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Short Communication

Antiserum against the conserved nine amino acid N-terminal peptide of influenza A virus matrix protein 2 is not immunoprotective

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The recent emergence and rapid spread of the pandemic H1N1 swine influenza virus reminded us once again of the need for a universal influenza vaccine that can elicit heterosubtypic protection. Here, we show the superior immunogenicity and immunoprotective capacity of the full-length matrix protein 2 ectodomain (M2e) peptide coupled to keyhole limpet haemocyanin (KLH) compared with the N-terminal 9 aa residues of M2e (SP1). Immunization with M2e–KLH protected mice against a lethal challenge with influenza A virus and significantly reduced weight loss and lung virus titres. In addition, passive transfer of serum raised in rabbits against M2e–KLH protected mice against a lethal influenza virus challenge, whereas serum from rabbits immunized with SP1–KLH did not. Nevertheless, immunofluorescence staining revealed that rabbit serum raised against SP1–KLH bound specifically to infected Madin–Darby canine kidney cells. We conclude that the peptide SP1 contains an immunogenic epitope that is not sufficient for immunoprotection.

Influenza is possibly the most important respiratory infectious disease in humans worldwide. Yearly epidemics, and more importantly occasional pandemics, often cause considerable morbidity and mortality. Vaccination is the most important protective measure against seasonal influenza epidemics (Fiore *et al.*, 2009). However, effective vaccination against influenza is severely hampered by the structural variability of haemagglutinin (HA) and neuraminidase, the two major antigens of the virus. This frequent change in antigenic appearance is referred to as 'drift', while 'shift' refers to the emergence of a new HA gene in the human influenza gene pool and is usually the starting point of a new pandemic. Avian species are the major reservoir of different HA genes. Occasionally, avian influenza A subtypes cross the species barrier and might initiate a pandemic, such as the highly pathogenic Spanish flu during 1918–1919 and the recent, fairly mild, H1N1 swine influenza virus (H1N1v) pandemic (Dawood *et al.*, 2009). Different approaches have been followed to obtain intra- or even heterosubtypic protective immunity, that is, immunity to all influenza A strains regardless of subtype, mainly by exploiting more-conserved influenza targets for the development of novel vaccine candidates (Grebe *et al.*, 2008; Mozdzanowska *et al.*, 2003; Neirynck *et al.*, 1999).

We previously described an influenza vaccine based on the highly conserved external domain of influenza matrix protein 2 (M2e) linked to an appropriate carrier, such as hepatitis B virus core particle (Neirynck *et al.*, 1999). The protection provided by M2e-based vaccination presumably targets infected cells by a mechanism that is antibody- and Fc receptor-dependent (Jegerlehner *et al.*, 2004; Wang *et al.*, 2008).

Lamb *et al.* (1985) were the first to describe the influenza A M2 protein as an 'infected-cell surface antigen' with a minimum of 18 aa exposed on the cell surface. M2 is an essential integral membrane protein of influenza virus that forms a highly selective and pH-regulated proton channel (Pinto *et al.*, 1992). Although M2 protein is scarce on virus particles, it is expressed abundantly on the surface of infected cells (Lamb *et al.*, 1985). These authors also described an immune serum raised in rabbits by injecting a 9 aa peptide (SP1) that was chemically fused to the carrier keyhole limpet haemocyanin (KLH). SP1 corresponds to the N terminus of the mature influenza M2 protein. The antiserum specifically bound to influenza-infected cells.

In this study, we compared the immunogenic and immunoprotective properties of the oligopeptide, SLLTEVETP

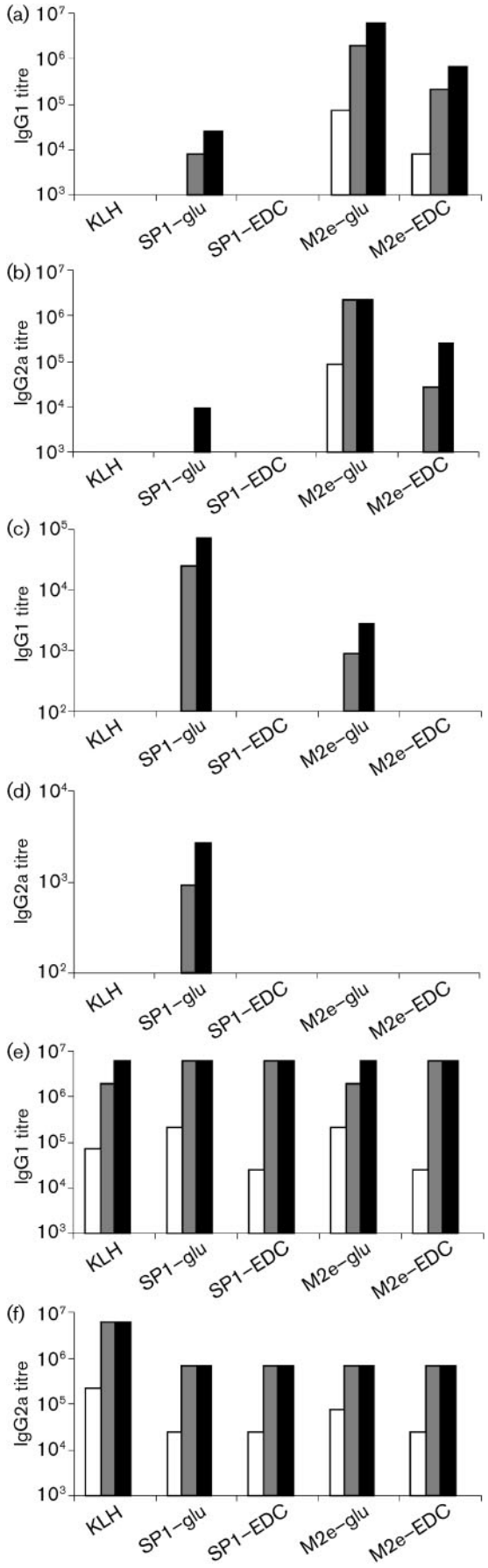

Fig. 1. Immunization of mice with SP1– or M2e–KLH conjugates induces SP1- and M2e-specific serum IgG antibodies. Glutaraldehyde (glu) and EDC, were used to link the SP1 and M2e peptides to KLH. Hyperimmune serum was generated by three immunizations at 3-week intervals. Results are expressed as antigen-specific end point IgG1 (a, c and e) and IgG2a (b, d and f) titres in pooled serum samples, as determined by M2e peptide ELISA (a and b), SP1 peptide ELISA (c and d) and KLH ELISA (e and f). White bars, First immunization; grey bars, first boost; black bars, second boost.

(SP1), which consists of amino acids 2 to 10 of M2, with the properties of the full-length M2e peptide (SLLTEVETPIRNEWGCRCNDSSD). Each peptide was coupled to KLH by using two different chemical conjugation reagents: glutaraldehyde or 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), as described by Lamb *et al.* (1985). Sequence analysis of human isolates had revealed the strongest conservation in the region of amino acids 2 to 10 of M2e. Therefore, in this study we investigated whether antibodies to this small region are not only immunogenic but also immunoprotective.

The SP1–KLH and M2e–KLH conjugates were used to immunize BALB/c mice. The SP1–KLH and M2e–KLH conjugates were supplemented with complete Freund's adjuvant for the first immunization and incomplete Freund's adjuvant for the second immunization. Mice were immunized three times at 3-week intervals and monitored for seroconversion to SP1 and M2e by using a peptide ELISA (De Filette *et al.*, 2005). Both M2e–KLH conjugates induced serum IgG1, -2a, -2b and -3 antibodies that specifically bound to SP1 peptide and M2e peptide in an ELISA assay (Fig. 1 and data not shown). Also, immunization with SP1 peptide fused to KLH with glutaraldehyde-induced serum IgG1 and IgG2a antibodies against peptides SP1 and M2e (Fig. 1a–d). M2e–KLH conjugates induced high titres of anti-M2e IgG1 and IgG2a, while the SP1–KLH glutaraldehyde conjugate induced considerably lower serum titres of anti-M2e IgG. The glutaraldehyde coupling method resulted in a conjugate that was far more immunogenic compared with EDC coupling, and similar results have been reported for other antigens (Mera *et al.*, 2008). It is possible that EDC, which reacts with both carboxylate and amino groups, generates various polymerized and randomly oriented conjugates, whereas glutaraldehyde, which is a bifunctional coupling reagent, links two compounds through their amino groups. Serum IgG responses against the carrier KLH were similar in all groups, indicating that the same amount of vaccine antigen had been administered to the animals (Fig. 1e and f).

We next assessed the ability of SP1– and M2e–KLH conjugates to elicit protection against a potentially lethal influenza A virus challenge. BALB/c mice were immunized as described before and 3 weeks after the last immunization they were challenged with 4 LD_{50} of a mouse-adapted PR8 strain. As expected, the challenge resulted in 80–100 %

mortality in the control group, which was immunized with KLH carrier and adjuvant (Fig. 2a). In contrast, vaccination with M2e–KLH conjugate resulted in complete protection. The mortality rate of the SP1–KLH-vaccinated mice was not significantly different from that of the KLH control group, and the survival rates of these groups were significantly lower than that of M2e–KLH-vaccinated mice, all of which survived ($P<0.001$, Kaplan–Meier). Following infection, all M2e–KLH vaccinated mice showed less morbidity than the SP1–KLH and KLH groups (Fig. 2b). The difference in body weight loss on day 6 after challenge, between the SP1–KLH and KLH groups on one hand and the M2e–KLH groups on the other, was highly significant ($P<0.005$, Tukey test). We also determined viral titres by the method of Reed and Muench (1938) in lung homogenates prepared 6 days after infection with 1 LD_{50} of mouse-adapted PR8. Mice immunized with either of the M2e–KLH conjugates had significantly lower lung viral loads ($P<0.05$, Tukey test) than the SP1–KLH vaccinated mice (Fig. 2c).

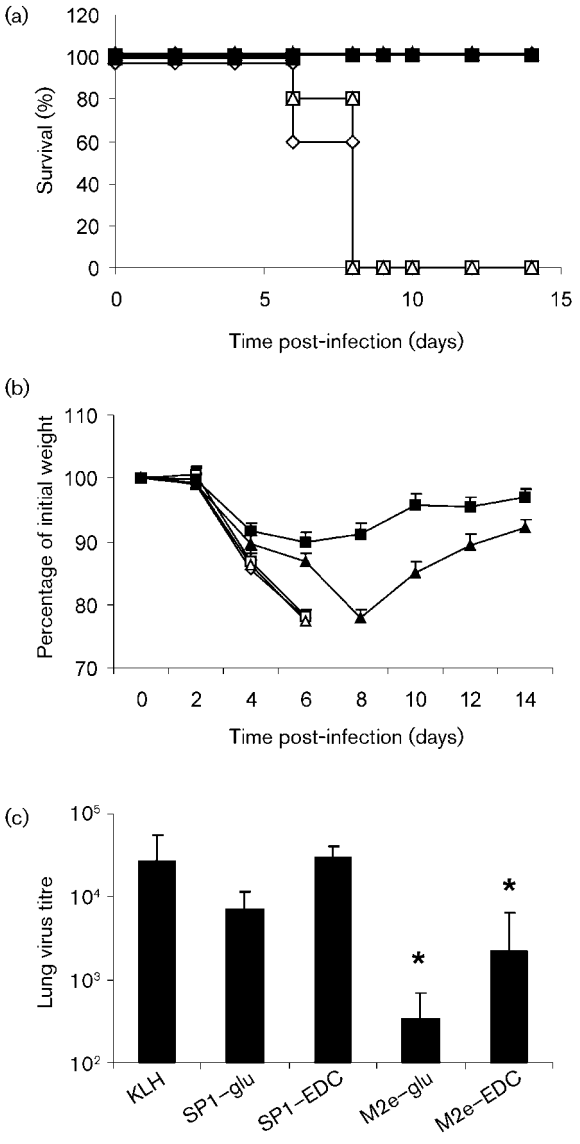

Fig. 2. Full-length M2e peptide coupled to KLH induces protective immunity against PR8 influenza virus challenge, but SP1 peptide coupled to KLH does not. Mice were immunized with SP1–glu (□), SP1–EDC (Δ), M2e–glu (■) or M2e–EDC (▲) peptide–KLH conjugates, or with KLH (◇) as control, and challenged with 4 LD_{50} of mouse-adapted PR8 virus. Survival (a) and body weight (b) were monitored for 2 weeks after challenge. (c) Virus titre (expressed as mean $\log_{10}$ $TCID_{50}$ ml^{-1}) in lung extracts prepared 6 days after challenge. Differences in lung viral load between mice vaccinated with M2e–KLH or SP1–KLH were statistically significant (*, $P<0.05$, Tukey test).

Since the immunogenicity of peptide antigens can differ in different animal species (Darnule *et al.*, 1980; Hanly *et al.*, 1995), we assessed the immunogenicity of SP1– and M2e–KLH conjugates in rabbits. For the primer and first boost injections, the peptide conjugates were supplemented with Freund's adjuvant. The second boost was composed of a combination of free peptide and the corresponding peptide–KLH conjugate. The last two boost immunizations were with adjuvanted peptide alone as described by Lamb *et al.* (1985). ELISA-based analysis of the serum IgG responses against SP1 and M2e peptides revealed responses of different strengths among the individual animals. The strongest peptide-specific IgG responses were obtained after the second immunization. Additional boosts with peptide resulted in a slight decrease in serum IgG titres in three of four animals (Fig. 3a and b). The antibody titres against SP1 peptide were greater in SP1–KLH immunized rabbits compared with M2e–KLH immunized rabbits. These results, when compared with the data from the active immunization of mice described above, indicate that the SP1–KLH glutaraldehyde peptide conjugate is more immunogenic in outbred New Zealand white rabbits than in inbred BALB/c mice.

To test whether the anti-M2e and anti-SP1 antibodies elicited in rabbits were protective, we injected naive BALB/c mice intraperitoneally with these sera. Twenty four hours after this passive transfer, the mice were challenged intranasally with 4 LD_{50} of mouse-adapted PR8 virus (Fig. 3c and d). All mice that received immune serum from M2e–KLH-immunized rabbits survived the challenge, while all those receiving anti-SP1–KLH immune serum died (Fig. 3c). The difference in survival rate between the M2e–KLH immune-serum recipients and the other two groups was highly significant ($P<0.001$, Kaplan–Meier). In addition, mice receiving anti-M2e–KLH rabbit serum displayed limited morbidity (Fig. 3d, as compared with the control group on day 6, $P<0.001$, Student's *t*-test). The protection was also reflected in the lung virus load (data not shown). Based on these passive experiments and the active immunization experiments described above, we conclude that M2e peptide, when chemically linked to KLH, can induce protective M2e-specific humoral responses. In contrast, SP1–KLH fusions fail to elicit a

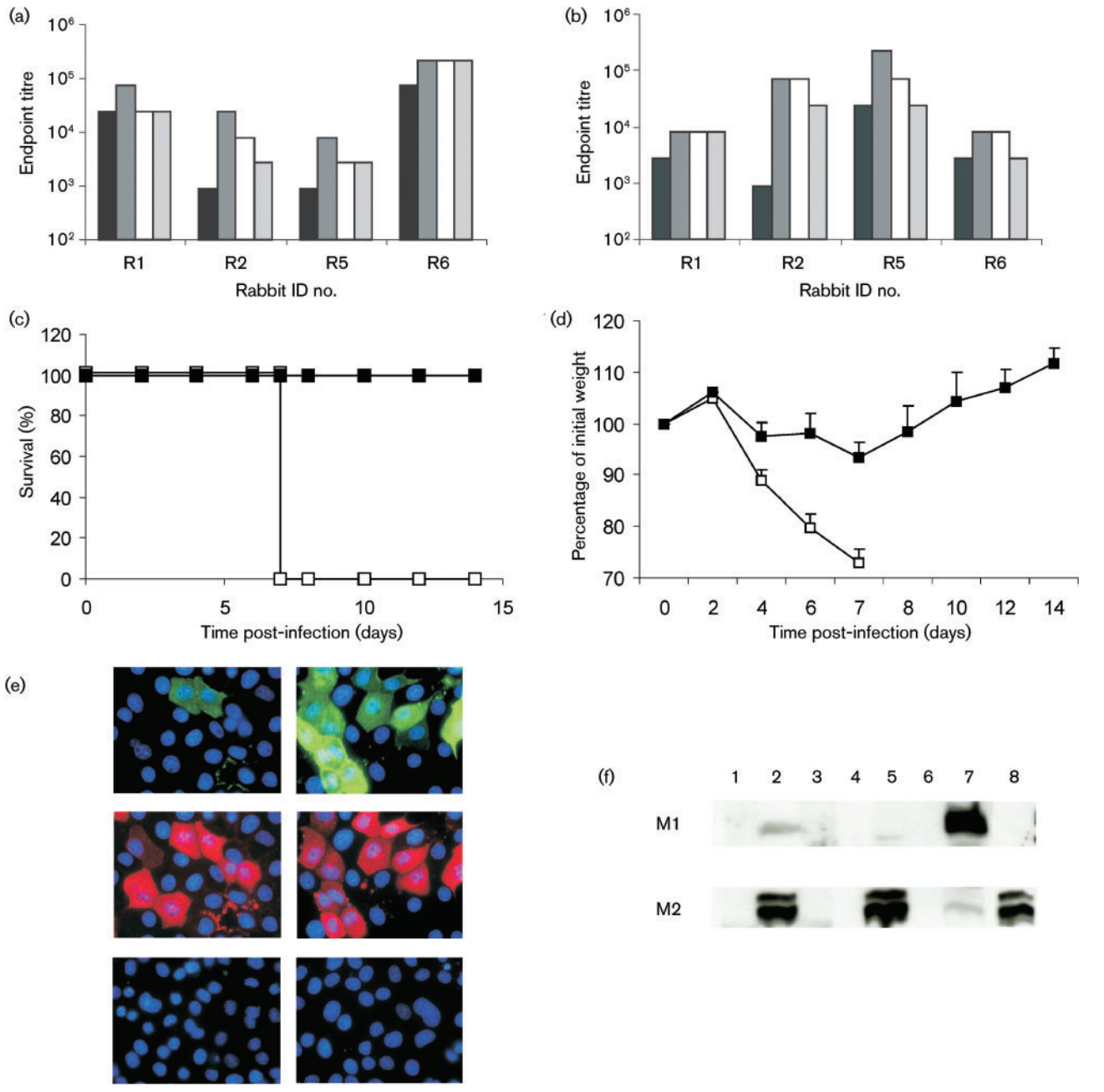

Fig. 3. Comparison of the antigenicity of SP1 and M2e and the protectiveness of SP1 and M2e immune sera. SP1 peptide coupled to KLH induces fairly strong SP1-specific antibody responses but poor M2e-specific responses in rabbits. Rabbit serum-IgG titres against M2e (a) and SP1 peptide (b) in hyperimmune antisera obtained after the first (black bars), second (dark grey bars), third (white bars) and fourth (light grey bars) boosts. Rabbits 1 and 6 (R1 and R6) were immunized with M2e–KLH, while rabbits 2 and 5 (R2 and R5) received SP1–KLH. The third and fourth boosts were with peptide only in the presence of adjuvant. (c, d) Protection against a lethal influenza A virus challenge in mice after passive transfer of hyperimmune M2e–KLH (■) or SP1–KLH (□) rabbit serum. After challenge with mouse-adapted PR8 virus, survival (c) and body weight (d) were recorded for 2 weeks. (e) Indirect immunofluorescence staining of M2 on PR8 virus-infected MDCK cells. The upper and lower panels show the surface fluorescent pattern after staining with rabbit anti-SP1–KLH (left) and rabbit anti-M2e–KLH IgG (right), both revealed with donkey anti-rabbit IgG labelled with Alexa Fluor 488. The middle panel shows immunostaining of infected cells with a goat anti-RNP serum obtained from the NIH Biodefence and Emerging Infections Research Resources Repository from the National Institute of Allergy and Infectious Disease, National Institutes of Health, USA [polyclonal anti-influenza virus RNP, A/Scotland/840/74 (H3N2), NR-3133]; Alexa Fluor 556-labelled donkey anti-goat was used as secondary antibody. The lower panels show uninfected cells stained with SP1– and M2e–KLH immune sera. Exposure times for the SP1– and

M2e–KLH immunostainings were adjusted to be the same as for the reactivity with uninfected control cells, and was set 3.5× higher for SP1–KLH than for M2e–KLH immunostaining. (f) Reactivity of mouse and rabbit anti-M2e and anti-SP1 immune sera with M1 and M2 proteins analysed by Western blot of a total lysate of PR8 virions in Laemmli sample buffer. The antisera used were pre-immune rabbit (1), rabbit anti-M2e–KLH (2), rabbit anti-SP1–KLH (3), pre-immune mouse (4), mouse anti-M2e–KLH (5), mouse anti-SP1–KLH (6), goat anti-M1 (Biodefence and Emerging Infections Research Resources Repository from NIAID, NR-3134) (7) and the M2e-specific 14C2 mAb (8) (Zebedee & Lamb, 1989).

detectable protective immune response, even though they are immunogenic in mice and rabbits.

It is possible that SP1–KLH immune serum does not protect against influenza A virus challenge because the anti-SP1 IgG fraction does not bind to natural M2, as expressed on infected cells. To test this possibility, Madin–Darby canine kidney (MDCK) cells were infected at an m.o.i. of 1 with PR8 virus; mock-infected cells served as negative controls. Twenty-four hours later we confirmed infection by immunostaining with an antiserum specific for influenza A virus nucleoprotein. Expression of M2 on the surface of infected cells was confirmed by immunostaining with the M2e-specific mouse mAb 14C2 (not shown). In parallel, cells were immunostained with serum from SP1– or M2e–KLH-vaccinated rabbits. The SP1- and M2e-immune-serum concentrations that were used were normalized to obtain equal M2e-peptide-specific IgG titres, as determined by M2e-peptide ELISA. The binding of M2e-specific antibodies to the infected cells was revealed by using rabbit IgG-specific Alexa 488-labelled antibodies. M2e-specific antibodies in the rabbit M2e–KLH immune serum readily bound to M2e expressed on the surface of PR8-infected cells (Fig. 3e). In contrast, $<1\%$ of PR8-infected cells could be immunostained with the rabbit SP1–KLH immune serum, a result that is in agreement with published data (Lamb *et al.*, 1985).

The first 8 aa residues of SP1 and M2e are identical to the N terminus of M1 protein. The M1 protein is located inside virus particles (Schulze, 1970) and virus-infected cells (Bucher *et al.*, 1989), and humoral immunity against this protein does not protect mice against viral challenge (Webster & Hinshaw, 1977). Therefore, it is possible that the failure of SP1–KLH serum to protect could be due to a relatively stronger reactivity of this serum with viral M1 protein compared with M2. Therefore, we examined whether anti-SP1 antibodies react preferentially with M1 protein rather than with M2 protein (Fig. 3f). We separated PR8 virion proteins by SDS-PAGE and compared the reactivity of SP1– and M2e–KLH immune sera with M1 and M2 proteins by Western blot analysis. Binding of SP1–KLH serum to M2 could only be detected after overexposure of the film (data not shown) and no reactivity was observed with M1 protein (Fig. 3f). Rabbit and mouse anti-M2e–KLH serum reacted strongly with M2 protein and the M2e–KLH rabbit serum also reacted with M1 protein (Fig. 3f). This result indicates that the antiserum elicited by M2e–KLH immunization also contains antibodies that specifically react with the SP1 region.

In summary, immune sera induced by SP1–KLH can specifically bind to M2e exposed on the surface of influenza virus-infected cells. However, all evidence obtained so far indicates that this specific response fails to confer protection against a lethal influenza A virus infection, but the reason for this is not clear. The titre of SP1-specific antibodies might not have been sufficiently high. Also, the avidity of the average SP1–antibody–M2 interaction may have been too weak to provide protection through antigen-dependent cellular cytotoxicity. It is also possible that SP1 rarely adopts a conformation similar to that of the native M2e on influenza virus-infected cells or at least a conformation that leads to the generation of immunoprotective antibodies. Another possibility is that a fraction of the SP1-specific antibodies bind to M2e sites but do not protect, while another fraction is protective but is competed out by the former, more abundant, fraction. However, we found no evidence for the supposition that SP1–KLH and M2e–KLH conjugates lead to different populations of IgG-subtypes. Some of these hypotheses can be tested if sufficient quantities of SP1–KLH immune antiserum are available. To do this in mice would require an unrealistically large number of animals, but another experimental animal system might make such studies possible. We conclude that, although the peptide comprising amino acids 2 to 10 of the M2 protein is highly conserved, it is not suitable for the development of a universal influenza A vaccine because it does not elicit protective antibodies.

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