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A universal influenza A vaccine based on the extracellular domain of the M2 protein

SABINE NEIRYNCK, TOM DEROO, XAVIER SAELENS, PETER VANLANDSCHOOT, WILLY MIN JOU & WALTER FIERS

Department of Molecular Biology, University of Ghent and Flanders Interuniversity Institute for Biotechnology, K.L. Ledeganckstraat 35, 9000 Ghent, Belgium
P.V. present address: Center for Vaccinology, Department of Clinical Biology, Microbiology and Immunology, University Hospital, De Pintelaan 185, 9000 Ghent, Belgium
Correspondence should be addressed to W.F.; email: fiers@dmb.rug.ac.be

The antigenic variation of influenza virus represents a major health problem. However, the extracellular domain of the minor, virus-coded M2 protein is nearly invariant in all influenza A strains. We genetically fused this M2 domain to the hepatitis B virus core (HBc) protein to create fusion gene coding for M2HBc; this gene was efficiently expressed in *Escherichia coli*. Intraperitoneal or intranasal administration of purified M2HBc particles to mice provided 90–100% protection against a lethal virus challenge. The protection was mediated by antibodies, as it was transferable by serum. The enhanced immunogenicity of the M2 extracellular domain exposed on HBc particles allows broad-spectrum, long-lasting protection against influenza A infections.

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Influenza is one of the main viral diseases in man. The infection of the respiratory tract is usually accompanied with coughing, high fever and myalgia. Influenza infection often causes severe morbidity and is occasionally even lethal. Some strains, such as the 'Spanish flu' of 1918–1920, have demanded an exceptionally heavy toll, but every epidemic is a real risk for immunocompromised persons, including the elderly. Influenza A virus expresses on its membrane two highly immunogenic, but very variable proteins: hemagglutinin and neuraminidase. New epidemic strains arise every 1 or 2 years because of selected point mutations in these two surface glycoproteins, a phenomenon called drift[1,2]. More rarely, gene exchange with an animal virus occurs, known as antigenic shift[1,3], and this may result in a pandemic. The conventional influenza vaccine must be adapted almost every year to follow the antigenic drift and shift of the virus. In these circumstances, the vaccine can protect about 80% of the immunized persons. Because of the high variability of hemagglutinin and neuraminidase, a broad-spectrum vaccine against influenza A has so far not been developed. Therefore, we evaluated whether a minor but evolutionarily constant protein of the virus could be enhanced in its immunogenic potential, such that it can be used as a vaccine.

Like hemagglutinin and neuraminidase, the M2 protein is an integral membrane protein of the influenza A virus[4,5]. However, the protein is much smaller, only 97 amino acids long. Of these, 24 amino acids at the N terminus are exposed at the membrane surface, 19 amino acids span the lipid bilayer, whereas the remaining 54 residues are located on the cytoplasmic side of the membrane. The extracellular part of the M2 protein is remarkably conserved, as shown by alignment of the sequences of M2 proteins, isolated from different human strains of influenza A virus[6,7]. No amino-acid change has been found in the extracellular domain of the M2 protein, from the first human influenza A strain isolated (in 1933), A/WS/33 (H1N1), to the most recently sequenced virus, A/Guangdong/39/89 (H3N2). Two virus strains do not fit this conserved pattern: A/PR/8/34 (H1N1), which has one amino-acid change, and A/Fort Monmouth/1/47 (H1N1), which has three amino-acid differences. These two strains probably represent side branches in the evolutionary tree.

The M2 protein is abundantly expressed at the cell surface of influenza A-infected cells[4]. The protein is also found on the membrane of the virus particle itself, but in much smaller quantities (14–68 M2 molecules per virion; ref. 5). The M2 protein is a homotetramer composed of two disulfide-linked dimers, which are held together by non-covalent interactions[8,9]. Amantadine (1-amino-adamantane hydrochloride) and its structural analog rimantadine (α-methyl-1-adamantane methylamine hydrochloride) are at present the only available drugs to treat influenza infections[10,11]. They inhibit the replication of influenza A viruses, but are not always effective, because some influenza A strains are resistant. This resistance resides in mutations in the transmembrane region of the M2 protein and in hemagglutinin[12–15]. Thus, the M2 tetramer

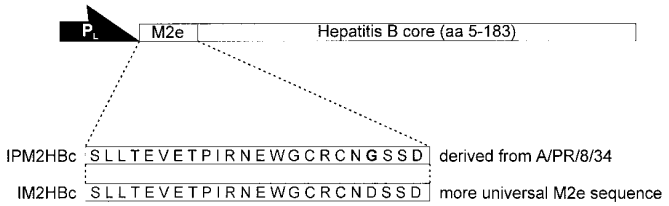

Fig. 1 M2HBc fusion proteins. The extracellular part of the M2 protein (M2e), genetically coupled to the N terminus of HBc. P_L, leftward promoter of bacteriophage λ; M2e, extracellular part of the M2 protein; amino-acid sequences, single-letter code. Top, M2e sequence as present in most vaccine strains; bottom, universal M2e sequence, present in almost all influenza A field strains (bold, single residue difference). The initiating methionine was completely removed after expression in *E. coli*, as shown by N-terminal sequencing.

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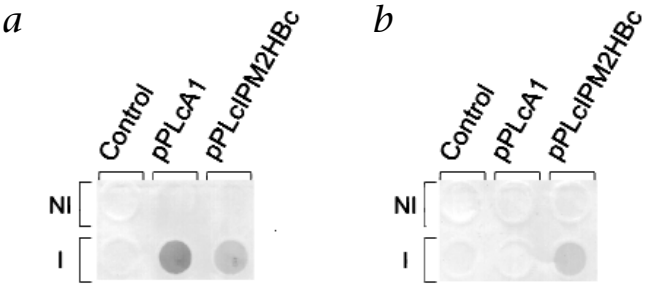

Fig. 2 Expression of M2e on the surface of HBc particles. Cultures of E. coli containing empty plasmid pPLc245 (control), pPLcA1 (*hbc* gene) or pPLcIPM2HBc (fusion of *m2-hbc*) were either not induced (NI) or induced (I), then cell supernatants were analyzed by dot-blot, detected with a monoclonal antibody against HBc (***a***) or M2e (***b***).

is important in the infectious cycle of influenza A virus.

Experimental data have shown that M2 can give rise to antibodies, which protect against a virus infection. A monoclonal antibody directed against the extracellular part of the M2 protein (14C2) has been shown to diminish the spread but not the infectivity of the virus *in vitro*[5]. Passive administration of the 14C2 monoclonal antibody inhibits viral replication in the lungs[16]. Antibodies against the M2 protein are also found in sera of patients after they recover from an influenza infection[17]. Mice are protected against an infection with homologous or heterologous influenza A virus after vaccination with a preparation containing complete M2 protein expressed in *Spodoptera frugiperda* cells[18]. The protein in those experiments[18] was partially purified, and the preparation was administered to mice in combination with incomplete Freund's adjuvant.

Here, we used a pure antigen, M2HBc, based on the fusion between the extracellular, 23-amino-acid M2 peptide (called M2e) and the hepatitis B virus core (HBc) protein. The latter part of the fusion protein forms particles, whereas the N-terminal M2e peptide is exposed on the surface. In this way, we mimicked the wild-type structure of the M2 protein in viral particles and infected cells, in which the free N terminus extends towards the extracellular environment. The HBc protein has already proven its efficacy as a carrier molecule for heterologous epitopes in eliciting a high titer antibody response to the genetically linked peptide[19–22]. The M2HBc particles elicited long-lasting protective immunity against a broad range of influenza A viruses. Immunization was either intraperitoneal in combination with a mild adjuvant, or intranasal without adjuvant. Serum from immunized mice provided protection in passive immunization experiments.

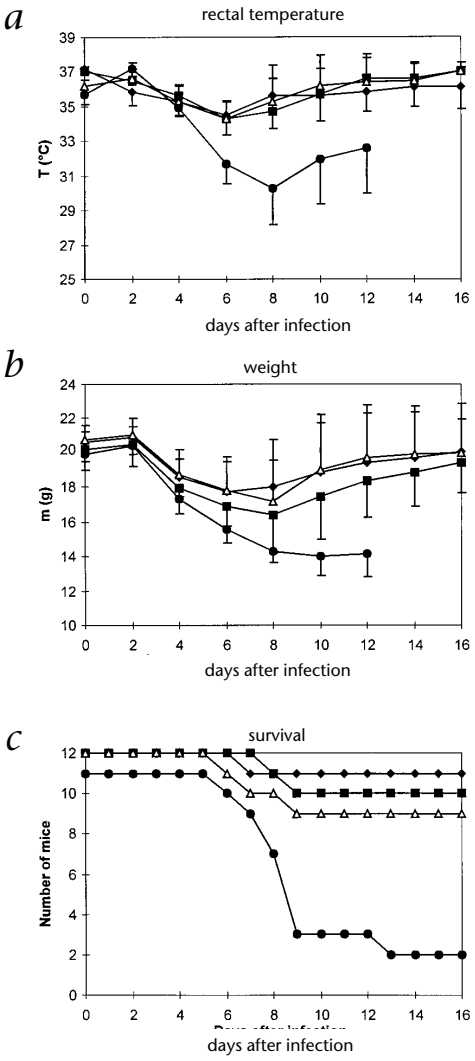

Fig. 3 Vaccination with IPM2HBc particles administered intraperitoneally. Rectal temperature ***a***, weight ***b*** and survival ***c*** of mice vaccinated with IPM2HBc (doses: 5 µg, △; 10 µg, ■; 50 µg, ◆) or buffer (●) and adjuvant alone, followed by lethal challenge with 5 LD_{50} mouse-adapted influenza A/PR/8/34.

Preparation of M2e hepatitis B core fusion proteins

We used HBc as carrier to present M2e as an antigenic epitope. The M2e genetic information was derived from the influenza strain A/PR/8/34 and inserted after the initiation codon of the gene for the hepatitis B core protein. The tightly regulated, leftward promoter P_L of phage λ (ref. 23) controlled the expression of the fusion gene (Fig. 1). The resulting protein, IPM2HBc, was efficiently expressed in *Escherichia coli* and had the expected N-terminal sequence. We also constructed a similar fusion gene, which corresponds to the more universal sequence of the extracellular part of the M2 protein; it codes for the protein M2HBc (Fig. 1). We purified both M2HBc particles to homogeneity and used them for vaccination studies.

HBc, also when expressed in *E. coli*, spontaneously associates to form particles, indistinguishable from the viral core particles isolated from the liver of hepatitis B-infected patients[24]. Electron micrographs showed that the IPM2HBc fusion protein formed similar particles. The insertion of an epitope at the N terminus of HBc results in a surface localization of that epitope[19]. To determine whether the fused M2e domain was also exposed at the surface of the particle, we loaded the soluble fraction from induced bacteria containing IPM2HBc in a native state onto a nitrocellulose membrane. An antibody directed against M2e indeed reacted with the native IPM2HBc fraction (Fig. 2*b*). Therefore, we concluded that the epitope was located on the surface of these HBc-like particles.

Immunization

We used purified IPM2HBc particles to immunize 7-week-old female Balb/c mice. We evaluated four groups of 12 mice: the first received 50 µg IPM2HBc; the second, 10 µg; the third, 5 µg; and the fourth (control), only buffer. We gave a total of three injections in-

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traperitoneally, each with the appropriate adjuvant, at 3-week interval. At 3 weeks after the last inoculation, we challenged the mice with 5 LD_{50}; dose producing 50% lethality) of mouse-adapted A/PR/8/34 (H1N1). We assessed morbidity by measuring rectal temperature (Fig. 3*a*) and weight (Fig. 3*b*) every other day. All mice immunized with IPM2HBc showed a significant degree of protection against the subsequent influenza challenge ($P < 0.005$, except $P < 0.05$ at the 5-µg dose). Depending on the dose administered, 9–11 immunized mice of 12 survived the influenza infection, in contrast to only 2 of 11 control mice (Fig. *3c*). Although the immunized mice still showed a high morbidity (Fig. 3*a* and *b*), the difference between the vaccinated groups and the control group was statistically highly significant; for example, on day 6, $P < 0.00005$ for rectal temperature and $P < 0.005$ for weight loss. There were no statistically significant differences between the various vaccinated groups.

As a dose of IPM2HBc ranging from 5 µg to 50 µg could protect the mice against a lethal influenza challenge, we used 10 µg antigen for subsequent vaccination studies. In a second experiment, using the standardized conditions described above, all vaccinated mice (14 of 14) survived a lethal challenge, whereas all mice in the control group died (0 of 16 survived by day 9).

Analysis of serum samples

We obtained blood samples 1 day before the first injection and 2 weeks after each of the three subsequent injections. We obtained a final blood sample 3 weeks after the challenge, when the mice had recovered. We analyzed the serum by ELISA to identify IgG antibodies directed against M2e and the carrier protein HBc. A considerable antibody response against M2e developed in the mice that received three injections with the vaccine (Fig. 4). The titer in the serum increased further after the challenge. The anti-HBc titer in the serum was usually considerably higher than the anti-M2e titer; in a typical experiment, the former amounted to 2.7×10^6 and the latter, to 4×10^4. It is not known whether this affects negatively the anti-M2e response. However, the anti-HBc titer can be largely reduced by disruption of the main epitope in the dominant antigenic loop of HBc (ref. 21).

A broad-spectrum vaccine

Influenza A strains, developed for efficient production in fertilized chicken eggs, are usually obtained by gene reassortment and often contain the *m2* gene from A/PR/8/34. The extracellular part of M2 from A/PR/8/34 differs at one position from the conserved common M2e sequence. Therefore, we compared the fusion proteins IPM2HBc and IM2HBc for their protective potential against a challenge with mouse-adapted X47 (H3N2). This virus contains hemagglutinin and neuraminidase from A/Victoria/3/75, whereas the M2 protein is derived from A/PR/8/34 (ref. 25). Thus, immunization with IPM2HBc and challenge with mouse-adapted X47 is a model for a homologous system, whereas vaccination with IM2HBc and challenge with the same virus corresponds to a heterologous infection.

Table 1 Survival of mice vaccinated intraperitoneally followed by a potentially lethal homologous or heterologous influenza A challenge

Immunization	Surviving mice	*P*
IPM2HBc	8/12	< 0.05
IM2HBc	12/12	< 0.0001
Control	2/11	

P, statistical significance of the protection, compared with the control group; Fisher's exact test.

The mice were immunized as described above, except that the first group (homologous) received 10 µg IPM2HBc; the second (heterologous), 10 µg IM2HBc; and the third group (control), only buffer (all supplemented with the standard adjuvant). At 3 weeks after the last immunization, we challenged the mice with 5 LD_{50} mouse-adapted X47. In the homologous group, 8 mice of 12 survived, whereas all 12 mice survived the heterologous challenge, in contrast to only 2 of 11 for the control group (Table 1). The difference in response between the former two groups was statistically not significant. The level of IgG antibody against M2e produced after the different immunizations were similar to the results in Fig. 4.

Virus clearance from the lungs

We injected groups of 30 mice using the protocol described above, with 10 µg IM2HBc or buffer and adjuvant. At 3 weeks after the third immunization, we infected mice with 5 or 0.5 LD_{50} mouse-adapted X47. As expected, after the high-dose challenge (5 LD_{50}), all mice in the mock-vaccinated control group died, whereas all the vaccinated mice survived. We used mice that received a sublethal challenge (0.5 LD_{50}) to determine viral clear-

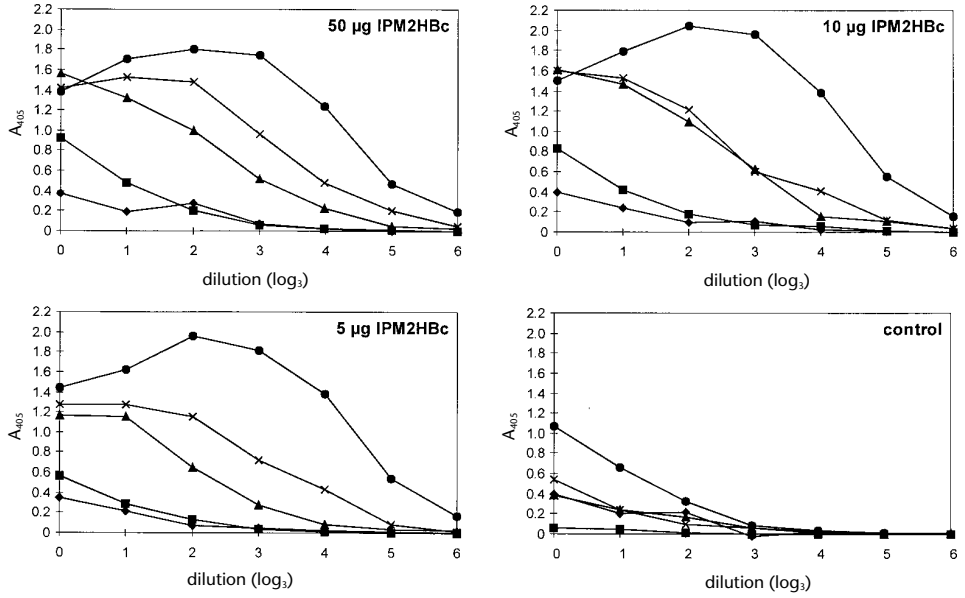

Fig. 4 Titer of antibody against M2e. Serum samples from the four treatment protocols in Fig. 3 were used. Preimmune serum (◆), and serum obtained after the first (■), second (▲) and third (X) immunization and after challenge (●) were initially diluted 1:50, then serially diluted 1:3. A_{405}, absorbance (corrected value).

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Table 2 Survival of mice after passive immunization followed by a potentially lethal heterologous influenza A challenge

Immunization	Immunizing antigen	Surviving mice	*P*
Normal	IM2HBc	11/12	< 0.0001
	control	1/12	
Passive	IM2HBc	12/12	< 0.0001
	control	1/12	

Normal immunization, intraperitoneally vaccinated mice challenged with mouse-adapted X47 (H3N2) 3 weeks after the last inoculation; passive immunization, naive mice injected intraperitoneally with serum from immunized mice 24 h before challenge with mouse-adapted X47 (H3N2). *P*, statistical significance of the protection, compared with the control group; Fisher's exact test.

ance from the lungs. At 7 days after infection, we killed five mice from each group, prepared lung homogenates, and titrated the virus in embryonated chicken eggs. There was a 98% reduction in the vaccinated group (Fig. 5). These results demonstrate that vaccination with IM2HBc particles considerably enhances virus clearing from the lungs.

Passive immunization

We used passive transfer of serum to evaluate whether the antibodies produced by the vaccination with IM2HBc could explain the observed protection. Therefore, we immunized two groups of 40 mice as described above, except that the first group received 10 μg IM2HBc; and the second, only buffer (all supplemented with the standard adjuvants). At 2 weeks after the third immunization, we killed 28 mice, and used the other 12 mice from each group as control. We obtained blood by heart puncture and prepared the serum, and the concentration of IgG antibodies against M2e was determined by ELISA. We injected 12 naive mice intraperitoneally with 'pooled' serum; each mouse receiving 550 ng IgG antibody against M2e (corresponding approximately to serum from two mice). Another group of 12 naive mice received a corresponding quantity of serum from the mock-immunized control group. Then 24 h later, we challenged the mice with 5 LD_{50} mouse-adapted X47. The results (Table 2) show that the circulating antibodies against M2e present in the immunized animals are sufficient to explain the observed protection.

Route of administration

We compared two routes of administration: intraperitoneal and intranasal. We immunized mice intranasally with 10 μg purified IPM2HBc or IM2HBc after anesthetizing them with ether; control mice received only buffer. No adjuvants were used for intranasal administration. The immunizations, three in total, were given with an interval of 3 weeks. As before, 3 weeks after the last administration, we challenged the mice with 5 LD_{50} mouse-adapted X47. After immunization with IPM2HBc (homologous infection), all 12 mice survived, whereas 11 of 12 mice treated with IM2HBc, survived the heterologous challenge; only 2 of 11 from the control group survived (Table 3). The intranasal route of immunization seems even more promising than intraperitoneal injection, given that adjuvants were not required to obtain protection.

We determined the level of circulating IgG antibody against M2e produced 2 weeks after the first, second and third immunizations and after challenge for the IM2HBc vaccination (Fig. 6). The antibody response to the extracellular part of the M2 protein was as high for the intranasal route without adjuvant as for the intraperitoneal route with adjuvant.

Table 3 Survival of mice vaccinated intranasally followed by a lethal homologous or heterologous influenza A challenge

Immunization	Surviving mice	*P*
IPM2HBc	12/12	<0.0001
IM2HBc	11/12	<0.001
Control	2/11	

P, statistical significance of the protection, compared with the control group; Fisher's exact test.

Long-term protection

We immunized two groups of 24 mice as described above, except that the first group received 10 μg IM2HBc; and the second, only buffer (all supplemented with the standard adjuvants). At 3 weeks after the last immunization, we challenged 12 mice from each group with 5 LD_{50} mouse-adapted X47. We challenged the other mice with the same dose of virus, but at 26 weeks after the last immunization. The results show that protective immunity was still present half a year after the last immunization (Table 4).

Discussion

At present, influenza vaccines are mainly based on viral hemagglutinin as an antigen[26]. The licensed vaccines are usually a mixture containing 15 μg of each hemagglutinin derived from two influenza A strains and one B strain. These strains are chosen based on prediction of forthcoming epidemics and must be adapted annually or biannually. Much effort is being made to improve the efficacy, for example by using improved adjuvants[27], and/or to broaden the specificity. Neuraminidase, either virus-derived or recombinant, which does not neutralize the virus but blocks the infectious cycle, can confer complete protection against a lethal challenge with a homologous viral strain in mice[28,29]. Its addition to existing hemagglutinin vaccines may increase the coverage and provide some cross-protection, as a new epidemic strain may contain a less drifted neuraminidase[30]. A completely different approach involves vaccination with a DNA plasmid or a viral RNA that expresses an influenza gene. This offers the advantage of also generating an MHC I-restricted cytotoxic T-cell response. Furthermore, because of prolonged expression, it may extend the normally short half-life of a cellular immune response. Particularly worthwhile are experiments involving influenza nucleoprotein either as such or in combination with M1 protein[31–34]. These are quasi-invariant internal influenza A proteins, and the immune protection elicited is broad-spectrum but purely cellular (both $CD4^+$ and $CD8^+$ memory T cells; ref. 32). Fairly large amounts of injected DNA are needed, as experiments with lower doses of DNA have shown failure to protect[35]. Further preclinical and clinical research is needed to evaluate whether such a DNA-based approach is safe, effective and persistent.

The M2 protein, although a weak antigen, is a promising candidate to develop a broad-spectrum vaccine against influenza A. Its amino-acid sequence is evolutionary constant, and there is experimental evidence that M2-specific humoral immunity can prevent viral replication in the lungs. Here we have described a prototype of such a vaccine, based on the highly conserved extracellular part of the influenza A virus M2 protein, enhanced in its immunogenicity by linkage to the hepatitis B virus core protein. We made the linkage at the N terminus of the carrier protein to retain a free N terminus of the M2e domain and in this

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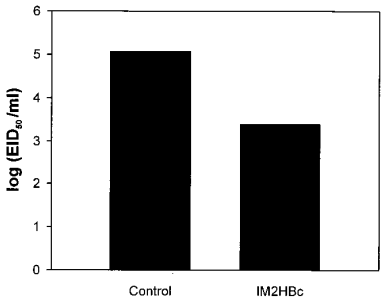

Fig. 5 Virus clearance from the lungs. After intraperitoneal immunization with IM2HBc or PBS and adjuvant (control), mice were challenged with 0.5 LD_{50} mouse-adapted X47; 7 d after challenge, the 50% egg infectious dose (EID_{50}) was determined for homogenized lung tissue titrated in embryonated chicken eggs.

way mimic the wild-type conformation of M2.

Vaccination studies with the resulting IPM2HBc particles showed that apparently the administered dose was not a very essential parameter, as doses ranging from 5 μg to 50 μg protected the mice. The immunized mice still showed a quite high morbidity, but presumably this was because of the large amount of challenge virus that was necessary to reach complete lethality in the unvaccinated controls. In a natural influenza infection, the initial number of infecting virus particles would be much less, so that it is reasonable to assume that the morbidity would decrease accordingly.

After three intraperitoneal or intranasal administrations of 10 μg purified M2HBc particles, a high degree of protection was obtained against both a homologous and a heterologous (one amino-acid change) influenza infection. Given the nearly invariable amino-acid sequence of the extracellular part of the M2 protein, it is reasonable to assume that the immune protection would be effective against all influenza A virus strains. Intranasal administration of antigen did not even require adjuvants to stimulate the immune response. Furthermore, we demonstrated that the mice were still protected half a year after the last immunization. Indeed, B-cell-dependent immunity is usually long-lasting, and can persist for a full human life-span, even in the absence of renewed antigen exposure[36].

Analysis of the serum of immunized mice showed a substantial antibody response to the extracellular domain of the M2 protein. After a viral challenge, this serum titer was further increased, presumably because of a more complete inflammatory response against the incoming virus, for example in the form of released cytokines. In a passive immunization experiment, we demonstrated that the antibodies against M2e produced were sufficient for protection against a lethal influenza challenge. This indicates that cellular immunity had no or a minor role in clearing the virus after the challenge.

Table 4 Protective immunity is long lasting

	Challenge after 3 weeks		Challenge after 26 weeks	
	Surviving mice	*P*	Surviving mice	*P*
M2HBc	11/11	<0.0001	9/12	= 0.0001
control	1/12		0/12	

P, statistical significance of the protection, compared with the control group; Fisher's exact test.

Broad-spectrum, protective immunity has been described in mice after vaccination with a preparation containing full-length M2 protein, produced in a recombinant baculovirus–insect cell system, together with incomplete Freund's adjuvant[18]. The vaccine described here, however, has several advantages. The relative purity of the baculovirus-derived M2 protein preparation was not reported[18]; indeed, membrane-anchored proteins are difficult to purify. In contrast, the well-defined M2HBc particles used here were purified to near-homogeneity. As M2HBc particles are made in *E. coli*, they can be produced efficiently at low cost and the process can be scaled up readily. Moreover, we intentionally avoided using an inflammatory adjuvant like Freund's, which is unlikely to become approved for use in humans. For immunization by intranasal administration of M2HBc particles, there was not even the need for an adjuvant. Finally, it is not apparent to what extent the protective immunity previously described[18] can be compared with that achieved here with pure M2HBc particles. Indeed, the previous experiment failed to demonstrate transfer of the protective immunity by serum[18], whereas we have documented here successful passive immunization.

In conclusion, we have described a vaccine that provides long-lasting, protective immunity in mice against all influenza A virus strains. The vaccine is based on the genetic fusion of the 23 external amino acids of the viral M2 protein to the N terminus of the hepatitis B virus core protein. HBc particles are formed in *E. coli*, which carry on their surface the M2e epitope exposed to the outside, as on influenza virus particles or on infected cells. The vaccine can be administered intraperitoneally with an approved adjuvant, or intranasally. Soluble antibodies can fully explain the observed protective immunity, as shown by passive transfer to naive mice. It is possible that the vaccine can be further improved, for example, by the inclusion of influenza-specific T-helper or CTL epitopes into the fusion protein. Moreover, the need for three immunizations may be reduced by immobilization of the vaccine in slow-release microspheres.

Methods

Virus. A/PR/8/34 (H1N1) and X47 were adapted to mice by several lung passages. After adaptation, virus was grown in fertilized chicken eggs[37] and purified. The 50% egg infectious dose (EID_{50}) and the lethality in mice were determined. For mouse-adapted A/PR/8/34, 1 LD_{50} corresponded to 4 10^6 EID_{50}. For mouse-adapted X47, 1 LD_{50} corresponded to 1.8×10^4 EID_{50}.

Expression of M2HBc particles in *E. coli* and analysis by dot-blot. Cultures of *E. coli* MC1061 (pcI857), containing pPLc245 (control) (ref. 38), pPLcA1 (carrying the gene for hepatitis B core protein, called *hbc* here; from M. Nassal, Zentrum für Molekulare Biologie, Heidelberg, Germany) or pPLcIPM2HBc (containing the fusion gene *ipm2hbc*), were grown for about 4 h at 28 °C (not induced) with shaking. When a density of 4.5×10^8 to 5.5×10^8 bacteria/ml was reached, the cultures were split; one half was incubated for 4 h at 28 °C (induced) and the other half was switched to 42 °C (induced) . The bacteria were concentrated by centrifugation and opened by sonication (Vibra cell; Sonics & Materials, Danbury, Connecticut). After centrifugation, the supernatants were filtered through a nitrocellulose membrane (pore diameter, 0.45 μm; Schleicher & Schuell, Keene, New Hampshire). The filter was blocked for at least 2 h in PBS pH 7.4 (14.5 mM phosphate buffer, pH 7.4, and 150 mM NaCl) containing 2.5% skim milk powder and 0.1% Triton-X100 (blocking buffer). Incubation with the primary antibody, diluted in blocking buffer, was done at room temperature for 30 min. Excess unbound antibody was removed by three washings with blocking buffer. The bound antibodies were detected with an alkaline phosphatase-conjugated antibody against mouse (Sigma). Subsequently, the filter was washed twice with PBS, pH 7.4, and 0.1% Triton-X100. A third

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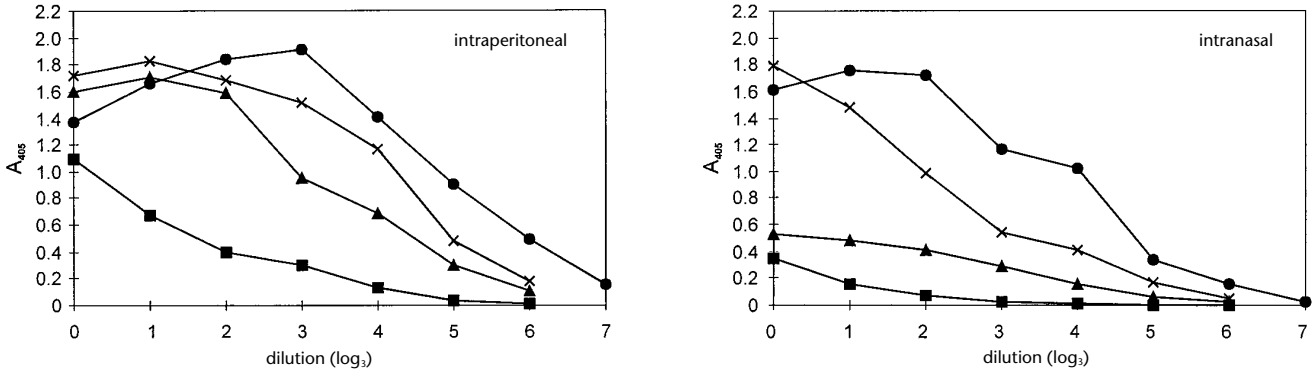

Fig. 6 Titer of antibody against M2e in serum samples after intraperitoneal or intranasal immunization with IM2HBc. Serum obtained after the first (■), second (▲) and third (X) immunization and after the challenge (●) was initially diluted 1:50, then serially diluted 1:3. A_{405}, absorbance (corrected value).

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washing step was done in substrate buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl and 5 mM $MgCl_2$). The filter was then incubated in substrate buffer with 165 µg/ml nitroblue tetrazolium and 165 µg/ml 5-bromo-4-chloro-3-indolylphosphate until a clear signal appeared. The blot was finally washed thoroughly with tap water and dried.

Construction of pATIPM2m1. RNA segment 7 of the influenza A virus, A/PR/8/34 (H1N1), was cloned as described for RNA segment 4 (ref. 39). The resulting plasmid was named pATIPMA. As the mRNA of the M2 protein is not a collinear transcript of RNA segment 7, an intron of 689 nucleotides had to be removed[40]. Therefore the first exon of the *m2* gene was made synthetically and fused directly to the second exon. By site-directed mutagenesis[41], a *Bcl*I site was introduced at the junction between the extracellular part and the membrane anchoring region of the M2 protein, resulting in plasmid pATIPM2m1. The information coding for the extracellular part of the M2 protein was isolated as a 72-bp *Bam*HI–*Bcl*I fragment.

Construction of IPM2HBc. The plasmid pPLcA1 contains the *hbc* gene under control of the P_L promoter of bacteriophage (ref. 42). The 346-bp *Nco*I–*Xba*I *hbc* fragment, isolated from pPLcA1, was inserted into pMa581, a derivative of pMa58, linearized by digestion with *Nco*I and *Xba*I. The resulting plasmid was called pMaHBc. By site-directed mutagenesis[41] we introduced a *Bam*HI site at the 5′ end of *hbc*, directly after the start codon. The resulting plasmid was called pMaHBcm. The information coding for the extracellular part of the M2 protein was isolated from pATIPM2m1 and cloned into pMaHBcm linearized by digestion with *Bam*HI, resulting in the vector pIPM2HBc. The *hbc* gene in the expression vector pPLcA1 was then replaced by the 418-bp *Nco*I–*Xba*I *m2hbc* fragment, creating pPLcIPM2HBcp. Because of the construction, four extra amino acids between the first methionine and the start of the extracellular part of the M2 protein had to be removed. This was achieved by 'looping-out' mutagenesis[43]. The resulting plasmid was called pPLcIPM2HBc.

Construction of IM2HBc. The extracellular part of the M2 protein from A/PR/8/34 differs by only one amino acid from most other viruses sequenced so far. By site-directed mutagenesis[43], the sequence of the extracellular part of the M2 protein in pPLcIPM2HBc was changed to the more universal M2 sequence (Gly20 to Asp20). The new plasmid was called pPLcIM2HBc. The sequence, determined on a model 373A sequencer (Applied Biosystems, Foster City, California), contained the desired mutation.

Expression and purification of IPM2HBc and IM2HBc. Expression of proteins under control of the P_L promoter was accomplished by shifting an exponentially growing culture from 28 °C to 42 °C (ref. 23). MC1061[pcI857,pPLcIPM2HBc] or MC1061[pcI857,pPLcIM2HBc] were grown in a BioFlo IV fermentor (New Brunswick Scientific, Edison, New Jersey). When the cultures reached a density of 4.5×10^8 to 5.5×10^8 bacteria/ml, they were shifted to 42 °C. After 3 h of induction, the bacteria were collected and resuspended in a volume of buffer (in ml) (50 mM Tris-HCl, pH 8,150 mM NaCl, and 5% glycerol with one protease inhibitor 'cocktail' tablet (Complete; Boehringer) per 25 ml) corresponding to twice the weight (in grams) of the pelleted bacteria. This suspension was treated with 1 mg/ml lysozyme (freshly dissolved in 25 mM Tris-HCl, pH 8) for 30 min on ice. Subsequently, the bacteria were lysed with 0.2% Triton-X100 in the presence of 25 mM EDTA, pH 8. After 30 min of incubation on ice, the lysates were centrifuged for 1 h at 48,000*g*. The pellet was removed and the concentrations of Tris-HCl, pH 8, and NaCl in the supernatant were adjusted to 20 mM and 50 mM, respectively. This solution was loaded on a DEAE Sepharose column (diameter, 2.5 cm and height, 5.5 cm; Pharmacia), equilibrated with 20 mM Tris-HCl, pH 8, and 50 mM NaCl. The fusion protein was not retained on the column. To the flow-through, 3.8 M $(NH_4)_2SO_4$, pH 7, was added to a final concentration of 1.2 M. The precipitate was collected over a CF11 cellulose column (diameter, 2.5 cm and height, 3.5 cm; Whatman International, Maidstone, UK). The column was eluted with PBS, pH 7.4. The eluate of about 50 ml was concentrated in a Centiprep 30 (Amicon, Danvers, Illinois) to 5 ml and loaded on a Sephacryl S-300 column (diameter, 2.5 cm and height, 91 cm; Pharmacia), which was equilibrated with PBS, pH 7.4. The peak fractions were 'pooled' and the concentration of IPM2HBc or IM2HBc was determined by ELISA. The LPS content was assayed (LAL Coatest Endotoxin; Endosafe, Charleston, South Carolina) and was sufficiently low (5–9 ng per 50 µg IPM2HBc) not to interfere with the experiments.

Immunization. Female Balb/c mice (Charles River Wiga, Sulzfeld, Germany) 7 weeks old were injected three times intraperitoneally with 10 µg (except where indicated otherwise) purified IPM2HBc (homologous) or IM2HBc (heterologous) in the presence of adjuvant (total volume, 200 µl). Where indicated, doses of 5, 10 and 50 µg IPM2HBc were used. Control mice received only adjuvant in PBS, pH 7.4. For the first immunization, half a dose of Ribi adjuvant (Ribi Immunochem Research, Hamilton, Montana) was used: 25 µg monophosphoryl lipid A + 25 µg synthetic trehalose-6,6-dimycolate. In the second and third injections, 25 µg monophosphoryl lipid A (Ribi Immunochem Research, Hamilton, Montana) and 25 µg muramyl dipeptide (N-acetylmuramyl-L-alanyl-D-isoglutamine; Sigma) were used. The injections were given at 3-week intervals. For intranasal immunization, the antigen, without adjuvants, was administered to mice anesthetized by ether. A total volume of 50 µl was administered in one nostril.

Challenge. Three weeks after the last inoculation, mice were challenged with mouse-adapted X47 (H3N2). In one experiment in which the longevity of the protection was determined, half the mice from each group (immunized or control) were challenged 3 weeks after the last inoculation and the other half were challenged 26 weeks after the last immunization. The virus was administered intranasally in a total volume of 50 µl to mice anesthetized by ether.

Serum preparation. Five blood samples were obtained from every mouse: before immunization, after the first, second and third immunizations, and after challenge. For the preparation of serum, the blood was incubated for 30 min at 37 °C; the samples were then placed on ice for at least 1 h and centrifuged twice for 5 min at 16,000*g*. Equal volumes of sera obtained from different mice were 'pooled' for analysis of antibody production. For

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isolation of blood by heart puncture, mice were anesthetized with 160 mg/kg pentobarbital (Nembutal; Sanofi, Brussels, Belgium). Serum was prepared as described above.

Analysis of production of antibodies against M2e and HBc. Microtiter plates (type II F96 Maxisorp; Nunc, Roskilde, Denmark) were used. A 0.1% casein solution for blocking and dilution of antibodies was used, as recommended by the manufacturer of the plates. To identify IgG antibodies directed against M2e, we made use of another fusion protein, IPM2hB2Mm, which consists of the extracellular part of M2 linked to human β_2-microglobulin (S.N. *et al.*), unpublished results). One half of the microtiter plate was coated with human β_2-microglobulin, and the other half, with 1 μg/ml IPM2hB2Mm, both as crude bacterial culture supernatant. The same concentration of total protein was used in both. Therefore, the hB2M content of the culture supernatant of bacteria secreting hB2M had to be adjusted to 1 μg/ml by the addition of purified hB2M (Sigma). A series of 1:3 dilutions of the different serum samples, starting with a 1:50 dilution, were loaded on the hB2M- and IPM2hB2Mm-coated wells. The bound antibodies were detected with an alkaline phosphatase-labeled antibody against mouse IgG (γ-chain-specific; Sigma). The microtiter plates were incubated for 1 h with substrate buffer (10% diethanolamine, 0.5 mM $MgCl_2$ and 0.02% NaN_3, pH 9.8) containing 1 mg/ml p-nitrophenyl phosphate before the absorbance was measured. To obtain the value for the specific reactivity to the extracellular part of the M2 protein, the absorbance of hB2M at a given dilution was subtracted from the absorbance of IPM2hB2Mm of the corresponding dilution. The antibodies against M2e were quantified using human β_2-microglobulin (Cymbus Bioscience, Southampton, UK) as a standard. Microtiter plates were coated with 1 μg/ml purified HBc to determine the level of IgG antibodies directed against the carrier protein. The plates were blocked as described above, and series of 1:2 dilutions of the different serum samples, initially diluted 1:1000, were loaded on the wells. The ELISA was then developed as described for the antibodies against M2e. Purified recombinant HBc and monoclonal antibody against HGc were gifts from H. Claeys (Bloedtransfusiecentrum, Leuven, Belgium).

Production of monoclonal antibodies against influenza A M2 protein. Balb/c mice were immunized three times with 2.5 μg purified IPM2hB2Mm (S.N. *et al.*, unpublished results). For the first injection, a complete dose of Ribi adjuvant was used; the second and third immunizations were done in the presence of 50 μg monophosphoryl lipid A. The injections were given with an interval of 3 weeks. Three days after the last immunization, spleen cells were isolated and fused with SP2/0-AG14 myeloma cells using standard protocols[44]. The isotype of the isolated monoclonal antibodies was determined (Isostrip; Boehringer). Two different immunoglobulin subtypes that recognized the extracellular part of the M2 protein were obtained: an IgM and an IgG2a.The IgG2a antibody was usually used here.

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