Synthetic recombinant influenza vaccine induces efficient long-term immunity and cross-strain protection

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Synthetic vaccines utilize specific antigenic epitopes in order to elicit a protective immune response. In this work we examined the immunogenicity of chimeric proteins expressing influenza epitopes and their ability, as single products or in various combinations, to protect mice from viral challenge. Oligonucleotides coding for three epitopes (HA91-108, NP55-69 and NP147-158) stimulating B cells, T helper cells and cytotoxic T lymphocytes (CTLs), respectively, were individually inserted into the flagellin gene of a Salmonella vaccine strain. Immunization of mice with the resultant hybrid flagella resulted in a specific humoral or cellular response. The protective efficacy of the chimeric flagella was evaluated by intranasal immunization of mice, without any adjuvant, and subsequent challenge with infectious virus. The construct containing the B-cell epitope by itself led to partial protection. However, the addition of the two T-cell epitopes augmented the protection in a significant manner. The protective immunity conferred by this combined vaccine, comprising the three epitopes, persisted for at least 7 months after the last boost, and was effective against several influenza A strains. Furthermore, this vaccine fully protected mice from a lethal challenge, and enhanced their recovery process. Our results indicate that stimulation of the different arms of the immune system is required for effective anti-influenza response, and demonstrate the applicability of such synthetic recombinant approach for preparing a broad spectrum influenza vaccine.

Keywords: Influenza; synthetic vaccine; recombinant; epitope; Salmonella flagellin

The influenza A viruses are responsible for many millions of infected individuals worldwide and tens of thousands of deaths annually, as well as causing considerable economic burden. The rapid changes in the viral surface antigens (denoted shifts and drifts) render the currently used vaccines strain-specific and only partially effective. Hence, new strategies for influenza vaccination are sought, among those the development of synthetic vaccines based on short epitopes, that should elicit protective immunity. Two major issues that are crucial for the success of this approach are the identification of the exact epitopes that stimulate the desired response and the design of carrier and adjuvant that will efficiently present these epitopes to the immune system. Cumulative results have shown that synthetic peptides could be successfully employed in various vaccination studies.

In the case of influenza infection, neutralizing antibodies directed to the haemagglutinin (HA) were shown to be the major factor responsible for neutralizing the virus, whereas cytotoxic T cells (CTLs), directed mainly towards the nucleoprotein (NP), are the key to viral clearance and the recovery process, once an infection is established. The interaction between the various cell types of the immune system was shown to be significant for the efficiency of the overall immune response. Several HA neutralizing and T-cell epitopes have been identified including the region 91–108, which is conserved in all A influenza H3 strains. Previous studies in our laboratory showed that a synthetic peptide corresponding to this sequence, conjugated to tetanus toxoid, and administered in CFA or as a conjugate with the synthetic adjuvant muramyl dipeptide, elicited neutralizing antibodies and conferred partial protection against viral challenge in mice. The NP, which is highly conserved in all A/influenza strains, was also screened for potential epitopes among which the peptide corresponding to amino acid residues 55–69 in its sequence was reported to be a T helper (Th) epitope, and a synthetic peptide corresponding to the sequence 147–158 was shown to stimulate CTLs. The latter, when coupled to a lipoprotein carrier, was used to immunize mice, and induced effective anti-influenza cellular response.

Synthetic peptides as such are usually poor immunogens and have to be administered when coupled to an appropriate carrier. In the present study the possibility of...
presenting the epitopes expressed in a chimeric Salmonella flagellin protein was explored. Attenuated Salmonella strains were used to express and present foreign epitopes from various antigens such as cholera toxin20, malaria circumsporozoite protein21, hepatitis B surface antigen22, tetanus toxin23 and streptococcal M protein24. Immunization with the recombinant bacteria induced a specific immune response directed against these antigens. Furthermore, a recombinant S. typhimurium vaccine strain expressing the intact influenza NP was shown to elicit an anti-influenza cellular response25-27.

We have previously described the expression of the B-cell epitope HA91-108 in the flagellin protein of an Aro A mutant of S. dublin, and reported that intranasal vaccination with the isolated chimeric flagella led to neutralizing antibodies that could partially protect mice from viral challenge22.

In the present work, we report the expression of the NP epitopes NP55-69 and NP147-158 in the flagellin of S. dublin, and the cellular response induced by these chimeric proteins. We also describe the evaluation of the capacity of these products, alone or in combination with the previously described B-cell epitope HA91-108 construct, to protect mice from viral challenge infection. We demonstrate that immunizing with the triple combination led to efficient long-term (7 months) immunity as well as cross-strain protection.

Our data demonstrate the feasibility of using synthetic recombinant vaccines and provide evidence that simultaneous triggering of pathogen-specific CTLs, T helper cells and antibody-secreting B plasma cells is advantageous for effective protective immunity.

MATERIALS AND METHODS

Synthetic peptides

Peptides corresponding to influenza NP epitopes, namely NP55-69 (RLIQNSLTIERMVLS) and NP147-158 (TYQRTRALVRTGD), were synthesized in a 430A peptide synthesizer (Applied Biosystems). After cleavage from the resin, the peptides were purified on a Sephadex G-25 column. The purity of the peptides was evaluated by HPLC using a Supelco LC-308 column and peptide purity was found to be over 98%. The synthesis of peptide corresponding to influenza HA91-108 (SKAFSNCYPYDVPDYASL) was described previously25.

Animals

BALB/c female mice were obtained from Olac (Blackthorn, Bicester, Oxon, UK). The mice were used at the age of 8–12 weeks.

Viruses

Several influenza strains: A/Japan/305/57 (H2N2); A/Aichi/68, A/England/42/72, A/Texas/1/77, were all (H3N2) grown in the allantoic cavity of 11-day-old embryonated hen eggs (Kfar Bilu Hatchery, Israel), according to standard procedures26. The virus titre in the allantoic fluid was determined by haemagglutination assay29 and presented as haemagglutination units (HAU).

Preparation of oligonucleotides, recombinant bacteria and chimeric flagellins

The following synthetic oligonucleotides were prepared in a 380B Applied Biosystems DNA synthesizer.

(a) The oligonucleotides coding for the epitope NP55-69:

\[ \text{ATC CGT CTG ATT CAG AAT TCT CTG ACT ATT GAA CGT ATG GTC CTA TCT I R L I Q N S L T I E R M V L S} \]

(b) The oligonucleotide coding for the epitope NP147-158:

\[ \text{T Y Q R T R A L V R T G D} \]

Codon usage was according to the sequence of the flagellin gene, with minor modifications in order to create Ava II and EcoRV restriction sites in the two oligonucleotides. A stop codon was designed to prevent the formation of inserts which are in the wrong orientation.

The synthetic oligonucleotide coding for the peptide HA91–108 was prepared according to similar guidelines, as described in detail in a previous publication28.

The plasmid vector carrying the flagellin gene from S. munchen (pLS408) was kindly provided by B. Stocker of Stanford University, and was used for the expression of the influenza epitopes. The recombinant bacteria were prepared as previously described. Briefly, the recombinant plasmids were transformed into E. coli IM101 competent cells. Plasmids from positive colonies were purified and used to transform Salmonella typhimurium LB5000 competent cells and were then transferred to a flagellin negative live vaccine strain (an Aro A mutant) of S. dublin SL5928 by transduction using the phage P22HT 105/1 int. The transformed S. dublin were selected for Ampicillin resistance, motility under the light microscope and growth in semisolid 1 R agar plate20,28. The flagella comprising the hybrid flagellins were detached from the bacteria using acidic cleavage, as described by Ibrahim et al.40. The recombinant flagellin harboring the T helper epitope NP55-69 was denoted Fla-55, the flagellin carrying the CTL epitope NP147-158 was termed Fla-147 and the one carrying the epitope HA91 108 was denoted Fla-91. Fla-control stands for the native flagellin.

Immunization procedures

For determination of cellular immunity, BALB/c mice were immunized once at the base of the tail with either 10 µg synthetic peptides or 50 µg hybrid flagellins, emulsified in complete Freund’s adjuvant. In protection experiments, recombinant flagellins were administered to mice intranasally in 20–30 µl PBS, under light ether anesthesia. Mice immunized with Fla-control received 50 µg protein, whereas those immunized with the different chimeric flagellins received 25 µg of the...
corresponding recombinant protein. The schedule of immunization consisted of three administrations at 3-week intervals.

Proliferative response of Ag-specific T cells

The ability of cells to proliferate in vitro in response to incubation with the various peptides was monitored as previously described. Briefly, lymphocytes from the inguinal lymph nodes were removed aseptically 14 days after the immunization, and a single cell suspension was prepared in RPMI 1640 medium containing 2 mM L-glutamine, antibiotics, 5 x 10^{-5} M 2-mercaptoethanol, 1.5% FCS and 0.5% normal mouse serum. The cells were cultured in 0.2 ml in the presence of the antigens, and the proliferative response was evaluated 3 days later by pulsing the cells for 18 h with [3H]thymidine and monitoring the incorporated radioactivity. Each test was performed in triplicate.

Complement-mediated lysis

P815 target cells were labeled with 51Cr for 1 h at 37°C in RPMI+HEPES and simultaneously infected with influenza A/Texas/77, washed and suspended in the same medium at a concentration of 2 x 10^6 cells ml^{-1}. Serial twofold dilutions were prepared from the serum samples in RPMI 1640 medium containing 2 mM L-glutamine, antibiotics, 5 x 10^{-5} M 2-mercaptoethanol, 10% FCS, from which 50 μl were added to 50 μl cells suspension (10^5 cells tube^{-1}) and incubated for 30 min on ice with occasional shaking. Then, 10 μl of guinea pig complement (GPC) were added for 60 min at 37°C. The reaction was terminated by addition of cold PBS, the tubes were centrifuged for 10 min at 1000 revs min^{-1}, supernatant samples were collected and counted in a γ-counter (Riastar; Packard Instruments Company, Downers Grove, IL). For controls, the spontaneous and total release were monitored, as well as the background release induced by incubation of infected target cells with complement alone or serum samples alone. The release of uninfected targets incubated with serum and complement was also monitored.

Histological assay

The mice were sacrificed and their lungs were removed and fixed in Bouin’s solution (75% saturated picric acid, 20% formaldehyde and 5% glacial acetic acid) for 24 h, rinsed with H_2O and moved to 70% ethanol until processing. After dehydration, the lungs were embedded in paraffin blocks, 5 μm sections prepared and stained with modified trichrome Light Green stain (hematoxylin–eosin–phosphomolybdic acid). The slides were examined under a light microscope.

Viral challenge and virus titration

Viral challenge was performed by placing 1600 EID_{50} units of A/Aichi, A/England or A/Japanese or 12.5 EID_{50} units of A/Texas, respectively, in the nostrils of ether anesthetized mice (in 50 μl PBS). Three days after virus inoculation the animals were sacrificed, their lungs removed, and blood was collected. All lung samples were snap-frozen and stored at −70°C. Immediately prior to the assay, the samples were thawed, homogenized in PBS 0.1% BSA (10% w/v) and centrifuged in order to remove debris.

Virus titres were determined by injection of 100 μl of serial tenfold dilutions, from the individual lung homogenates, into the allantoic cavity of 11-day-old embryonated eggs (two eggs per dilution). Following 48 h incubation at 37°C and overnight at 4°C, allantoic fluid was removed and virus presence was determined by hemagglutination in microtitre wells (50 μl of the allantoic fluid and 50 μl of 0.5% RBC in saline). For each group, the virus titre dose that would give a mean proportional infectivity of 0.5 EID_{50} was calculated according to the moving average method developed by Thompson, as described in detail by Barrett and Inglis. The results are presented in log of the EID_{50} dose.

In lethal challenge experiments, 1250 EID_{50} units of A/Texas/77 were inoculated to the nostrils of the mice, and the survival rates as well as the weight of the mice were monitored daily.

Statistical analysis

The percent of virus-positive lungs from each group in every dilution was determined from the results of the haemagglutination assay that was used to calculate virus titre, as described in the previous section. The efficiency of the various vaccine preparations in reducing virus titre was ranked, when rank 1 was assigned to the most effective vaccine, and the highest rank to the least effective. A two-way analysis of variance on ranks was performed with these results, using a multicomparison test on Friedman’s nonparametric method. First, the null hypothesis of no treatment differences was examined, and once rejected, the different treatments were rated according to their performances.

RESULTS

We have previously demonstrated that intranasal immunization of mice with Fla-91—a recombinant flagellin containing the influenza B-cell epitope HA91–108—led to partial protection of mice from viral challenge. In an attempt to augment the protective effect by the addition of other influenza epitopes to the B-cell epitope, the two recombinant bacteria carrying a Th epitope (NP55–69, denoted Fla-55) or a CTL epitope (NP147–158, denoted Fla-147) were constructed, and the flagella expressing the two peptides were isolated as described above.

The ability of these epitopes to prime for a cellular response was evaluated by the injection of either free synthetic peptides corresponding to the three epitopes or the hybrid flagella carrying these epitopes emulsified in CFA, at the base of the tail of BALB/c mice, and monitoring the proliferation of lymphocytes from these animals in response to in vitro stimulation with the three synthetic peptides. The results, presented by thymidine incorporation (Table 1) indicated that NP55–69 was indeed a strong T-helper epitope, eliciting highly significant stimulation and that NP147–158 also elicited cellular response, albeit a weaker one. Similar results were obtained when the epitopes were presented as free peptides or as part of chimeric flagellins, which...
Table 1 Cellular response of mice immunized with synthetic influenza peptides or with recombinant flagellins

<table>
<thead>
<tr>
<th>Immunizing agent</th>
<th>c.p.m. response to stimulating synthetic peptides:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Medium</td>
</tr>
<tr>
<td>NP55-69 *</td>
<td>7036</td>
</tr>
<tr>
<td>HA91-108 *</td>
<td>11064</td>
</tr>
<tr>
<td>NP147-158 *</td>
<td>13753</td>
</tr>
<tr>
<td>Fla-control *</td>
<td>5432</td>
</tr>
<tr>
<td>Fla-55 *</td>
<td>5607</td>
</tr>
<tr>
<td>Fla-91 *</td>
<td>5586</td>
</tr>
<tr>
<td>Fla-147 *</td>
<td>9553</td>
</tr>
<tr>
<td>NP55-69 a</td>
<td>4631</td>
</tr>
<tr>
<td>HA91-108 a</td>
<td>17842</td>
</tr>
<tr>
<td>NP147-158 a</td>
<td>15963</td>
</tr>
<tr>
<td>Fla-control b</td>
<td>21714</td>
</tr>
<tr>
<td>Fla-55 b</td>
<td>5840</td>
</tr>
<tr>
<td>Fla-91 b</td>
<td>4865</td>
</tr>
<tr>
<td>Fla-147 b</td>
<td>12012</td>
</tr>
</tbody>
</table>

*Synthetic peptide corresponding to the sequence 55-69 of the nucleoprotein, reportedly a T-cell epitope. aSynthetic peptide corresponding to the sequence 147-158 of the nucleoprotein, reportedly an epitope. bFlagella cleaved from Salmonella vaccine strain. cRecombinant flagellins, expressing the sequences NP55-69, HA91-108 and NP147-158, respectively. The values considered significant are printed in bold numbers.

The local cellular immune response in the lungs was studied after intranasal immunization of BALB/c mice either with the individual hybrid flagella, or with their various combinations. The antigens (25 μg of each recombinant flagellin) were administered in PBS three times at 3-week intervals, following which the mice were sacrificed, their lungs removed and stained for histological evaluation, as described in Materials and Methods. Two groups were examined, 1 week and 2 months, respectively, after the last boost. One week after the last boost, all the mice exhibited massive perivascular and peribronchial lymphocyte follicular infiltration. In contrast, only mice vaccinated with the two T-cell epitopes, and particularly those given the combination of Fla-55+Fla-147 and Fla-55+Fla-91+Fla-147 had significant lymphocytic infiltrations also 2 months after the last boost, whereas mice receiving other combinations exhibited a histological picture very similar to that of untreated mice.

The protective effect of the recombinant flagellins was evaluated by vaccinating mice intranasally with Fla-55, Fla-91, Fla-147, individually and in various combinations, and subsequent challenge with A/Texas/77. As shown in Figure 1, when challenge was given 7 days after the last boost (light bars), some reduction in influenza titre was observed in animals vaccinated even with Fla-control. This effect might be due to antiflagellin activated cells, as supported by the observation of massive perivascular and peribronchial lymphocyte follicular infiltration in the lungs of these mice. A similar effect was obtained with the individual recombinant flagellins. The mixed preparations of Fla-55+Fla-91 and Fla-91+Fla-147 reduced the viral titre somewhat further, and the preparations consisting of Fla-55+Fla-147 or the triple combination, namely, Fla-55+Fla-91+Fla-147, exhibited the most effective protection. On the other hand, when the mice immunized with various combinations of hybrid flagellins were challenged one month after immunization (Figure 1, dark bars) the Fla-control lost its ability to reduce viral titre, so as some of the other preparations. The only preparations that showed appreciable protective capacity were Fla-91+Fla-147, and to a larger extent, the triple combination Fla-55+Fla-91+Fla-147, that reduced viral titre by 1.6 and 2.5 orders of magnitude, respectively.

In order to evaluate the capability of the vaccines to induce long-term memory and protection, groups of mice were immunized with Fla-control, Fla-91+Fla-147 or Fla-55+Fla-91+Fla-147 and were challenged with A/Texas/77 influenza virus 1, 4 or 7 months, respectively, after the last boost. The results, presented in Table 2, indicated that protective effect persisted at least 7 months after the last immunizing boost. The preparation containing all three epitopes was the most efficient, and it was apparent that the addition of the Th epitope to the other two, significantly augmented the protection.

Since the T-cell epitopes we used are from the internal viral nucleoprotein, it is most likely that antibodies against these epitopes will not inhibit haemagglutination and infectivity, but operate through other mechanisms, such as complement-mediated lysis. To look for such an effect, samples of serum, pooled from the different groups of the experiment that studied the protection seven months after the last boost, were tested for their ability to induce lysis of target cells infected with influenza virus in the presence of complement. The results (Figure 2) showed that serum from mice immunized with
Table 2 Duration of the protective immunity afforded by the recombinant preparations

<table>
<thead>
<tr>
<th>Immunizing agent:</th>
<th>1 month</th>
<th>4 months</th>
<th>7 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>time after last Fla-91+ Fla-55+ Fla-91+ immunization</td>
<td>Fla-control 10.56 10.31 10.07</td>
<td>Fla-91 10.17 8.91 9.49</td>
<td>Fla-91+Fla-147 8.86 7.3 9.2</td>
</tr>
<tr>
<td>titre in log EID,, , log EID,,</td>
<td>n.s.</td>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>Statistical significance</td>
<td>-0.39 1.76</td>
<td>-1.4</td>
<td>-</td>
</tr>
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</table>

*Significance level of difference from control, according to multicomparison test on Friedman's nonparametric method. n.s.: not significant.

The recombinant flagellin preparations was effective in inducing lysis of infected target cells, while noninfected cells were not lysed. The most effective lysis was obtained with serum from mice immunized with the triple combination, suggesting that antibodies to both NP and HA are involved in this effect.

The epitopes used in this study were conserved ones. HA91–108 is present in all A/H3 strains, and the NP is conserved in all A strains. Hence it was of interest to determine whether the use of these epitopes could induce cross-protection. BALB/c mice were immunized with Fla-control, Fla-91, Fla-91+Fla-147 or with the triple combination, and one month later challenged with one of three A/influenza strains: Two H3N2 subtypes (A/ Aichi and A/England) and one H2N2 subtype (A/ Japanese). As shown (Table 3), Fla-91 vaccination was protective only against H3 subtype viruses. The addition of Fla-147 (CTL epitope) to Fla-91 improved the protection of Fla-91 alone, and led to a protective effect also against the H2 strain. When the flagellin carrying the Th epitope (Fla-55) was added to the mixture, a further increase of the protective capacity was observed against the A/Aichi strain.

In the previous experiments the protective effect was manifested by reduction of viral titre. The next experiment evaluated the potential of the vaccine to protect mice against lethal viral challenge. Mice were immunized with the same preparations as above, and one month later the animals were inoculated with a lethal dose of A/Texas/77 (10−2 HAU) animal−1. Survival, as well as body weight, were recorded daily. As shown in Figure 3A, vaccination with the triple combination led to 100% survival, whereas immunization with Fla-91 and Fla-91+Fla-147 resulted in 10 and 67% survival, respectively, compared to 15% survival of the untreated control. The weight loss (indicating disease severity) of the surviving mice (Figure 3B) showed that mice immunized with the triple combination did lose some weight (less than 18% of their body weight) during the first week after the viral challenge, but resumed normal weight at 20 days after the infection. In contrast, animals from all other treatments continued to lose weight for an additional 34 days (up to one-third of their body weight) and did not regain their initial weight until 40 days after the challenge. This was also the case for the mice vaccinated with Fla-91+Fla-147, even though they exhibited 67% survival. The lungs of the surviving mice were examined histologically 45 days after the infection. Lungs from mice immunized with Fla-91, Fla-91+Fla-147 and the control group displayed
scattered focal inflammatory foci of granulocytes, lymphocytes and macrophages, necrotic exudate as well as abscesses and massive parenchymatic damage. In contrast, the lungs of mice immunized with the combination of the three epitopes appeared normal, with limited perivascular and peribronchial foci of infiltrating lymphocytes. These results suggest that local immunity in the lungs might have curtailed the viral spread and allowed rapid and efficient recovery.

In order to determine whether this protective immunity was long-lasting, mice were vaccinated with the triple combination and challenged 7 months after the last boost. The results demonstrated that 100% of the immunized mice survived, compared to 0% survival in the control group (Figure 3A, closed symbols), indicating that effective long-term protective memory was indeed established by this vaccine.

DISCUSSION

The purpose of this study was to develop and evaluate a novel approach to vaccination, based on the employment of a synthetic recombinant vaccine. Using influenza in mice as a model system, we investigated the prospect of such a vaccine to provide protection against viral challenge. In a previous paper we described a chimeric vaccine based on the employment of flagellin, which acted both as a carrier and as an adjuvant, and hence no external adjuvant was required. These properties of the flagellin vaccine may be interpreted in view of previous reports that this protein encompasses several T-helper epitopes, which implies that it should induce activated T cells. Indeed, our results indicate a massive lymphocyte infiltration in the lungs of mice immunized with the Fla control seven days after the last boost. These responses could account also for the low levels of protection observed in this control group at that time (Figure 1), which were also reported in a previous publication, but did not persist for more than a few weeks.

These results suggest that for long-term reactivity it is important to induce a good cellular response, that is protective on its own, and probably optimizes the humoral response as well. Although it was considered the role of Th cells to deliver help to B cells in the production of antibodies and for CTL activation, it was demonstrated here that activated B cells can also deliver help to antiviral CTLs and that even anti-influenza HA-specific IgG Abs enhance killing. Thus, the results obtained in this study further demonstrate that eliciting a multicomponent immune response results in a synergistic protective effect, which is in agreement with the model proposed by Liu and Müllbacher that argued for reciprocal help delivered between the different subpopulations of lymphocytes.

The route of administration employed throughout this study was the intranasal one. This is based on the presumption that it will be effective in inducing local response in the lung and nasal airways, which are the foremost targets in influenza infection. Indeed, resistance to infection in humans has been correlated with anti-HA in nasal washings of either the IgG or the IgA...
isotypes 42, 43. It was also reported for several respiratory tract diseases, including influenza, that protection was correlated with increased respiratory tract secretory-IgA (s-IgA) 44–50, and that intranasal vaccination induces local IgA memory 31–33. It is noteworthy that an advantage of the intranasal administration, in addition to its high efficacy, is the ease of application, which is an important consideration in vaccine development.

One of the most acute problems related to influenza vaccines is the narrow range of their specificity and their restricted strain-specific activity. The rapid variation in the viral surface antigens, the haemagglutinin and the neuraminidase, denoted shifts and drifts, leads to multiple strains with high variability in their serospecificity, and thus limits the efficacy of the currently existing vaccines which are based on these two proteins. The HA epitope used in this study is a conserved region in all H3 subtypes of influenza A virus; the NP is highly conserved in all influenza A strains. Consequently, the vaccine combining all three influenza epitopes was effective against three different H3 influenza strains, as well as an H2 strain (Figures 1 and 3 and Table 3). The most significant protection was against the H3 strains, as was expected, since the B-cell epitope (HA91–108) is found on the HA of these viruses, and it was already demonstrated that antibodies directed against this epitope are involved in protection from influenza challenge 38. The protective response against the H2 subtype was most likely mediated by T-cell activity, and possibly by complement-mediated lysis.

Another parameter to be concerned with is the duration of immunity. The vaccine described in this paper was shown to induce both humoral and cellular immunity, local and systemic, with long-term memory. This was manifested by (1) the complement-mediated lysis, in response to viral inoculation (Figure 2); (2) the reduction of viral titres after challenge infection (Table 2); and (3) the ability of the immunized mice to survive lethal challenge (Figure 3A), all persisting seven months after the last boosting dose.

The survival of mice after a lethal dose challenge is the ultimate demonstration of the protective effect. Under these conditions, in which the challenge dose is orders of magnitude higher than that pertaining in natural infection, all the exposed mice are infected regardless of their immune status. However, whereas none of the control mice can survive infection of this severity, the immunized protected mice recuperate. The findings that Fla-91 alone did not provide protection and that only combinations containing Fla-147 were effective, emphasize again the role of CTL in the immune process leading to protection. Furthermore, although the combination Fla-91+Fla-147 was quite effective, leading to 67% survival, a significant difference in its efficacy from the triple combination (Fla-91+Fla-55+Fla-147) was observed, not only as compared to the 100% survival achieved with the latter, but also in the efficiency of the recovery process, as evident from the weight loss (Figure 3B) and lung damage experiments. These data indicate the role of the T-helper epitope 55–69 in the induction of protection from viral challenge.

In conclusion, our findings demonstrate the potential of a synthetic recombinant vaccine to induce local and systemic immunity, resulting in effective protection against influenza, which is cross-strain specific and of long-term duration.

ACKNOWLEDGEMENTS

The plasmid vector pLS408 was kindly provided by Professor B. A. D. Stocker from Stanford University. We wish to thank Dr Claude Leclerc, from the Pasteur Institute, for her help in the lymphocyte proliferation assay, and Dr Asher Mesheorer, from the Weizmann Institute of Science, for his help in the analysis of the histological sections.

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