Preparation and properties of a novel influenza subunit vaccine

H. BACHMAYER
Ph.D.

E. LIEHL
Ph.D.

G. SCHMIDT*
Ph.D.

Sandoz Forschungsinstitut Wien, Vienna, Austria

Summary
Haemagglutinin and neuraminidase have clearly been shown to be responsible for the stimulation of immunity against influenza.

In the approach described, these two immunogens are selectively solubilized, leaving an intact subviral particle which contains lipid and non-protective components. The methods used can readily be adapted to large scale production.

The immunogenicity of such antigen-preparation was studied in different animal species. Antibodies against haemagglutinin and neuraminidase were measured in mice, hamsters, guinea-pigs and rabbits. In addition, mice-protection experiments were performed.

The immune response of volunteers to this type of subunit vaccine has also been evaluated (Kunz and Hofmann, 1975). Results of a first field trial indicate that the solubilization procedure did not alter the immunogenic properties of the two antigens.

Introduction
Killed influenza vaccines currently in use contain either whole virions or a mixture of antigens obtained by disrupting the virus particles with detergents or organic solvents (Perkins and Regamey, 1973; Perkins, Regamey and Hennessen, 1975).

It is well established that antibodies against two of the viral proteins, haemagglutinin and neuraminidase, give optimal protection against influenza (Dowdle et al., 1973). None of the other viral proteins, which are located beneath a host-derived lipid membrane, is essential for immunity. An ideal influenza vaccine should, therefore, contain only these two essential immunogens, since the other protein and lipid antigens may cause undesired side reactions.

All methods for the preparation of purified influenza immunogens ('subunits') described so far utilize as a first step a more or less complete disruption of the virus particle (Laver, 1973). Thus, these procedures set free all the components, namely proteins, lipids and nucleic acids, of the virion.

A different approach was utilized in this study. The possibility was investigated of selectively solubilizing the haemagglutinin and neuraminidase from the virus particle. The pronounced difference in size and density of the components would allow the use of simple separation procedures.

This approach of 'selective' solubilization, as contrasted to 'complete' solubilization, is illustrated in Fig. 1.

Materials and methods
Viruses
Egg-grown viruses purified by zonal ultracentrifugation were used throughout these studies. The prototype strains were obtained from G. C. Schild (WHO World Influenza Centre, London).

Animal studies
Immunization schedule: Equal doses of vaccines (approx. 1400 i.u./ml A/Port Chalmers/73 whole virus and subunits) given on day 0 and day 28, blood samples drawn on day 28 and day 35. Route of immunization: i.p. for mice and hamsters, s.c. for guinea-pigs, s.c. and i.m. for rabbits.

Some of the vaccines studied contained 0.15% Al(OH)₃ as adjuvant.

For protection experiments thirty mice in each group were immunized (0.25 ml i.p.) with vaccines containing influenza A/Hong Kong/68 (X-31 recombinant, diluted to HA titres of 1:32) in 0.2% Al(OH)₃ as adjuvant. Animals were challenged with mice-adapted A/Hong Kong/68 virus (10³⁵ LD₅₀).

Further experimental details are either given in the legends to the figures or will be included in separate publications on the biochemical (Bachmayer, 1975), immunological and human field studies.

Results and discussion
Influenza virus labelled with ³²P phosphate during

* Present address: Sandoz AG Nürnberg, GFR.

Correspondence: Dr Helmut Bachmayer, Sandoz Forschungsinstitut, Brunnerstrasse 59, A-1235 Wien, Austria.
The radioactivity label is located completely within the 'core' of the virus particles. The surface projections with haemagglutinin and neuraminidase activities are devoid of radioactivity. The radioactivity is distributed between the phosphate groups of the ribonucleic acid and phospholipid components of the viral membrane.

The screening for detergents suitable for a selective removal of haemagglutinin and neuraminidase was performed in the following way: 32P-labelled virus was incubated with different concentrations of selected detergents. A control sample contained virus mixed with phosphate-buffered saline (PBS) instead of the detergent.

After a certain period of time (usually 30 min at room temperature or overnight at 4°C) aliquots of the incubation mixture were layered on to preformed density gradients. Rate zonal centrifugation was used to separate fast sedimenting intact virus particles or subviral 'cores' from slowly sedimenting solubilized material.

The fractions obtained were analysed for haemagglutinin and neuraminidase activities and their radioactivity determined.

The results obtained with different concentrations of the non-ionic detergent Neodol 23–6 (Shell) are illustrated in Fig. 2. Panel (a) shows the untreated control sample: haemagglutinin, neuraminidase and 32P radioactivity are located in a single peak representing the whole virus position.

A change in the distribution of the biological activities and radioactivity can be observed in the experiments shown in panels (b), (c) and (d): haemagglutinin and neuraminidase are solubilized completely if the detergent concentration is raised to approximately 0.2% (d). However, 32P radioactivity is also solubilized quantitatively under these conditions. Ten and 100-fold lower concentrations of the same detergent give intermediate results (c and b, respectively). Only part of the biological activities can be solubilized under conditions where the virus 'core' is still intact. At a Neodol 23–6 concentration of 0.02%, (c) haemagglutinin and neuraminidase as well as radioactivity are partially solubilized. Using even lower concentrations of detergent (0.002%) it seems to be possible to detach most of the biological activities from the lipid containing virus core.

However, the haemagglutinin and neuraminidase are highly aggregated and sediment almost as fast as the intact virus particle.

Results very similar to the ones just described are obtained when Neodol 23–6 is substituted by other non-ionic detergents or by anionic detergents such as sodium deoxycholate or sodium dodecyl sulphate. The haemagglutinin and the neuraminidase are either only partially released from the virus or—at higher detergent concentrations—the virus particle is further degraded as indicated by solubilization of 32P radioactivity.

In the course of our comparative studies with different groups of detergents, the use of cationic detergents was also investigated. The use of this group of surfactants for the solubilization of viral components has not been described so far. The results obtained on incubation of 32P-labelled influenza virus with different concentrations of hexadecyltrimethyl ammonium bromide (cetyl trimethyl ammonium bromide, CTAB) are given in Fig. 3.

At the highest concentrations used, haemagglutinin and neuraminidase are solubilized together with 32P label (Fig. 3d). The lowest concentration studied gives only partial release of haemagglutinin and neuraminidase, still highly aggregated (Fig. 3b). So far, the results parallel the observations made with the other group of detergents as described before.
However, with CTAB it was possible to find an intermediate surfactant concentration which allowed the selective solubilization of haemagglutinin and neuraminidase activities from the intact subviral particle, which still contained the bulk of the $^{32}$P label. A typical experiment of this type is shown in Fig. 3c.

Larger quantities of solubilized immunogens and subviral particles can be obtained by centrifugation in batch type zonal rotors or by employing differential ultracentrifugation. Recoveries of haemagglutinin and neuraminidase activities in the soluble fractions obtained regularly exceeded 80%.

The experiments with $^{32}$P-labelled virus suggest...
that the 'core' particles obtained with intermediate CTAB concentrations still contain RNA and phospholipids. In addition, lipid analysis has been carried out. No differences in the lipid patterns could be observed between CTAB subviral particles and whole virus (Bachmayer, 1975). Gel electrophoresis was used to characterize the protein composition of CTAB solubilized material and subviral particles as compared to the corresponding whole virus preparation. Essentially all glycoprotein polypeptides are recovered in the solubilized material, the subviral particle still containing all internal viral proteins (Bachmayer, 1975).

The intact state of the subviral particles was further confirmed by electron microscopy (Fig. 4).

It is clear that all the envelope spike proteins have
been removed yielding smooth-surfaced particles. Using rocket immunoelectrophoresis in the presence of SDS (Fig. 5) a reduction in the numbers of antigens present in the subunit preparation as compared to the whole virus can be clearly demonstrated.

Stimulation of antibodies against haemagglutinin and neuraminidase was studied in different experimental animals to establish the recovery of immunogenicity of the CTAB solubilized material. In addition, mice protection experiments were performed.

A standardized set of subunit vaccine and whole virus used for the production of it was directly compared in all these experiments.

The primary (day 28) and secondary (day 35) immune response to influenza A/Port Chalmers was studied in rabbits, hamsters, guinea-pigs and mice (Tables 1–4). Observed differences in the antibodies

**Table 1. Antibodies against haemagglutinin (HI) and neuraminidase (NI) in rabbits**

<table>
<thead>
<tr>
<th></th>
<th>HI*</th>
<th>NI*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 28</td>
<td>Day 35</td>
</tr>
<tr>
<td>Whole virus</td>
<td>640 2560</td>
<td>45 73</td>
</tr>
<tr>
<td>Subunits</td>
<td>80 2560</td>
<td>13 170</td>
</tr>
<tr>
<td>Control</td>
<td>20 20</td>
<td>0 0</td>
</tr>
</tbody>
</table>

* Reciprocal titre.

**Table 2. Antibodies against haemagglutinin (HI) and neuraminidase (NI) in hamsters**

<table>
<thead>
<tr>
<th></th>
<th>HI*</th>
<th>NI*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 28</td>
<td>Day 35</td>
</tr>
<tr>
<td>Whole virus</td>
<td>139 1575</td>
<td>&lt;10 20</td>
</tr>
<tr>
<td>Subunits</td>
<td>557 2560</td>
<td>10 120</td>
</tr>
<tr>
<td>Control</td>
<td>20 20</td>
<td>0 0</td>
</tr>
</tbody>
</table>

* Reciprocal titre.

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**Fig. 4.** Electron micrographs of influenza virus (MRC-11 recombinant) particles before and after treatment with CTAB. Untreated virus (a) is shown in comparison to solubilized material (insert b) and subviral particles (c) obtained after treatment with 0.03% CTAB. Bar equals 100 nm.

**Fig. 5.** Rocket immunoelectrophoresis of influenza X-31 recombinant whole virus and subunit. Samples contained equal HA and NA and were treated with 1%, 0.5%, 0.25%, 0.125%, and 0.06% SDS (wells 1–5). The agar contained rabbit antiserum against influenza X-31 whole virus in 1.5% Indubiose plus 0.2% Triton X-100. Migration towards the anode.
response of the animal species studied are pronounced. The best response both with whole virus and subunit vaccine was observed in guinea-pigs (Table 3).

In this animal model the subunit vaccine was equal or even superior to the whole virus vaccine. However, the secondary immune response stimulated by application of the subunit vaccine seems to exceed the response to the whole virus in the other animal species as well. From the data it can also be concluded that the responses to haemagglutinin and neuraminidase antigens do not necessarily go parallel.

The results of mice protection experiments performed with influenza A/Hong Kong/68 are shown in Fig. 6. Animals immunized with whole virus and subunit vaccines, respectively, were infected with mice adapted virus 3, 4 and 8 weeks after immunization. Protection rates were calculated from the degree of lung lesions scored at day 9 after challenge. Different kinetics were found for the protection conferred to the animals immunized with whole virus and subunits respectively. At week 4 or later the subunit vaccine seems to be at least equal to the whole virus vaccine.

To evaluate the immunogenicity of this novel type of subunit in man a field trial was organized by Professor C. Kunz, Institute of Virology, University of Vienna. Zonal purified influenza A/Port Chalmers/73 (MRC-11 recombinant) concentrate was prepared and adjusted to an antigen content of approx. 1400 i.u./ml using the WHO haemagglutinin standard (Krag and Bentzon, 1971).

Solubilized immunogens were prepared from half of this virus concentrate as outlined above. Adjuvant (0-15\% Al(OH)₃) was added to part of the whole virus and subunit preparations. The antigen content of these four vaccines (whole virus fluid, whole virus with adjuvant, subunit fluid, subunit with adjuvant) was again tested using the recently described single radial immunodiffusion technique (Schild, Wood and Newman, 1975) (Fig. 7).

Three of the vaccines were found to contain an equal antigen content of 1557 i.u./ml. With the adjuvant containing subunit vaccine only 65\% of the haemagglutinin content originally present could be found in the test. This would indicate that solubilized haemagglutinin and neuraminidase are much stronger absorbed to Al(OH)₃ than whole virus particles.

The field trial was performed in a double blind manner. Volunteers (forty-eight to sixty-five persons per group) received one of the four vaccines by random allocation. Blood samples were taken on the day of vaccination, 4 and 12 weeks later. Antibodies against haemagglutinin and neuraminidase were determined using standard methods. The distribution of haemagglutinin and neuraminidase inhibiting antibodies observed on day 0, 28 and 90 after vaccination is shown in Fig. 8 (HI) and Fig. 9 (NI) for the groups of volunteers who received the two adjuvant free fluid vaccines. No differences could be
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Fig. 7. Standardization of antigen content of vaccines using single radial diffusion test (Schild et al., 1975). X, reference antigen 74/560; ⊗, subunit fluid vaccine; △, subunit adjuvant vaccine; ○, whole virus fluid vaccine; ●, whole virus adjuvant vaccine.

Table 5. Summary of haemagglutinin and neuraminidase antibody responses in human study population receiving equal amounts of influenza whole virus and subunit vaccines without adjuvant

<table>
<thead>
<tr>
<th>Day</th>
<th>Subunit vaccine</th>
<th>Whole virus vaccine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HI-titre (GMT) (reciprocal)</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>24</td>
<td>18-36</td>
</tr>
<tr>
<td>28</td>
<td>290</td>
<td>204</td>
</tr>
<tr>
<td>90</td>
<td>151</td>
<td>136</td>
</tr>
<tr>
<td></td>
<td>Protective antibody level*</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>20%</td>
<td>9%</td>
</tr>
<tr>
<td>90</td>
<td>89%</td>
<td>70%</td>
</tr>
<tr>
<td></td>
<td>≥ Four-fold rise of HI-titres</td>
<td></td>
</tr>
<tr>
<td>0-28</td>
<td>89%</td>
<td>79%</td>
</tr>
<tr>
<td></td>
<td>NI-titre (GMT) (reciprocal)</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>1·9</td>
<td>2·1</td>
</tr>
<tr>
<td>28</td>
<td>10·2</td>
<td>9·6</td>
</tr>
</tbody>
</table>

* HI-titres ≥ 1:64.

observed in the titre distributions obtained after application of the whole virus or subunit vaccines. It is interesting to note that the adjuvant in the vaccines did not increase the level or duration of antibody titres (Kunz and Hofmann, 1975).

The serological findings are summarized in numerical form in Table 5. Geometric mean titres (haemagglutinin and neuraminidase inhibition) are listed together with the levels of protective antibody (HI titres ≥ 1:64) on day 0, 28 and 90. According to all these criteria the subunit vaccine was found to be at least as effective as the standardized whole virus vaccine used for comparison.

Questionnaires were used to determine the degree of side reactions observed by the volunteers. A significantly lower number of side reactions was found after application of the subunit vaccine without adjuvant, as compared with any of the other formulations tested (Kunz and Hofmann, 1975).
Novel influenza subunit vaccine

Fig. 9. Distribution of reciprocal neuraminidase inhibition antibody titres before and after vaccination of volunteers with influenza (b) whole virus and (a) subunit vaccines without adjuvant (Kunz and Hofmann, 1975). ▲—▲, Day 0; ●—●, day 28.

Acknowledgment
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References