VIRAL VACCINES

Comparative antibody responses and protection in mice immunised by oral or parenteral routes with influenza virus subunit antigens in aqueous form or incorporated into ISCOMs

H. O. GHAZI, C. W. POTTER, T. L. SMITH and R. JENNINGS

Summary. The total and subclass antibody responses of mice and protection of these animals against live influenza A/Sichuan/2/87 virus challenge infection were determined after immunisation with homologous A/Sichuan/87 aqueous or ISCOM-formulated surface glycoprotein subunit antigens administered by either the oral or intramuscular routes. The results show that the greatest systemic and local antibody responses were elicited in mice immunised with A/Sichuan ISCOMs by the intramuscular route; protection against homologous virus challenge was also effective in these animals, particularly after two doses of the vaccine. However, relatively high immune responses and protection were also elicited by the A/Sichuan/87 ISCOM vaccine administered orally. Immunisation of mice by the intramuscular route resulted in levels of serum IgG2a subclass antibody significantly greater than those induced by the same preparation given by the oral route, or by the aqueous A/Sichuan/87 subunit antigen preparation administered by either route. The findings indicate that the ISCOM delivery system can be used for immunisation by the oral route, although in mice, under the conditions used, this strategy compares unfavourably with the intramuscular route in terms of both local and systemic immune responses and protection against homologous challenge virus infection.

Introduction

Despite a long history of research, inactivated whole, split or subunit influenza vaccines given by injection induce less than satisfactory immunity to challenge virus infection, and studies have indicated only 60–90% immunity following challenge infection of vaccinees. Although this may be due in part to antigenic shift and drift and inter- and intra-antigenic variation, the use of embryonated eggs for vaccine preparation or the failure of vaccine to stimulate mucosal IgA antibody, the conclusion is a need for better vaccines. Of the methods used to enhance immunity to influenza, inactivated vaccines have been tested orally and intranasally or incorporated into various delivery systems to enhance the immune response. These latter include Quil A to produce ISCOMs of virus subunits, and studies of ISCOMs incorporating influenza virus subunits, herpes simplex virus, measles virus fusion protein, feline leukaemia virus and Epstein-Barr virus have all produced enhanced antibody response to the viral components.

Furthermore, ISCOM-vaccine preparations induce cell-mediated immunity and an IgG subclass antibody response which resembles more closely that after live virus infection than that seen after immunisation with viral proteins alone. Studies with ISCOM preparations have demonstrated an immune response when given orally, and studies with ISCOM-influenza virus proteins given orally or intranasally have also reported an immune response. Thus, it is possible that oral or intranasal ISCOM-influenza virus vaccine offers the potential for an enhanced humoral antibody response, a cellular immune response, local IgA antibody formation and a more acceptable method of administration, compared to currently available vaccines. This strategy may improve immunity to influenza.

In the present study, influenza subunit proteins in an ISCOM formulation were given to mice orally or by the intramuscular route; the serum antibody and nasal wash IgA antibody responses were measured by
enzyme-linked immunosorbent assay (ELISA) and immunised animals were challenged to determine protective efficacy.

The challenge experiments were performed 3 and 7 weeks post-immunisation to assess the duration of the immune response. In addition, the IgG subclass antibody response was measured, since previous reports have indicated that ISCOM vaccines induce a high IgG2a antibody response\(^{17,18}\) which has been reported to have greater neutralising activity than other IgG subclass antibodies.\(^{23,24}\)

**Materials and methods**

**Viruses**

Seed influenza virus A/Sichuan/2/87 (H3N2) was kindly supplied by Dr J. Skehel, World Influenza Centre, London. Virus pools were prepared by inoculating 0.2 ml of a 10\(^{3}\) dilution of seed virus into the allantoic cavity of 10-day embryonated eggs. After incubation at 33°C for 72 h, allantoic fluids were harvested and stored at -80°C, as previously described.\(^{25}\)

**Influenza A/Sichuan subunit vaccine**

Influenza virus A/Sichuan/87 in allantoic fluids was clarified by centrifugation at 360 g for 15 min, and the virus was pelleted by centrifugation at 2200 g for 2 h. The pellet was then reconstituted in phosphate-buffered saline (PBS), pH 7.4, and centrifuged at 4°C through a sucrose gradient of 10–60% at 80000 g for 90 min; fractions with peak haemagglutinating (HA) activity were pooled, centrifuged at 60000 g for 2 h and the pellet was reconstituted in PBS to give purified influenza A/Sichuan virus concentrate. Haemagglutinin (HA) and neuraminidase (NA) subunits from influenza A/Sichuan/87 virus were obtained by treating purified virus with zwitterionic detergent, Empigen BB, as described previously.\(^{26,27}\) Briefly, virus concentrates were suspended at a concentration of 0.5% v/v in Empigen BB for 18 h, centrifuged at 39000 g for 1 h to remove core particles and undisrupted virus particles and the supernate was dialysed against PBS for 48–72 h at 4°C. After dialysis, the virus subunits were purified by centrifugation through a sucrose 10–60% density gradient at 80000 g for 90 min, and the fractions containing high titres of HA were pooled to give a purified subunit preparation.

**A/Sichuan subunit ISCOM vaccine**

A/Sichuan subunit ISCOM vaccine was prepared by the method of DeVries et al.\(^{28}\) A mixture of one part l-\(\alpha\) phosphatidyl ethanolamine and one part of cholesterol were dissolved in chloroform which was then removed in a stream of nitrogen. Octylglycoside (Sigma) 1% in PBS was added to the lipid mixture and the preparation was shaken vigorously to give a 1 mg/ml stock lipid solution. ISCOMs were prepared by mixing the stock lipid solution, A/Sichuan antigen preparation and the glycoside Quil-A (kindly donated by Dr B. Morein, National Veterinary Institute, Division of Vaccine Research, Uppsala, Sweden) in proportions of 1, 2 and 4, respectively, and shaking vigorously for 1 h at 4°C. The mixture was then dialysed against PBS to allow ISCOMs to form, and this was confirmed by electronmicroscopy.

**HA and protein estimations**

The HA content of A/Sichuan subunit and ISCOM-A/Sichuan/87 subunit vaccine preparations was determined by single radial diffusion, as described previously;\(^{29}\) Standard antigens were kindly supplied by Dr J. Wood, National Institute of Biological Standards and Control, Potters Bar, London. Protein content was estimated by the method of Bradford et al.\(^{30}\)

**Animals**

BALB/c mice were obtained from a closed, random-bred colony held in the University of Sheffield laboratories. Mice were used at age 8–9 weeks when the weight was c. 25 g.

**Infectivity of A/Sichuan virus for BALB/c mice**

To determine the infectivity of influenza A/Sichuan/87 virus for mice, 0.1 ml of serial, 10-fold dilutions of stock virus were inoculated intranasally into groups of three mice. Seventy-two h later, nasal washings were collected and lungs were removed and processed to give a 40% w/v extract in PBS from each mouse.\(^{11,17}\) These were tested for virus by inoculating 0.2 ml into the allantoic sac of 10-day embryonated eggs, and testing the allantoic fluids for HA after incubation for 72 h at 33°C. From these results, the infectivity of the virus for nasal and lung infections was calculated independently, and expressed as 50% mouse infectious doses (MID50).

**Experimental protocols**

Groups of 15 BALB/c mice were test bled and inoculated with one of the vaccines to be compared; these were A/Sichuan subunit ISCOM vaccine given orally or intramuscularly; A/Sichuan/87 subunit vaccine in PBS given orally or intramuscularly; or PBS. In each case, vaccines were administered in a 0.5-ml volume containing 0.25 μg of HA. At 21 days post-immunisation, serum and nasal washings were collected from each mouse and tested for specific IgG and IgA antibody, respectively. At this time five or six animals from each group were inoculated intranasally with 10\(^3\) (nasal infection) MID50 A/Sichuan/87 virus.
**Table I.** Antibody response and protection against homologous challenge virus infection of BALB/c mice given influenza A/Sichuan/87 vaccines

<table>
<thead>
<tr>
<th>Vaccine given (0.25 µg HA in 0.5 ml)</th>
<th>Route of inoculation</th>
<th>Number of mice</th>
<th>3 weeks post-immunisation</th>
<th>7 weeks post-immunisation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mean ELISA absorbance values (SD)</td>
<td>Number of infections on challenge/total</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>N/W</td>
<td>Lung</td>
</tr>
<tr>
<td>A/Sichuan ISCOMs</td>
<td>Oral</td>
<td>12</td>
<td>0.74 (0.37)</td>
<td>3/6</td>
</tr>
<tr>
<td>A/Sichuan subunits</td>
<td>Oral</td>
<td>10</td>
<td>0.37 (0.15)</td>
<td>4/5</td>
</tr>
<tr>
<td>A/Sichuan ISCOMs</td>
<td>i/m</td>
<td>10</td>
<td>1.28 (0.23)</td>
<td>2/5</td>
</tr>
<tr>
<td>A/Sichuan subunits</td>
<td>i/m</td>
<td>10</td>
<td>0.59 (0.11)</td>
<td>4/5</td>
</tr>
<tr>
<td>Control</td>
<td>i/m</td>
<td>10</td>
<td>0.21 (0.03)</td>
<td>5/5</td>
</tr>
</tbody>
</table>

i/m, intramuscular; N/W, nasal washing.

**Table II.** Antibody response and protection against homologous virus challenge of BALB/c mice given two doses of A/Sichuan/87 vaccines

<table>
<thead>
<tr>
<th>Vaccine given (0.25 µg HA in 0.5 ml)</th>
<th>Route of inoculation</th>
<th>Number of mice</th>
<th>Mean ELISA absorbance values (SD) at 3 weeks post-immunisation</th>
<th>Mean ELISA absorbance values (SD) at 7 weeks post-immunisation</th>
<th>Number of infections on challenge/total</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>3 weeks post-immunisation</td>
<td>7 weeks post-immunisation</td>
<td>N/W</td>
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<tr>
<td>A/Sichuan ISCOMs</td>
<td>Oral</td>
<td>12</td>
<td>0.54 (0.40)</td>
<td>0.84 (0.31)</td>
<td>3/9</td>
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<tr>
<td>A/Sichuan subunits</td>
<td>Oral</td>
<td>10</td>
<td>0.37 (0.12)</td>
<td>0.75 (0.44)</td>
<td>5/8</td>
</tr>
<tr>
<td>A/Sichuan ISCOMs</td>
<td>i/m</td>
<td>10</td>
<td>1.53 (0.19)</td>
<td>2.62 (0.05)</td>
<td>0/9</td>
</tr>
<tr>
<td>A/Sichuan subunits</td>
<td>i/m</td>
<td>10</td>
<td>0.57 (0.16)</td>
<td>2.00 (0.21)</td>
<td>4/8</td>
</tr>
<tr>
<td>Control</td>
<td>i/m</td>
<td>10</td>
<td>0.28 (0.06)</td>
<td>0.28 (0.04)</td>
<td>5/5</td>
</tr>
</tbody>
</table>

i/m, intramuscular; N/W, nasal washing.

vaccine by the intramuscular route. Significantly lower responses were observed in mice given the same vaccine orally and mice given subunit vaccine intramuscularly; these responses were marginally higher than those observed in mice given subunit vaccine by the oral route. The response to the A/Sichuan subunit ISCOM...
in a 0.1-ml volume of PBS; 3 days after challenge nasal washings and lungs were collected from each infected mouse and tested for virus, as described above. The remaining mice were left for a further 4 weeks, at which time nasal washings and sera were again collected, and the mice were tested for protection by inoculating 10⁵ MID₅₀ of A/Sichuan/87, as described above.

In a separate experiment, groups of 15 BALB/c mice were inoculated with the same virus vaccine preparations by the same routes as above; 3 weeks later, nasal washes and serum samples were collected to measure the antibody response and several mice within each group were tested for susceptibility to challenge virus infection as described above. At this time the remaining mice were inoculated with a second dose of the same vaccine, and 4 weeks later nasal washes and serum were again collected, and the mice were tested for resistance to challenge virus infection.

Enzyme linked immunosorbent assay (ELISA)

The serum IgG antibody response of mice to A/Sichuan/87 virus and the IgA antibody present in mouse nasal secretions were measured by an ELISA procedure, as described previously. Briefly, the wells of ELISA microtitration plates were coated with 200 µl of carbonate buffer, pH 9.6, containing 2 µg of HA of purified, whole A/Sichuan/87 virus, and left at 4°C for 24 h. After washing with Tween-20 0.05% v/v in PBS, 200 µl of test or standard sera in PBS-Tween plus bovine serum albumin BSA 1% were added in duplicate to appropriate wells. After incubation for 1 h at 37°C, the wells were again washed and 100 µl of goat anti-mouse IgG or anti-mouse IgA Fc-specific antibody, each conjugated to horse-radish peroxidase, were added to the wells; the concentrations of these antibodies were established by prior titration. After incubation for 1 h at 37°C, the plates were again washed, and 100 µl of o-phenyl ethyldiamine in citrate-phosphate buffer, pH 5, were added. After incubation for 30 min in the dark at room temperature, the reaction was stopped by addition of 2 N H₂SO₄, and the absorbance values were read at 492 nm. Initially, 32 sera were tested at a range of doubling dilutions from 1 in 50 to 1 in 1600. Endpoint titres were determined as the serum dilution that gave an absorbance value of 0.1. Titres were compared with all sera tested r = 0.942 (fig. 1). Eleven of these sera had ELISA titres >10⁵ (absorbance values >1:1). When these sera are excluded from the regression analysis, correlation is improved further (r = 0.971).

Antibody response and protection against challenge infection after one dose of vaccine

The results obtained for mice given a single dose of vaccine at 3 and 7 weeks after immunisation are shown in table I. At 3 weeks post-immunisation (p.i.), the highest serum antibody titres were seen in animals given A/Sichuan subunit ISCOM vaccine by the intramuscular route; the second highest were observed in mice receiving the same vaccine given orally. A/Sichuan subunit ISCOM vaccine given by either route induced higher ELISA antibody titres as indicated by absorbance values at 492 nm than the subunit vaccine (table I). When animals were challenged, some degree of protection was seen in all groups compared to the controls. Although the numbers were small and not statistically significant, fewer infections were seen in mice given ISCOM-vaccine preparations than in those given subunit vaccines. These results correlated with the antibody response.

When serum was collected from mice at 7 weeks p.i. and tested by ELISA, all antibody levels as indicated by the absorbance values had increased, the highest values being observed in mice given the ISCOM vaccine preparation by either route, compared to subunit vaccine. Again, some degree of protection was seen in all test groups compared to control, with the least number of infections seen in mice given the A/Sichuan subunit ISCOM vaccine by either intramuscular or oral routes. The critical comparison was for ISCOM-vaccine given orally with subunit vaccine given intramuscularly; at both 3 and 7 weeks p.i., slightly higher ELISA antibody levels and increased protection against challenge infection were seen in mice given the ISCOM vaccine preparation orally.

Antibody responses and protection against challenge infection after two doses of vaccine

The results obtained at 3 and 7 weeks after two doses of vaccine, respectively, are given in table II. At 3 weeks p.i., the highest serum IgG antibody responses were seen in mice given A/Sichuan subunit ISCOM
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3 weeks

7 weeks

Fig. 2. Serum and nasal wash antibody responses of mice at 3 and 7 weeks after immunisation with influenza A/Sichuan vaccines. A/Sichuan ISCOM vaccine given by the intramuscular route (A) or oral (C) routes; A/Sichuan subunit vaccine given by the intramuscular (B) or oral (D) routes; E, PBS given intramuscularly. ■, serum IgG; ○, nasal wash IgA.

vaccine given by the intramuscular route was significantly (p < 0.001) greater than all other ELISA antibody responses. Four weeks after the second immunisation, the highest antibody levels were again observed in mice given A/Sichuan subunit ISCOM vaccine by the intramuscular route. Protection against challenge infection again corresponded approximately to antibody responses; thus, no mice were infected with challenge virus after immunisation with two doses of A/Sichuan subunit ISCOM vaccine by the intramuscular route. Relatively low numbers of mice showed no evidence of protection after immunisation with the A/Sichuan subunit vaccine preparation given intramuscularly or the A/Sichuan subunit ISCOM vaccine given orally; the lowest degree of protection was seen after two doses of the A/Sichuan subunit vaccine given orally (table II). Again, the critical comparison was for A/Sichuan subunit ISCOM vaccine given orally and A/Sichuan subunit vaccine given intramuscularly; and the results indicate a similar degree of protection against challenge virus infection. However, the higher antibody level after two doses of subunit vaccine given intramuscularly does not result in improved protection, in contrast to that observed with ISCOMs given by this route.

Local IgA antibody responses after immunisation

Responses to one dose of vaccine. Nasal washes collected from immunised BALB/c mice 3 and 7 weeks p.i. with the various vaccine preparations were tested for IgA antibody by ELISA. The results are shown in fig. 2 and the serum total IgG antibody levels are also included for completeness. At 3 weeks p.i., significant increases in serum IgG antibody above baseline values were seen in mice given A/Sichuan subunit ISCOM vaccine by either the oral or intramuscular routes and A/Sichuan subunit vaccine intramuscularly; however, significant increases above baseline were not seen in mice given A/Sichuan subunit vaccine by the oral route. At this time, nasal wash IgA antibody was not detectable in any mice in any group.

At 7 weeks p.i., serum IgG antibodies had risen in all immunised groups (fig. 2). However, no significant increase in detectable IgA antibody was found; although some slight increases were seen, these were not significant compared to background ELISA absorbance levels. The results indicate that neither type of vaccine, given by either route, induce significant IgA antibody responses.

Responses to two doses of vaccine. For mice given two doses of vaccine 3 weeks apart, the serum IgG antibody responses at 4 weeks after the second immunisation rose significantly for mice given A/Sichuan subunit ISCOM vaccine preparation by both the oral and intramuscular routes (fig. 3), and for mice given A/Sichuan subunit vaccine by the intramuscular route; no significant increase in IgG antibodies were seen for mice given two doses of A/Sichuan subunit vaccine by the oral route (fig. 3).
Fig. 3. Serum and nasal wash antibody responses of mice 7 weeks after immunisation with two doses of influenza A/Sichuan vaccines. A/Sichuan ISCOM vaccine given by the intramuscular (A) or oral (C) routes; A/Sichuan subunit vaccine given by the intramuscular (B) or oral (D) routes; E, PBS given intramuscularly. ■, serum IgG; □, nasal wash IgA.

At this time levels of nasal wash IgA antibody significantly above baseline values were observed in mice given two doses of A/Sichuan subunit ISCOM vaccine or A/Sichuan subunit vaccine by the intramuscular route; however, this response was not detected in mice given either of these vaccine preparations by the oral route (fig. 3).

**Serum IgG subclass antibody response**

**Response to one dose of vaccine.** Serum samples from mice obtained 3 and 7 weeks after immunisation with a single dose of the various vaccines were tested for IgG subclass antibody responses by the ELISA procedure; the availability of specific mouse IgG subclass antibody allowed quantitative estimation of the IgG1, IgG2a, IgG2b and IgG3 responses. These results are shown in fig. 4a and b. At 3 weeks p.i., IgG subclass antibody response were proportional to the total IgG antibody response (see table I). Thus, the largest responses in the IgG1, IgG2a, IgG2b and IgG3 subclasses were seen after intramuscular inoculation with A/Sichuan subunit ISCOM vaccine whereas generally lower responses were seen after immunisation with the A/Sichuan subunit vaccine given intramuscularly or orally and the A/Sichuan subunit ISCOMs given orally (fig. 4a). However, a significantly (p < 0.001) high IgG2a antibody response was seen for all mice given A/Sichuan ISCOMs by the intramuscular route, compared to that seen for all other vaccine protocols. When serum samples were examined 7 weeks p.i. (fig. 4b), the subclass antibody responses were again relative to those observed for the total IgG antibody response for each vaccine type and immunisation route. However, the IgG2a antibody response at 7 weeks had increased significantly for mice given A/Sichuan subunit vaccine intramuscularly and A/Sichuan subunit ISCOM vaccine orally, compared to mice which had received A/Sichuan subunit ISCOM vaccine intramuscularly (fig. 4b). The results indicate that the A/Sichuan subunit ISCOM vaccine given intramuscularly induces an earlier disproportionately high IgG2a antibody response, compared to other vaccines, and that this response was delayed for mice given the same vaccine orally or the A/Sichuan subunit vaccine intramuscularly.

**Response to two doses of vaccine.** The IgG subclass antibody responses were also examined 3 weeks after first inoculation, and 4 weeks later, after a second vaccine dose; these results are shown in fig. 4c and d. The responses at 3 weeks after a single dose are not significantly different from those shown in fig. 4a and b with the same protocols. Thus, all four subclass antibody responses were similar and proportional to the total IgG response except for a significantly (p < 0.01) high response for IgG2a antibody in mice given A/Sichuan subunit ISCOM vaccine intramuscularly. Four weeks after the second dose of vaccine, the picture was essential similar to that seen 7 weeks after a single immunisation (fig. 4a); however, after two doses, significantly (p < 0.001) enhanced IgG2a and IgG3 antibody responses were seen in mice given A/Sichuan subunit ISCOM vaccine intramuscularly, but not for the other groups (fig. 4d). Mice were not
tested 7 weeks after the second vaccine dose to
determine if there was also a delayed response to these
IgG subclasses in the other vaccine groups.

Discussion

The increasing use of ELISA for the detection of
antibodies to virus antigens has led to much discussion
of the most appropriate method of expressing the
results obtained. There have been mathematical
calculations advanced to aid in the expression of these
results; the relationship of ELISA to other well-
established techniques has been explored also. The
ELISA has been used widely for the evaluation of vaccines, particularly influenza, in animal
models, and in vitro splenocyte culture. In man, specificity of the ELISA can be a problem in the
evaluation of the serum antibody response to influenza
vaccines, due to background immune responses ac-
quired through natural infection but this is not a
problem when testing for antibodies by ELISA in
laboratory animal sera.

The use of multiple dilution assays is both labour-
intensive and costly. Here we have shown, with a small
number of mouse sera tested by both multiple and
single dilution assays, that a single-dilution assay is a
valid measure of the serum IgG antibody status in an
animal model.

Studies with ELISA to detect IgG to chlamydial
antigens report that absorbance values ≥ 1.6 do not
reflect accurately the concentration of IgG present in
the sample. The initial studies presented here indicate
that ELISA absorbance values are directly propor-
tional to the log_{10} of the ELISA titre as defined earlier.
Consequently, the overall data presented here rely on
the single dilution assay as a valid measure of antibody
status.

Studies have shown that immunisation of mice with
subunit influenza virus vaccine induces a serum anti-
body response and relative protection against challenge
virus infection; these results are confirmed in the
present study. Furthermore, mice inoculated with
the same vaccine incorporated into ISCOMs have
been found to give a significantly increased antibody
response and protection against challenge virus
infection, and again these results are confirmed in
the present study. Thus, the ISCOM delivery system
enhanced immune responses to influenza virus
antigens in mice, and these results are paralleled by
similar studies with other virus antigens.

ISCOM incorporated vaccines have been shown also
to induce cellular immunity and stimulate high
IgG2a antibody responses. Besides the implication
that Th1 cells, and hence CMI, are activated, the
stimulation of a relatively high IgG2a response is
important, as this is the IgG subclass antibody
reported to have the greatest neutralising property.
and the preferential subclass antibody induced by live virus infection which provides the more solid immunity against challenge infection.\textsuperscript{17, 44} Furthermore, IgG2a antibodies have been reported to have an important role in protection against tumours and some other infectious agents. These results indicate that ISCOM vaccines represent a potent delivery system for virus antigens and their further development and use could enhance the immune response to many virus infections.

Earlier studies have shown that virus antigens formulated into ISCOMs cross cell membranes and this property may be important in the enhanced immune response observed.\textsuperscript{15, 45} In particular, ISCOM vaccines induce immune responses when given orally.\textsuperscript{19} In the present studies, ISCOM-influenza vaccine was given orally to mice to determine the immune reaction, and the results were compared to those with the same vaccine given intramuscularly and aqueous subunit vaccine given by both routes. The results indicate that the best immune responses were seen in mice given ISCOM vaccine intramuscularly. However, although the prescribed route of inoculation for influenza subunit vaccine is intramuscular, the present studies show that ISCOM vaccine given orally produced relatively high immune responses and protection against challenge virus infection; this was found after both one and two doses of vaccine. Although results obtained in mice cannot be used to predict the effect in other species, the results suggest that the ISCOM delivery system represents a method for immunising by the oral route, a method of immunisation which would probably be more acceptable. However, the results do not suggest that the immune response to oral immunisation is increased compared to intramuscular immunisation, and the former route of inoculation cannot be considered antigen-sparing.

In the critical comparison of aqueous subunit vaccine given intramuscularly and ISCOM-vaccine given orally, no claim can be made for the former inducing significantly increased local antibody responses. Thus, after one dose of either vaccine, no significant detectable IgA was seen at 4 weeks; and after a second dose of vaccine, only relatively small amounts of IgA antibody were induced in both groups.

IgG subclass antibody responses for the two vaccines given orally were marginally lower than that seen for A/Sichuan ISCOM vaccine given intramuscularly, although of much the same order. Thus, after a single dose of vaccine the subclass antibody response at 3 and 7 weeks was similar in both vaccine groups, and this was seen also 4 weeks after the second dose of vaccine. The results suggest that immune reactions to aqueous A/Sichuan subunit vaccine given intramuscularly could be improved if the material was incorporated into ISCOMs, and these results confirm previous studies.\textsuperscript{17, 44} Furthermore, ISCOM vaccine gives similar antibody response and immunity to challenge virus infection in mice when given orally. Although no immunological advantage has been found by giving the vaccine by this route, the delivery method would be more acceptable, and oral administration offers the possibility of overcoming definite but mild reactions seen following intramuscular inoculation.\textsuperscript{47}

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