogenic particles, the anti-M2e response after the second boost equaled (particle 1817) or even surpassed (particles 1818 and HBc/K77-M2e) the anti-HBc response (Fig. 8). The anti-HBc IgG1 titer declines in the order 1604 > 1817 > HBc/K77-M2e > 1818. A similar pattern was observed for total IgG. In particle HBc/K77-M2e, the immunodominant loop had been modified by a substitution, which may explain the lower HBc immunogenicity relative to particle 1604. But the HBc spikes of particles 1817 and 1818 remained unchanged, which means there must be another reason for the reduced anti-HBc response to these particles.
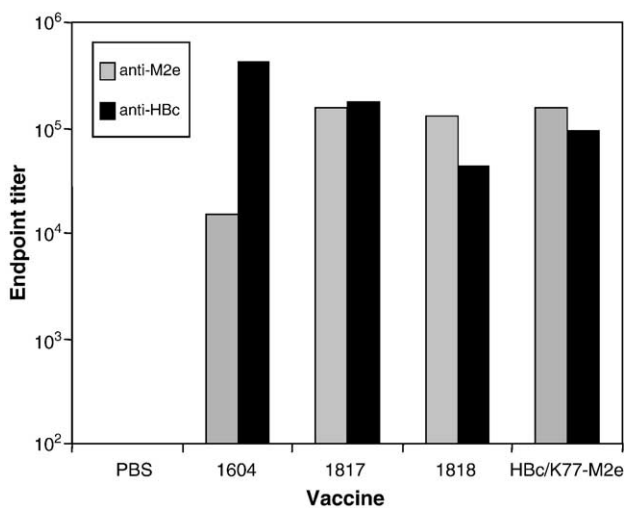

Fig. 8. Comparison of IgG1 response directed to either M2e or HBc in sera of vaccinated BALB/c mice after the second boost (sera from same experiment as shown in Fig. 6).

Particles 1604 and 1818 were also compared in an experiment involving only two immunizations. All the mice vaccinated with 1818 were protected against a lethal challenge while three out of ten 1604-vaccinated mice succumbed to the viral challenge (Table 3; the difference in survival was significant, $P < 0.05$ by the Kaplan–Meier method). The IgG1 and IgG2a titers in the 1818-vaccinated group were significantly higher than the titers obtained for the 1604-vaccinated mice ($P < 0.025$, two-sample t test). Four more mice from each group were euthanized on day 3 post-challenge to determine the extent of virus replication in the lungs. Three days after challenge, the 1818-vaccinated mice had 10-fold lower titers of virus in their lungs compared to the 1604-vaccinated mice (Table 3).

Discussion

The hallmark of influenza virus is the remarkable variability of its major surface glycoproteins, HA and NA, which allows the virus to evade existing anti-influenza immunity in the target population. Amino acids exposed on the outside of these major antigenic determinants can mutate almost at random without destabilizing the scaffold of the protein or interfering with the active center, sialic acid receptor, and neuraminidase activity, respectively. M2e, a small peptide localized close to the membrane of the virus and the infected cells, is presumably shielded from efficient interaction with immune effector cells. Indeed, despite frequent exposure to influenza infections, antibodies directed towards M2e are absent or barely detectable in the serum of humans (Black et al., 1993, Liu et al., 2003). Clearly, M2e does not take part in the drift/shift versus population immunity competition. This is one of the reasons why the sequence has remained nearly unchanged since the first human influenza A strain was isolated in 1933. When an occasional mutation did occur, as in the PR8-strain, it was inconsequential for the induced protective immunity (Neirynck et al., 1999). In addition, M2e sequence conservation may also be imposed by its genetic link to a second protein. The influenza A segment 7 codes for both M1- and M2-protein, which are produced by differential splicing (Lamb et al., 1981). The result is that the first 9 amino acids of M2e, including the initiator methionine, are shared with M1 and, therefore, function in two capacities. The next 15 amino acids of M2e are coded for by a segment of RNA that is also translated in a different reading frame to produce the C-terminal sequence of M1-protein. Again, the dual function of this RNA-sequence segment severely constrains possible mutations. M2e is, therefore, an ideal target for a broad-spectrum, recombinant influenza A vaccine, as it is almost completely conserved across all human virus strains.

We have reported previously that M2e becomes highly immunogenic when linked to an appropriate carrier such as hepatitis virus B core, resulting in protection against a

potentially lethal influenza challenge (Neirynck et al., 1999). This protection is due to antibodies as it is transferable by passive immunization (Neirynck et al., 1999). The HBc particle is an excellent carrier for a number of reasons. It is highly immunogenic (Salfeld et al., 1989; Wynne et al., 1999), is both a T cell-independent and a T cell-dependent antigen (Milich and McLachlan, 1986), exhibits a lack of genetic restriction (Milich and McLachlan, 1986), and is able to transfer these attributes to fused epitopes (Ulrich et al., 1998). In addition, HBc particles are remarkably tolerant to modifications such as substitutions, additions, and deletions, making it a versatile carrier of heterologous epitopes (Kratz et al., 1999; Murray and Shiau, 1999; Pumpens and Grens, 2001; Ulrich et al., 1998). Furthermore, HBc particles can be produced efficiently in *E. coli*, obviating the need for biological materials that may be in short supply or may contain biohazards.

As mentioned above, we routinely used a fairly severe viral challenge to evaluate the efficacy of the M2e-based vaccination. Though this challenge with a highly lethal dose is undoubtedly more severe than a person encounters upon natural infection, it has the advantage that a clear-cut protection against mortality can be demonstrated. It may be noted that the challenge conditions used by various authors to score protection after vaccination with an M2e-based vaccine vary widely. A mild challenge dose does not allow to score for survival, but the protection can be assayed, e.g., by reduction of virus titer in lung homogenates. Frace et al. (1999) used 100 MID_{50} of a m.a. flu strain for challenge, and Mozdzanowska et al. (2003) administered 1000 MID_{50} of a low pathogenic flu strain. The ratio LD_{50}/MID_{50} varies according to the virus preparation, but the latter authors reported that 1000 MID_{50} corresponded to 0.01 LD_{50}. In contrast, in the present study, we used mostly 4 LD_{50}, which is in range of those used by other investigators who also scored for mortality, viz. 10 LD_{50} by Jegerlehner et al. (2002) and 1 LD_{90} by Fan et al. (2004). These differences in vaccine dose and in influenza strain used for challenge preclude meaningful comparison of the effectiveness of the various M2e-vaccines reported by different investigators.

In order to improve the M2e-HBc vaccine, we removed the C-terminal domain, which normally interacts with the internal viral nucleic acid. A single cysteine residue was added to the C-terminus of HBc. This resulted in higher expression levels, more easily characterized particles, and increased purity of the final product. Removal of the C-terminal nucleic acid binding domain did not affect the protective efficacy of the particles. Also, Jegerlehner et al. (2002) used particles derived from HBc lacking the basic C-terminal domain. In some particles, the two cysteine residues in M2e were replaced by other small residues such as alanine or serine to avoid potential protein aggregation by disulfide bridging (Table 1); these substitutions had no detectable effect on efficacy of protection or immunogenicity. As mentioned above, the spikes on the HBc particles

are the major antigenic determinants. Nevertheless, insertion of the M2e-sequence in the spike loop (particles 1473 and 1569) did not improve the efficacy relative to the particles with an M2e-sequence fused to the N-terminus. Possibly, a more constrained conformation of the insert could limit the potential benefit of enhanced accessibility.

Adjuvants stimulate innate immunity, and, when co-administered with an antigen, boost immunogenicity. The result is not only a much enhanced cellular and humoral immune response, but also a biasing of the responses towards either a more Th-1 or a more Th-2 type immunity, depending on the adjuvant used. The Th immunity type is indicated by the relative levels of induced immunoglobulin isotypes, IgG1, which is indicative of Th-2 activity versus IgG2a and IgG2b, both indicative of Th-1 effects. In previous studies involving i.p. administration, we used MPL-TDM emulsion for priming, followed by MPL-MDP for booster injections (Neirynck et al., 1999). Alhydrogel is the most widely used adjuvant in human vaccines. It has a long record of safety, but it does not have the efficacy of other, newer adjuvants. Alhydrogel functions mainly by prolonging the half-life of adsorbed antigens in vivo. Adjuvants such as MPL and CpG oligonucleotides are derived from natural pathogens and their mechanism of action involves stimulation of specific Toll-like receptors (TLR) on immune effector cells. Further characterization of MPL led to the synthetic RC-529, which has already been used in a number of clinical trials (Persing et al., 2002). It retains the high efficacy of MPL but its side-effect profile is similar to that of Alhydrogel. The results reported here show that Alhydrogel provides important adjuvant effects to adsorbed M2e-HBc particles, but that this activity can be further enhanced by other adjuvants such as RC-529, which directly targets the TLR4 Toll-like receptor (Evans et al., 2003). Unlike the TLR agonists, Montanide ISA-720 is a water-in-oil emulsion that is believed to function as a reservoir, slowly releasing the antigen, and facilitates the uptake of antigen by antigen presenting cells (Aucouturier et al., 2002). As both types of adjuvants have already been used in human clinical trials, they are obvious candidates for further development as possible components of a universal flu vaccine.

There is widespread interest in needle-free vaccination as many people have an aversion to needle injection. Vaccines can be safely and rapidly administered to a large number of recipients through the i.n. route, which may be more efficient in stimulating nasal lymphoid tissues and leads to improved mucosal immunity in the respiratory tract region (Liang et al., 2001; Tamura et al., 1998). *E. coli* and *Vibrio cholerae* enterotoxins are very potent adjuvants for oral and nasal administration, but their toxicity limits their application. However, mutants such as LTR192G that have much reduced toxicity while retaining adjuvancy have been generated by site-directed mutagenesis (Dickinson and Clements, 1995; Freytag and Clements, 1999). LTR192G has been used extensively as an adjuvant for oral and

intranasal vaccination in experimental animals, including in combination with conventional influenza vaccines (Lu et al., 2002; Tumpey et al., 2001). It has also been tested in several clinical trials (Kotloff et al., 2001; Losonsky et al., 2003). In agreement with previous results (Neirynck et al., 1999), M2e-HBc administered i.n. leads to effective protection (Figs. 1 and 5A), but the addition of LTR192G provides a more solid protection in terms of mitigating morbidity (Fig. 5B) and considerably enhancing the titers of IgG1, IgG2a, and IgG2b (Table 2). IgA was also clearly present in circulation, but at levels presumably too low to play a significant role in protection; its presence, however, confirms induction of localized, mucosal immunity. IgA was not detected in circulation when the i.p. vaccination route was used.

A major improvement of the vaccine particle was achieved when the copy number of M2e at the N-terminus was increased from 1 to 2 or 3 (particles 1604, 1817, and 1818, respectively). Repeats of B-cell epitopes have often been used to increase the immune response to vaccines, including for M2e-based vaccines (Liu et al., 2004; Neirynck et al., 1997). By using 2 and a fortiori 3 copies of M2e instead of one, more complete protection was achieved against mortality and morbidity after a severe challenge. But the effect is most quantifiable by considering the IgG titers. After the second immunization with particle 1817 or 1818, the titers of IgG1 and IgG2a increased by a factor of 80 and 30, respectively, over those observed using the 1604 particle. There was an inverse correlation with the HBc titers, which decreased in the order 1604 > 1817 > 1818 (Fig. 8). This is remarkable as most of the HBc immune response is directed toward the immunodominant loop (Kratz et al., 1999; Pumpens and Grens, 2001; Wynne et al., 1999), which remained intact in these three particles. It is possible that the longer extension of the N-terminus physically interferes with access of immune effector cells to the normally immunodominant HBc spikes. Alternatively, there may be competition between the anti-HBc specific B-cells and the more abundant anti-M2e-specific cells for cytokines and other growth factors; this may deprive the former of optimal growth and stimulatory factor concentrations. The end result is that the immune response against M2e, as it is displayed in particles 1817

Table 2
IgG1, IgG2a, IgG2b, and IgA M2e-antibody titers in serum collected after the second boost[a]

	IgG1	IgG2a	IgG2b	IgA
Control (PBS)	<100	<100	<100	<100
1559	100	300	300	100
1559 + LTR192G	72,900	72,900	72,900	300

[a] Pooled serum from control mice (PBS treated), mice treated with particle 1559 alone, and mice treated with 1559 in combination with 5 μg LTR192G was analyzed. All vaccinations were i.n. Same mice from experiment shown in Fig. 5.

Table 3
Survival, lung virus titer, IgG1 titer, and IgG2a titer of mice immunized twice with M2e-vaccine and challenged with m.a. influenza A virus[a]

Vaccine[b]	Survival[c]	Lung virus titer[d]	IgG1[e]	IgG2a[e]
PBS	0/10	4.23	0	0
1604	7/10	3.33	2700	300
1818	10/10	2.26	218,700	8100

[a] Fourteen mice were immunized twice intranasally at 3-week intervals with 10 μg M2e-vaccine formulated with 1 μg LTR192G.
[b] Control (PBS) and M2e-vaccine particles.
[c] Number of surviving mice over a 14-day post-challenge period.
[d] Four mice were euthanized 3 days post-challenge, and lung virus titers were determined (expressed as mean $\log_{10}$ EID_{50}/ml).
[e] IgG-isotype M2e-antibody titers of serum samples collected 14 days after the second immunization.

and 1818, is even stronger than against the highly immunogenic HBc. It may be noted here that the immune response to HBc is at least 80-fold, and in BALB/c mice even 300-fold higher than against the envelope antigen HBs, which is the basis for the highly effective and successful vaccine against human hepatitis B virus infection (Milich and McLachlan, 1986). We also found that the 1818 vaccine is capable of conferring complete protection after only 2 injections instead of 3 (Table 3). Jegerlehner et al. (2002) reported that a modified HBc particle displaying an M2e peptide chemically coupled to the immunodominant loop induced a higher M2e-specific immune response and was more protective in vivo than an 1559-like particle made by genetic fusion according to our previous description (Neirynck et al., 1999). The results shown here demonstrate that the recombinant particle 1818 is at least as immunogenic and protective as the HBc/K77-M2e chemical conjugate, which was made according to the description reported by the aforementioned authors.

M2e is scarcely present on virus particles, but fairly abundant on virus-infected cells (Lamb et al., 1985). It is therefore more likely that the latter are the targets for protective antibodies. The mechanism of protection by the antibodies induced by M2e vaccination has not yet been fully elucidated. Undoubtedly, NK-dependent antigen-dependent cell cytotoxicity (ADCC) plays an important role (Jegerlehner et al., 2004; De Filette et al., unpublished results). But other protective mechanisms may also be involved.

In conclusion, the M2e-HBc vaccine can be administered either parenterally or intranasally in combination with an adjuvant; effective adjuvants suitable for human clinical trials have been identified. The results reported here document that vaccination of mice with an M2e-HBc universal flu vaccine confers full protection against a severe (4-fold 50% lethal dose) challenge with a mouse-adapted influenza A strain. The optimized vaccine particle, 1818, is clearly superior in terms of conferring improved protection, reducing morbidity, decreasing virus replication in the lungs, and inducing higher antibody

titers. Further evaluation of this optimized, universal human influenza vaccine in other animals is in progress.

Materials and methods

Mice

Pathogen-free, female BALB/c mice were obtained from Charles River (Charles River Wiga, Sulzfeld, Germany). The animals were housed in a temperature-controlled environment with 12-h light/dark cycles; food and water were delivered ad libitum. Mice were immunized at 8 weeks of age after 1-week adaptation in the animal room.

Influenza A strain

The strain X47 [A/Victoria/3/75 (H3N2) × A/Puerto Rico/8/34 (H1N1)] was adapted to mice (m.a.) by a series of lung passages, and one passage in embryonated chicken eggs, as previously described (Neirynck et al., 1999). The sequence of the M2-protein in the final preparation (before aliquotting) was verified as identical to the original PR8-strain (Fiers et al., 2001). This m.a. X47 preparation was highly pathogenic; the dose lethal for 50% of the mice (LD50) was 0.02 HAU.

Construction of expression vectors

Plasmid pKK223-3 (Amersham Pharmacia, UK) was modified to include a unique *Nco*I restriction site, generating pKK223-3N, thereby enabling insertion of HBc genes as *Nco*I–*Hin*dIII restriction fragments. A truncated HBc gene (coding for amino acids 1 through 149) was modified by PCR to contain a single C-terminal cysteine following Val$_{149}$ and cloned into pKK223-3N to generate the plasmid pKK223-3N-1123, which expresses particle 1123. This plasmid was further modified to accept the directional insertion of synthetic dsDNA fragments. This was done by introducing a *Sac*I and an *Eco*RI restriction site between the codons for Asp$_{78}$ and Pro$_{79}$ in the immunodominant loop region (plasmid V16), or a *Sac*I site between the codons for Ile$_3$ and Asp$_4$ (plasmid V54). Particle 1559, which contains a single copy of M2e fused to the N-terminus of full-length HBc particles, is identical to IM2eHBc described previously (Neirynck et al., 1999); however, it was produced using the pKK223-3 expression system. A brief description of all the constructs used is shown in Table 1.

Immunodominant loop insertion constructs (1473 and 1569)

Synthetic oligonucleotide fragments coding for amino acids 2–24 of M2 were inserted into the immunodominant loop insertion vector V16; the two endogenous cysteines at positions 17 and 19 of M2e were mutated to either alanines (1473) or serines (1569).

N-terminal fusion constructs (1604, 1817, and 1818)

One, two, and three copies of M2e were fused to the N-terminus of particle 1123 to generate particles 1604, 1817, and 1818, respectively. In all three cases, only the N-terminal copy of M2e retained the initiator methionine at position 1; the carrier-proximal copy of M2e retained the cysteines at positions 17 and 19; all other copies contained the M2e variant with cysteines 17 and 19 mutated to serines (M2e(2C > 2S)). Plasmid V54 was digested with *Nco*I and *Sac*I to liberate the HBc sequence Met$_1$Asp$_2$Ile$_3$, which was replaced with the coding sequence for M2e(1–24) to yield plasmid V54.M2e, which expresses particle 1604. A second copy of M2e(1–24), in which the endogenous cysteine residues in M2e were replaced by serine residues, was fused to the N-terminus (without the initiator methionine) of the particle specified by the V54.M2e vector, using PCR, to yield the 1817-expressing plasmid V54.M2e(2C > 2S)/M2e. To produce the 1818-expressing plasmid, V54.M2e(2C > 2S)/M2e(2C > 2S)/M2e, PCR was used to fuse a third copy of M2e(1–24/2C > 2S) to the N-terminus (without the initiator methionine) of the hybrid gene encoded by V54.M2e(2C > 2S)/M2e.

Chemical conjugate (HBc/K77-M2e)

An expression vector encoding a modified 151 amino acid HBc particle was constructed. The HBc subunit contains a single lysine residue inserted between amino acids 76 and 77 (see US Patent 6,231,864); it terminates at position V149 of HBc, followed by a C-terminal cysteine residue. A synthetic peptide corresponding to M2(2–24/2C > 2S)–Gly–Gly–Gly–Cys (Multiple Peptide Systems, San Diego CA, USA) was conjugated to HBc/K77 to produce HBc/K77-M2e using published methods (Jegerlehner et al., 2002).

Expression and purification of recombinant particles

Methods for expression and purification of recombinant particles followed previously published protocols (Birkett et al., 2002). Recombinant particles used as vaccine preparations in the present studies are listed in Table 1.

Adjuvants

To provide a comparison with previous results, we immunized mice with particles formulated with MPL-TDM emulsion (25 μg monophosphoryl lipid A (MPL) and 25 μg synthetic trehalose-6,6-dimycolate (TDM) in an oil-in-water emulsion) and boosted them twice with particles formulated with 25 μg MPL and 25 μg muramyl dipeptide (MDP, *N*-acetylmuramyl-L-alanyl-D-isoglut-amine) (Neirynck et al., 1999). All products were obtained from Sigma-Aldrich (St. Louis, MO, USA). A detoxified mutant of heat-labile *E. coli* enterotoxin, LTR192G, was

used for intranasal (i.n.) administration; this preparation was generously provided by Dr. J. Clements (Department of Microbiology and Immunology, Tulane University Medical Center, New Orleans, LA) (Dickinson and Clements, 1995; Freytag and Clements, 1999). Montanide ISA-720 (SEPPIC SA., 75, Quai d'Orcay, 75321 Paris, France) was used as per manufacturer's directions to generate water-in-oil emulsions of M2e-HBc particles. For Alhydrogel (Superfos Biosector, Denmark) formulations, particles were completely adsorbed (>95%) using a 10% (v/v) solution of a 2.0% Alhydrogel stock solution. For another formulation, Alhydrogel + RC-529-AF, RC-529-AF (Corixa Corporation, Seattle WA, USA) was added to Alhydrogel to a final concentration of 50 μg/ml.

Immunization

Previous research had shown that within the range of 10–50 μg, the amount of antigen used was not critical (Neirynck et al., 1999). Therefore, in the present study, M2e-HBc vaccine was kept constant at 10 μg per injection for both the i.p. and the i.n. routes of administration. The total volume of the administered vaccine [M2e-HBc construct + adjuvant in PBS (phosphate buffered saline)], was 50 μl for i.n. administration (25 μl per nostril) and 100 μl for i.p. injection. Typically, an experiment involved 7 or 8 groups of 14–15 mice each. Three immunizations were administered at 3-week intervals. Before the first, and 2 weeks after each immunization, blood samples were collected by piercing of the ventral tail vein. The final bleeding of surviving mice (2 weeks after challenge) was performed by cardiac puncture. Blood clotting was allowed to continue for 30 min at 37 °C; samples were placed on ice, and serum was separated by collecting the supernatant of two consecutive centrifugations.

Challenge and follow-up

Three weeks after the last immunization (second boost), the mice were challenged with a 50-μl dose containing 4 LD50 (corresponding to 2.5×10^3 TCID$_{50}$) of m.a. X47 influenza virus in buffer. This challenge dose was administered i.n. to mice lightly anesthetized by ether (experiments shown in Figs. 1–5) or by isoflurane (experiments shown in Figs. 6–8). Mortality was monitored on a daily basis for 3 weeks. Morbidity was assessed by rectal temperature and body weight.

Immunology

Antibody titers were determined by ELISA using sera from individual mice, pooled sera from the group, or both. To determine M2e-specific antibody titers, microtiter plates (type II F96 MaxiSorp, Nunc) were coated with 50 μl of a 2 μg/ml M2e-peptide solution in 50 mM sodium bicarbonate buffer, pH 9.7, and incubated overnight at 37 °C. After

washing, the plates were blocked for 1 h with 200 μl of 1% BSA in PBS. After 1-h incubation, the plates were washed again. A series of 1/3 dilutions of the different serum samples, starting with a 1/100 dilution, were loaded on the peptide-coated plates. The bound antibodies were detected with a peroxidase-labeled antibody directed against mouse isotypes IgG1, IgG2a, IgG2b, or IgG3 (Southern Biotechnology Associates, Inc., Birmingham, AL, USA) and diluted 1/6000 in PBS + 1% BSA + 0.05% Tween 20. After washing, the microtiter plates were incubated for 5 min with TMB substrate (Tetramethylbenzidine, Sigma-Aldrich). The reaction was stopped by addition of an equal volume 1 M H$_3$PO$_4$ and the absorbance at 450 nm was measured. Endpoint titers are defined as the highest dilution producing an O.D. value twice that of background (pre-immune serum). A similar procedure was followed for the assay of M2e-specific IgA, except that the bound antibodies were detected with a peroxidase-labeled antibody directed against mouse IgA (Sigma-Aldrich), diluted 1/7000 in PBS + 1% BSA + 0.05% Tween 20, and the incubation with TMB substrate was 10 min.

The HBc-specific antibody titers were determined as follows. The microtiter plates (type II F96 MaxiSorp, Nunc) were coated with 100 μl of a 10 μg/ml solution of polyclonal rabbit antibody directed against HBc (DakoCytomation, Carpinteria CA, USA) in 50 mM bicarbonate buffer, pH 9.7, and incubated overnight at 4 °C. The plates were washed and then blocked with 1% BSA in PBS. After washing, capturing of HBc antigen (1 μg/ml in PBS + 1% BSA) was allowed to proceed for 1 h. The plates were again washed, and a series of 1/3 dilutions of the different serum samples, starting with a 1/100 dilution, were loaded on the plates. The bound antibodies were detected with an alkaline phosphatase-labeled antibody directed against mouse IgG (Sigma-Aldrich), diluted 1/10 000 in PBS + 1% BSA. After washing, the microtiter plates were incubated for 60 min with p-nitrophenyl phosphate, and the absorbance at 415 nm was measured. The endpoint titers are defined as the highest dilution producing an O.D. value twice that of background (pre-immune serum). A similar procedure was used for determination of the HBc-directed IgG1 titers.

Animal welfare

The animal facility operates under the Flemish Government License Number LA1400091. All experiments were done under conditions specified by law (European Directive and Belgian Royal Decree of November 14, 1993) and authorized by the Institutional Ethical Committee on Experimental Animals.

Data analysis

Comparison of antibody titers between two experimental groups was performed with the two-tailed Student's *t* test.

Comparison of more than two groups was done with the Tukey multiple comparison test. This test compares the difference between each pair of means with appropriate adjustment for the multiple testing (Altman, 1991). Comparison of survival of mice after challenge was carried out with the Kaplan–Meier test (Palmar and Machin, 1995).

Acknowledgments

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