Intranasal immunization with synthetic recombinant vaccine containing multiple epitopes of influenza virus

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1. Introduction

Infection with influenza virus results in an array of specific immune responses to their host. The first line of defense against influenza infection consists of neutralizing antibodies. Although antibodies are not essential for healing, they probably have a beneficial effect in the recovery process [1]. Influenza infection was shown to elicit antibodies against most influenza proteins [2], but those most significant for their protective capacity are the anti haemagglutinin (HA) antibodies. The neutralizing effect of anti-HA antibodies can be attributed either to the prevention of viral entry into susceptible cells by the blocking of virus binding and internalization, or to their acting on later stages of viral replication [1]. Resistance to influenza infection was shown to correlate with serum anti-HA antibodies levels [3,4] and passive transfer of immune serum provides protection against further challenge [5]. However, antibodies to HA are usually strain specific and offer little or no protection against heterotypic infection [6–8].

Cytotoxic T lymphocytes (CTLs) constitute the second line of defense against influenza. Since CTLs can recognize viral antigens only when they are presented by major histocompatibility complex (MHC) class I molecules on the surface of infected cells [9], they cannot prevent the infection. However, they contribute significantly to the process of recovery and viral clearance [10,11]. CTLs are produced against most of the influenza proteins [1], but it is known that the nucleoprotein (NP) is the major target for CTL response [12], which protects mice from viral challenge [11,13,14]. It has also been reported that anti-NP CTLs, that were able to protect mice from viral challenge, were generated by injection of plasmid DNA encoding the influenza A NP into the quadriceps of BALB/c mice [15].

CD4+ helper T-cells (Th) are also involved in immunity against influenza, as it has been established that they are important for efficient antibody formation [1,9]. Th cells are directed against internal proteins and act efficiently as help to B-cells that produce anti-HA antibodies [1,9]. CD4+ cells can also be helpers to CD8+ CTLs; however, this phenomenon is less clear than the help of Th cells to B-cells [9].

In our laboratory, we have investigated several influenza epitopes (HA91–108, NP55–69 and NP 147–158), each stimulating a different arm of the immune system, for the design of a synthetic vaccine [16,17]. In earlier studies, we showed that the 18-residue peptide corresponding to the sequence of the HA region 91–108 (HA91–108) is a very...
effect of epitope [18,19]. Since this region is common to all
H3 strains [20], the peptide elicited antibodies in mice which
reacted with the intact influenza virus of several type A H3
strains. In addition, the mice immunized with this peptide
were partially protected against challenge infection with
influenza virus [18,21]. Recently, we found that only the
91–97 residues are exposed in the 3D structure of HA. The
Th and CTL epitopes from NP (NP55–67 and NP147–158,
respectively) induce MHC-restricted immune responses
[22–24]. The H-2K<sup>d</sup>-restricted peptide NP147–158 was
employed in our studies, but according to several re-
ports, the actual epitope is the nonapeptide 147–155
[25,26].

Synthetic vaccines based on peptides which represent im-
munogenic epitopes hold several advantages over the tra-
ditional vaccines. They are chemically well-defined stable
antigens and are free of any hazardous effects. However,
they are usually poor immunogens and require appropriate
carriers in order to be effective. One of the efficient delivery
systems utilizes the flagellin gene of a Salmonella vaccine
strain [27,28]. We have previously described the evaluation
of the above epitopes when expressed in a chimeric flagellin
protein [17]. These peptide-based recombinant vaccines in-
duced efficient cross-strain protection and long-term immu-
nity against influenza virus in mice [29]. These success-
ful results were obtained by intranasal immunization with
recombinant flagellin without the aid of adjuvant [17,30].
Furthermore, the combined use of B- and T-cell epitopes
administered as a mixture of recombinant flagella, each ex-
pressing individually one epitope, significantly improved the
protective efficacy against viral infection, indicating the syn-
ergetic effect of priming both arms of the immune system
[16,17,29].

In the present study, we have constructed two versions
of a single recombinant plasmid which encodes these three
epitopes of influenza virus. One is a short version comprised
of the three epitopes spaced by two short segments from the
flagellin sequence, while in the second, the three epitopes
are inserted in the whole flagellin gene. We investigated the
efficacy of the resultant peptide constructs, for induction of
protective immunity against influenza.

2. Materials and methods

2.1. Mice and viruses

BALB/c mice at the age of 10–12 weeks were purchased
from Harlan Laboratories (Rehovot, Israel). Influenza strains
A/Texas/1/77 (H3N2) were grown in the allantoic cavity of
11-day-old embryonated hen eggs (Bar On Hatchery, Hod
Hasharon, Israel). Virus growth and purification were ac-
cording to standard method described by Barret and Inglis
[31]. Titration of virus in the allantoic fluid was performed
by the haemagglutination assay using 50 µl of allantoic fluid
serial dilutions and 50 µl of 0.5% chicken RBC in saline.

Titers were expressed as haemagglutination units (HAU).

For the construction of the plasmid containing the three
epitopes, namely, HA91–97 (SKAFSNC), NP55–69
(RLIQNLSLIERMVL), and NP147–155 (TYQRTRALV), we have prepared recombinant plasmid containing H-1<sup>d</sup>
flagellin gene from S. muenchen, in which the internal
EcoRV fragment (H-1<sup>d</sup> 211–228 residues) was replaced
with NP55–69 epitope [17]. This plasmid was then used as
a template for the PCR with the primers 1 (Fig. 1) which
encode for the flagellin H-1<sup>d</sup> 185–189 (5’ TCC AGC AAC
TGT CCG AAA GAA ACT GT 3’) and H-1<sup>d</sup> 294–298 (5’
GTT CCG CTG ATA AGT ATC AAC ACC CTC T 3’)
resides. The second PCR was performed using the two
primers (primers 2 in Fig. 1) encoding HA91–97 (5’ AAG
TCT AGC AAG GCT TTC AGC AAC AAC TGC TGT 3’) and
NP147–155 (5’ TGC AAC AGC TAA AGC ACG GGT
CCG CTG ATA 3’), which were overlapping with the first
primers. The resultant PCR product, denoted pHNN, was
confirmed by DNA sequencing analysis and cloned into the
pQE31 plasmid (Qiagen, Beer Sheva, Israel) for overex-
pression of their gene products (pHNN). The overexpressed
HNN peptide in E. coli by IPTG induction was purified
with Ni-NTA column (Qiagen) using their N-terminal 6-
histidine tag.

In addition to the pHNN, a second construct was prepared,
in which the three epitopes were expressed in the entire flag-
ellin molecule: the HindIII/PstI fragment of original flagellin
H-1<sup>d</sup> gene (pLS408) [27] was replaced by inserting with the
above PCR product containing three epitopes and the result-
tant plasmid was designated as pFla-HNN. This plasmid
was transferred to a flagellin negative vaccine strain (an Arora
mutant) of S. dublin SL5928 as described previously [17].
The Flagella comprising the hybrid flagellin were detached
from the recombinant bacteria using acidic cleavage, as de-
scribed by Ibrahim et al. [32]. The purity of isolated peptide,
denoted Fla-HNN, was assessed by SDS-PAGE.

2.3. Immunization and infection procedures

Groups of 8–10 mice were immunized intranasally (i.n.)
with the each of the peptides. An amount of 50 mg per animal
in 50 µl PBS were administrated to the nostrils of mice
lightly anesthetized with ether. The mice were boosted twice,
at 3-week intervals, using the same amount of antigen as for
the initial immunization. Infection of mice was performed by
inoculating i.n. infectious allantoic fluid containing 1 HAU
virus per mouse under light ether anesthesia, 1 month after
the last booster.
Fig. 1. Structure of HNN and Fla-HNN recombinant peptides containing three epitopes of influenza virus. (A) H-1d is the flagellin gene from S. munchen, and the nucleotide sequences were designed to code for epitope 91–97 of influenza A H3 subtype HA, and epitopes 55–69 and 147–155 from NP. Arrows indicate PCR primers shown in materials and methods. Primers 1: H-1d 185–189 and H-1d 294–298; 2: HA91–97 and NP147–155. (B) Purified peptides were analyzed by 15% SDS-PAGE. Lanes M: size marker; 1: HNN; 2: intact flagellin; 3: Fla-HNN.

2.4. Lung homogenates and serum preparation

In order to measure the production of specific anti-influenza antibodies by the immunized mice, the supernatant fluid from lung homogenates (suspended in 0.1% BSA in PBS) were collected for determination of the levels of IgA antibodies, as well as for HA assay. The samples were frozen in aliquots and kept at −70°C until use. Sera were prepared from the blood collected 3-weeks after the second and third immunizations and were assayed for their IgG antibody levels by ELISA.

2.5. Enzyme-linked immunosorbent assay (ELISA)

ELISA was used to assay for specific IgA or IgG antibodies, respectively, in lung homogenates and sera. The desired antigen was absorbed to ELISA microtitre plates (Immunoplate, Nunc, Denmark) overnight at 4°C in PBS. Allantoic fluid containing A/Texas/a/77 virus (100 HAU/ml) was added in aliquots of 100 µl well buffer and incubated overnight at 4°C. The plates were washed four times with PBS containing 0.1% Tween-20 (PBS-Tween). The wells were then blocked with 1% BSA in PBS for 90 min at 25°C, and washed with PBS-Tween. Serial dilutions of the different samples containing the antibodies as specified were added and incubated for 2 h at 37°C, and unbound antibody was washed off with PBS-Tween. Goat anti-mouse IgA or IgG antibodies, conjugated to horseradish peroxidase (HRP; Jackson Laboratories) were used as second antibodies. 3,3′,5,5′-tetramethylbenzidine (TMB, ZYMED, San Francisco) was added as substrate. Following the addition of the substrate (100 µl/well), the plates were incubated at 37°C until color was produced (5 min). The reaction was terminated by adding 50 µl of 1N HCl and the intensity of color was subsequently determined at 450 nm by an ELISA reader (Multiskan MCC/340 MK II, Lab System, Helsinki, Finland).

2.6. Splenocyte proliferation assay

BALB/c mice were immunized with 50 µg/50 µl of either HNN, Fla-HNN or Fla peptides. The spleen was dissected
14 days after third immunization and proliferative response to the each peptide was tested. The cells were cultured in 96-well flat-bottomed plates using triplicates of 0.2 ml cultures containing 5 x 10^5 cells/well in RPMI-HEPES. Splenocytes were stimulated with different concentrations of the various peptides or inactivated purified virus and cultured for 48 h. The cells were pulsed with 1 mCi (37 Bq) of \(^{3}H\) thymidine overnight. The thymidine incorporation was determined in a Packard \(\beta\)-counter.

2.7. Cytokine assay

All the antibodies required for the ELISA assay as well as the purified cytokines were obtained from Pharmingen (San Diego, CA). The purified anti-cytokine capture mAbs diluted to 2 \(\mu\)g/ml (rat anti-mouse IL-4) or 4 \(\mu\)g/ml (rat anti-mouse IL-2, IL-10, and IFN-\(\gamma\)) in carbonate buffer (0.1 M NaHCO\(_3\), pH 8.2) were adsorbed to ELISA plate, and incubated for overnight at 4 \(^\circ\)C. After wash with PBS-Tween, the plates were blocked with PBS/10% fetal calf serum at 200 \(\mu\)l per well for 2 h at room temperature. Standard and diluted samples were added to wells and incubated for overnight at 4 \(^\circ\)C. Plates were washed and biotinylated anti-cytokine detecting mAb in PBS/10% serum was added to each well for 1 h. The peroxidase-conjugated avidin was used as secondary antibody. The following steps for determination were the same as described in ELISA. The cytokines were quantitated by comparison with a standard curve of purified cytokines captured and detected as before.

2.8. Protection assay against viral challenge

Immunized mice received an i.n. inoculation of infectious allantoic fluid, and were sacrificed 5 days later. Lungs and blood samples were stored at \(-70^\circ\)C. Immediately prior to the assay, lungs were thawed, homogenized in PBS 0.1% BSA (10% w/v) and centrifuged in order to remove debris. Virus titers were determined by the whole egg titration method [33]. In brief, lung homogenates (100 ml of 10-fold serial dilutions) were injected into the allantoic cavity of 9–11 days old embryonated eggs. Following incubation for 48 h at 37 \(^\circ\)C and overnight at 4 \(^\circ\)C, allantoic fluid was removed and virus presence was determined by haemagglutination assay, in micro-titer plates containing 50 \(\mu\)l allantoic fluid and 50 \(\mu\)l 0.5% chicken erythrocytes in saline. The results of these assays are presented as log EID\(_{50}\) [31]. To examine the ability of immunized mice to resist lethal challenge, the group of immunized mice, as well as control groups, were inoculated with LD\(_{50}\) (400 HAU) of A/Texas/77 virus and their body weight as well as survival rates were monitored.

2.9. Statistical analysis

Statistical analysis was performed using the Stat-View II program (Abacus Concepts, Berkeley, CA). Fisher PLSD test was utilized to calculate probability (\(P\)) values. Results are presented as mean and S.E. of at least two repeated independent experiments.

3. Results

3.1. Cloning and purification of HNN and Fla-HNN

To investigate the effect of combined immunization of the B- and T-cell epitopes of influenza virus HA91–97, NP55–69 and NP147–155, the plasmid containing these three epitopes (pHNN) was constructed as shown Fig. 1. The PCR was performed using overlapping primers of epitopes and hypervariable region of flagellin H-1\(^{d}\) gene (185–211 and 228–298 amino acid residues) from Salmonella muenchen. The resultant PCR product encoding the recombinant HNN peptide was cloned into pQE31 vector (pHNN) for expression in E. coli, or into plS408 vector [27] for expression within the original flagellin gene of Salmonella vaccine strain (pFla-HNN) as described in Fig. 1A. The purified HNN and Fla-HNN peptides, subjected to SDS-PAGE, were of 17, and 56 kDa, respectively (Fig. 1B). The additional bands, smaller in size than the 56 kDa (Fig. 1B, lane 3), could represent some degraded forms of the Fla-HNN, due to the instability of this protein.

3.2. Humoral immune responses of recombinant HNN and Fla-HNN

BALB/c mice were immunized with purified HNN, Fla-HNN or native Flagellin (Fla) i.n. (50 \(\mu\)g/mouse) three times at 3-week intervals. The mice were bled 3 weeks following the third immunization and the samples of each group were pooled together. Serum IgG levels were assayed by ELISA for the presence of anti-peptide or anti-virus antibodies (Fig. 2). The Fla-HNN peptide elicited higher IgG antibodies against influenza virus, while HNN peptide induced relatively low antibody production (Fig. 2D). All the anti-sera recognized their own respective homologous peptide, and Fla and Fla-HNN peptides, showed high cross-reactivity between them (Fig. 2B and C). However, there is no cross-reactivity between HNN and Fla-HNN even though they share common epitopes of both the influenza and the flagellin.

Similar results were obtained concerning the lung IgA production (Fig. 3). However, in this case, the cross-activity between Fla and Fla-HNN was not so significant, although they recognize their own homologous peptide as well as the HNN peptide (Fig. 3B and C). In addition, the overall anti-viral recognition by all lung homogenates was low (Fig. 3D) compared to the IgG titre (Fig. 2D).
Fig. 2. Immunogenicity of HNN peptides demonstrated by binding in ELISA of IgG in sera of mice immunized i.n. with: HNN peptide (■); Fla-HNN peptide (●); native flagellin (○); or non-immunized mice (×). The antigen coating the ELISA plates were: HNN (A); Fla-HNN (B); flagellin (C); or A/Texas/77 influenza virus (D).

Fig. 3. Production of IgA antibodies against HNN (A); Fla-HNN (B); Fla (C); peptide and A/Texas/77 virus (D); following i.n. immunization of mice with each peptide as determined by ELISA in lung homogenates. Binding of anti-HNN peptide antiserum (■); anti-Fla-HNN peptide antiserum (●); anti-Fla peptide antiserum (○); and serum from non-immunized mice (×).
3.3. Cellular immune responses of the mice immunized with HNN

The two recombinant products were evaluated for their ability to prime T-helper activity by measuring thymidine incorporation in spleen cells of BALB/c mice immunized with the various constructs. The splenocytes were incubated in vitro with their respective homologous peptide (20 μg) or influenza virus (100 HAU). As shown in Fig. 4, each of the two peptides as well as the native flagella led to significant proliferation when stimulated with the homologous antigen (Fig. 4, left). Upon stimulation with the virus, however, only cells from mice immunized with the constructs containing the influenza epitopes (HNN and Fla-HNN) induced proliferation (Fig. 4, right panel).

These results are corroborated, even more clearly, by the cytokine secretion assay (Fig. 5). As shown, spleen cells from mice immunized with either HNN or Fla-HNN release significant levels of IL-2 and IFN-γ in response to the intact influenza virus. Thus, immunization with HNN and Fla-HNN induced Th1 type-cytokine release which is related to the antibody-dependent cell mediated cytotoxicity and clearance of infected cells. In contrast, the cells did not secrete IL-4 or IL-10 which represent a Th2 response (data not shown). The mice immunized with native flagellin or the non-immunized mice did not show any response.

![Fig. 4](image1.png)

Fig. 4. Proliferation of spleen cells from mice primed with various peptides in response to in vitro stimulation with each peptide or viral particles. The proliferation was monitored by thymidine uptake and represented as stimulation index compared to media control.

![Fig. 5](image2.png)

Fig. 5. Cytokine secretion by spleen cells in response to influenza virus stimulation. Mice were immunized by the various antigens three times at 3-week intervals and their spleen cells were stimulated in vitro with inactivated influenza virus. Results are performed as mean cytokine concentrations quantitated by comparison with a standard curve of purified cytokines detected as mentioned in materials and methods.
Fig. 6. Protection against sublethal influenza virus challenge infection of mice immunized with various peptides. The mice were challenged 4 weeks after the last immunization, and sacrifice 5 days later. The protective effect was shown as log EID₅₀. *: indicates a statistical significant difference ($P < 0.05$).

3.4. Protection conferred by vaccination with HNN

We have also investigated the capacity of HNN and the Fla-HNN to confer protective immunity against viral challenge. Following intranasal immunization, the mice were challenged with 1 HAU of A/Texas/1/77 influenza virus 1 month after the last boost. Five days later, the mice were sacrificed and the titre of infectious virus in the lungs was determined (Fig. 6). Only the mice immunized with HNN peptide showed protection against viral challenge. Fla-HNN peptide did not lead to reduction in virus titre even though, as shown in Fig. 3, it led to a significant level of lung IgA antibodies cross-reactive with intact virus, as well as to anti-viral cellular immune response (Fig. 4).

In view of the observation described before that only the HNN induced protective immunity against sub-lethal dose infection, we examined the ability of HNN immunized mice to resist also a lethal dose challenge. Groups of mice immunized with HNN and a control group were inoculated with LD₅₀ (400 HAU) of the virus and their body weight loss and survival rate were monitored for 3 weeks after challenge. The mice immunized with HNN peptide showed 100% survival compared to only 40% survived in the control mice (Fig. 7, right). In addition, the immunized mice lost less body weight and rapidly started gaining weight, which is an indication for a recovery process that began already 8 days after the viral challenge (Fig. 7, left).

4. Discussion

The flagella, a multimerized form of flagellin gene product of Salmonella, have been previously used in our laboratory to express influenza epitopes. Using recombinant methods, the B-cell epitope (HA91–108) from the HA as well as the T-helper (NP55–69) and CTL (NP147–158) epitopes from the NP were individually expressed in the flagellin [17]. To evaluate the ability of such a synthetic recombinant peptide-based vaccine, each of these flagellin constructs, or a mixture of all three, was used for intranasal immunization, followed by a challenge infection. The combined vaccine, comprising the three epitopes, induced long-term immunity and fully protected mice from a lethal challenge [17]. It should be noted that the recombinant flagella expressing the B-cell epitope alone did not provide significant protection, thus emphasizing the role of T-helper and CTL activity in the immune process [17]. These results indicate that stimulation of the different arms of the immune system is required for effective anti-influenza response and protection against challenge infection.

In the present study, we have tried to express all three epitopes in a single recombinant product and have investigated its efficacy for vaccination. For this purpose, we have constructed two types of plasmids, one encoding a short flagellin peptide (HNN) containing the three epitopes spaced by short segments of the flagellin, and the other—a long recombinant flagellin protein (Fla-HNN) encompassing the
HNN peptide in its correct internal position within the flagellin molecule (Fig. 1A). The purified HNN and Fla-HNN peptides were of 17, and 56 kDa, respectively, as expected (Fig. 1B). The HNN peptide was highly expressed in E. coli and easily purified. In contrast, the expression of Fla-HNN peptide was very low due to the structural abnormality and instability caused by inserting three foreign peptides in a relatively small molecule such as the flagellin. Moreover, the Salmonella expressing Fla-HNN lost their mobility, indicating the possibility that the flagella may have not formed properly.

Both recombinant products elicited significant humoral response against the respective homologous antigen. But, as regards the recognition of the intact influenza virus, the HNN peptide induced relatively low antibody level while the Fla-HNN construct elicited higher IgG response against the virus (Fig. 2D). Interestingly, even though HNN and Fla-HNN share common epitopes of the flagellin, there is no cross-activity between them. These results indicate that these short fragments of flagellin, namely Fla185–211 and Fla 228–298 of HNN (Fig. 1) are not efficient for B-cell induction. Similar results were obtained concerning the IgA production (Fig. 3), though the overall anti-viral activity of all lung antibodies was low compared to the IgG. It is expected that most of the antibodies are directed towards the B-cell epitope HA91–108, since the two NP epitopes are a T-helper and CTL-inducing epitopes, respectively (17). The most interesting point, however, is that these antibodies, induced by a single construct, encompassing the three epitopes, recognized the intact influenza virus.

Each of the two peptide products led to significant cellular immune response manifested in cell proliferation (Fig. 4), as well as Th1 type-cytokine (IL-2 and IFN-γ) release (Fig. 5), when stimulated with the virus. It is well known that IL-2 and IFN-γ are involved in the immune response induced by influenza virus [34,35]. The antiviral effects of IFN are mediated by induction of a set of IFN-stimulated genes which interfere with viral replication [36–38]. These results suggest that immunization with HNN and Fla-HNN peptides induces the (antibody-dependent) cell mediated cytotoxicity that could be instrumental in the clearance of infected cells and in the recovery from infection.

The recombinant peptide-based vaccine described in this paper was shown to induce both humoral and cellular immunity. More significantly, intranasal immunization with the HNN peptide reduced viral titres after challenge infection (Fig. 6) and endowed the immunized mice with the ability to survive a lethal challenge (Fig. 7). However, our findings suggest that the linking of multiple epitopes may be less protective against a sublethal dose of influenza infection when compared to the combined use of a mixture of flagellins expressing each epitope individually [17]. This is the observation with both the shorter construct HNN and the longer construct Fla-HNN which consists of the entire flagellin molecule with the three inserted influenza epitopes. In the case of HNN, this may be due to the insufficient capability of the short segments of flagellin to induce B-cell immune response (Figs. 2 and 3). Fla-HNN, however, did not lead to a reduction in virus titre even though it conferred good immunogenicity and led to antibodies cross-reactive with intact virus. It also induced significant cellular immunity. Hence, it is difficult to explain its low protective effect. 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 prevalent in natural infection, all the exposed mice are infected regardless of their immune state. However, whereas only 40% of the control mice survived infection of such severity, the immunized mice with HNN were completely protected. Our results indicate that the recombinant HNN peptide shows similar efficacy of protection to that observed after vaccination with the mixture of the three flagellins each expressing one epitope [17].

The role of Th cells in delivering help to B-cells for the production of antibodies and for CTL activation has been the subject of intense study (e.g. [39,40]). In addition, it has been suggested that activated B-cells can also deliver help to antiviral CTLs [41] and that even anti-influenza HA-specific IgG antibodies enhance killing [42]. The results obtained in this study provide further support to this notion, which is in agreement with the model produced by Liu and Müllbacher that argued for reciprocal help delivered between the different subpopulations of lymphocytes [43].

The future peptide-based vaccines will probably contain a mixture of several epitopes representing both B- and T-cell epitopes, overcoming the problem of hypervariable sequences of some viral proteins and MHC restriction. Although improvement is certainly needed, the possibility of using such a recombinant peptide vaccine containing multiple epitopes in a single construct, as employed in the present study, paves the way for the development of more efficacious synthetic vaccines in the future.

References


