Heterotypic Protection against Influenza by Immunostimulating Complexes Is Associated with the Induction of Cross-Reactive Cytotoxic T Lymphocytes

Suryaparaksh Sambhara, Samantha Woods, Rita Arpino, Anjna Kurichch, Alan Tamane, Brian Underdown, Michel Klein, Karin Lövgren Bengtsson, Bror Morein, and David Burt

Influenza immunostimulating complexes (flu-ISCOMs) and a monovalent subvirion vaccine prepared with an H1N1 strain of influenza virus were compared in mice for immunogenicity and protection against challenge with homologous and heterotypic influenza viruses. Flu-ISCOMs but not subvirion vaccine fully protected mice against homologous virus challenge after one immunization as assessed by measurement of virus lung titers. The improved protection induced by flu-ISCOMs was associated with a 10-fold higher prechallenge serum hemagglutination inhibition titer. Furthermore, only flu-ISCOMs fully protected mice against mortality and reduced morbidity following challenge with an influenza virus of the serologically distinct H2N2 subtype. This cross-protection correlated with the induction of virus cross-reactive cytotoxic T lymphocytes that recognized a known major histocompatibility class I (H2-Kd)–restricted epitope within the hemagglutinin of influenza virus that is conserved among the H1 and H2 influenza virus subtypes. Flu-ISCOMs may offer significant advantages over current commercial formulations as an improved influenza vaccine.

The development of improved vaccines against influenza virus is an acknowledged goal in vaccinology [1]. Current influenza vaccines are ~70% effective at preventing illness in healthy adults [2] and ~50% effective at preventing hospitalization and pneumonia in persons ≥65 years old [3]. The desired properties of an improved influenza vaccine are production of increased titers of virus-neutralizing antibodies against influenza hemagglutinin (HA) in the serum (IgG) and at mucosal surfaces (sIgA) to prevent infection [4, 5] and induction of virus cross-reactive T lymphocytes (CD8⁺ or CD4⁺) or their cytokines to facilitate recovery from infection [5–7]. Such characteristics should increase the proportion of vaccinated persons who are protected against infection with homologous influenza virus. Furthermore, the ability of a vaccine to induce cytotoxic T lymphocytes (CTL) that recognize highly conserved epitopes within proteins across the various influenza virus subtypes may reduce the severity of illness and promote faster recovery when the vaccine and circulating strains are serologically distinct.

Various strategies are being investigated to develop parenterally administered influenza subunit vaccines that are more efficacious than the current vaccines. These strategies include the use of alternative delivery vehicles for influenza virus antigens, such as liposomes [8] and oil-in-water emulsions [9], formulation in adjuvants [10], and cDNA encoding various viral proteins [11, 12]. An alternative approach would be to use immunostimulating complexes (ISCOMs), an adjuvanted particulate vaccine system comprising cholesterol, phospholipid, antigen, and the adjuvant Quil A [13]. Studies in various animals have demonstrated improved humoral and cell-mediated (CTL and T helper) immune responses against model antigens (e.g., ovalbumin) and viral proteins, human immunodeficiency virus gp120, influenza HA, measles fusion protein, and herpes and rabies viral proteins when vaccines were administered in the form of ISCOMs [14].

Various properties of ISCOMs contribute to these increased immune responses. Compared with soluble antigens, ISCOMs are more rapidly distributed from the site of injection to the draining lymph nodes and spleen, where they persist for longer periods of time [15]. After subcutaneous immunization, antigens formulated as ISCOMs are taken up by a subset of splenic macrophages distinct from those that take up soluble antigens [16]. Within antigen-presenting cells, intact ISCOMs associate with intracellular lipid membranes and localize within the cytosolic and vesicular compartments [17], enabling antigen processing for both class I and class II major histocompatibility complex (MHC)–restricted T cell responses. ISCOMs have been shown to up-regulate MHC class II antigen expression on monocytes [15] and enhance the production of granulocyte-macrophage colony-stimulating factor, tumor necrosis factor-α, interleukin (IL)-1 and IL-6 from macrophages as well as production of Th1 (interferon-γ) and Th2 (IL-4) cytokines from antigen-specific T cells [18]. Both IgG1 and IgG2a responses are induced in mice immunized with antigens formulated as ISCOMs [19].
In the present study, proteins from a recent vaccine strain of an influenza H1 virus were formulated as ISCOMs and examined in mice for their ability, compared with commercial vaccine derived from the same H1 strain, to induce serum antibodies with hemagglutination inhibition (HAI) activity, to generate CTL, and to protect against both homologous and serologically distinct heterotypic viruses.

Materials and Methods

Mice. Female BALB/c mice between 6 and 8 weeks of age were obtained from Charles River (Quebec).

Influenza viruses and vaccines. A/Taiwan/1/86 (A/Taiwan) (H1N1), A/Japan/305/57 (A/Japan) (H2N2), and A/Philippines/1/82 (A/Philippines) (H3N2) as live influenza viruses in egg-derived allantoic fluid; mouse-adapted A/Taiwan/1/86 (gift of M. Harmon, Pasteur Institute, Paris), and commercial A/Taiwan/1/86 monovalent subunit vaccine (Fluzone) were obtained from PM C (Swiftwater, PA); and commercial A/Taiwan vaccine containing 0.05±10 μg of HA in 0.1 mL of PBS. Control mice received either PBS alone or 200–400 hemagglutination units (HAU) of live A/Taiwan virus as allantoic fluid. Fourteen days later, mice were challenged intranasally while under anesthesia with 50 μL (5 LD50) of live mouse-adapted A/Taiwan or A/Japan in allantoic fluid.

Protection was assessed either by monitoring mortality daily and morbidity (weight change) every 2–3 days up to 14 days after challenge or by quantitation of residual virus in mouse lungs 4 days after challenge. Virus titers were determined on lung homogenates by plaque assay. Lungs dissected from individual animals were homogenized (Polytron; Brinkman, Ontario) in 2 mL of PBS containing 0.5% gelatin. The lung suspensions were centrifuged at 1250 g for 15 min, and supernatants were filtered through a 0.45-μm membrane (Millipore, Bedford, MA) and stored at −70°C until assayed. Serial dilutions of lung homogenates (100 μL) were incubated in duplicate with washed confluent MDCK cells in 24-well flat-bottomed tissue culture plates for 1 h at 37°C in 6% CO2. To each well was added 1 mL of overlay medium, comprising 2% wt/vol Noble agar (Difco, Detroit), 0.2% D(+)-glucose (Sigma), 0.02% diethylaminoethyl dextran (Pharmacia, Quebec), 2.0% BME vitamins (ICN, Mississauga, Ontario), and 2 μg/mL TPCK trypsin (Sigma) in 2X Dulbecco’s MEM (ICN). The plates were incubated for 3 days at 37°C (5% CO2), and then 1 mL of a 2% neutral red solution in PBS was added to each well and the plates were incubated overnight. Stain was then aspirated, and the plaques were counted and expressed as the mean logarithm of the number of plaque-forming units per milliliter.

HAI assay. Blood was obtained before and after immunization via the orbital sinus vein of 6 randomly selected mice. Serum samples were heated at 56°C for 30 min to inactivate complement. To 25 μL of serum was added 12.5 μL of trypsin (8 mg/mL) at 56°C for 30 min. Samples were cooled to room temperature and incubated for a further 15 min with 112.5 μL of 0.001 M sodium periodate. Excess periodate was neutralized with 225 μL of glyc erol (0.6% in 0.14 M NaCl). Serially diluted antisera were tested in duplicate for their ability to inhibit the agglutination of 1% chick red blood cells by 4 HAU of A/Taiwan or A/Japan virus in a standard HAI assay [25].

Determination of CTL activity. For CTL studies, mice were given a single immunization with either 1 μg of HA as A/Taiwan flu-ISCOMs or 10 μg of HA as monovalent A/Taiwan subvirion vaccine in 0.1 mL of PBS via the subcutaneous route. Ten days later, spleens were removed and splenocytes restimulated with live virus. Spleen cells (2.5 × 107) were incubated at 37°C, 6% CO2, in an upright flask in 20 mL of RPMI–10% fetal bovine serum with the same number of gamma-irradiated (30 Gy [3000 rads]) normal syngeneic spleen cells that were previously infected for 1
the absence of responder lymphocytes. The percent specific chromium release was calculated as (induced counts – spontaneous counts)/(total counts – spontaneous counts) × 100. The spontaneous release of 51Cr in the absence of effector cells was 5%–15% of the total counts. The percentage of lysis for individual replicates was within ±3%–10% of the mean.

Results

Serum HAI responses and protection against homologous virus. BALB/c mice were given one or two immunizations 3 weeks apart with 0.05, 0.5, or 5 µg of HA (measured by single radial immunodiffusion) in the form of A/Taiwan flu-ISCOMs or monovalent homologous subvirion influenza vaccine. Figure 1A–D show HAI titers in sera obtained 13 days after each immunization and virus lung titers 4 days after challenge with homologous mouse-adapted influenza virus. After the first immunization, HAI activity in sera from mice that received 5 µg of HA as flu-ISCOMs was 10-fold higher than that in sera from mice given 5 µg of HA as subvirion influenza vaccine or 0.5 µg of HA as flu-ISCOMs (figure 1A). These prechallenge HAI titers correlated with protection as determined by virus lung titers.

Virus could not be detected in the lungs of mice given one 5-µg dose of HA as flu-ISCOMs, indicating complete protection, whereas no protection was afforded by subvirion vaccine containing 5 µg of HA (figure 1B). Lower doses of flu-ISCOMs were not protective. Two immunizations with subvirion influenza vaccine were required to clear virus by day 4 after challenge (figure 1D). Serum HAI titers in mice immunized twice with 0.5 or 5 µg of subvirion vaccine (figure 1C) were at least 10-fold higher than those seen after primary immunization and gave partial (0.5-µg dose) and complete (5-µg dose) protection against virus challenge (figure 1D).

flu-ISCOMs containing 0.5 µg of HA induced prechallenge HAI titers comparable to those induced by 5 µg of HA as subvirion vaccine, while flu-ISCOMs at 5 µg of HA induced HAI titers that were at least 20-fold higher than those from the same dose of HA as subvirion vaccine. Mice immunized twice with 0.5 or 5 µg of HA as ISCOMs completely cleared virus from their lungs by day 4 after challenge. These mice were still fully protected against challenge with homologous virus up to at least 4 months after immunization. At the same time point, protection was less durable in mice that received 5 µg of HA as influenza subvirion vaccine, since virus could be detected in 20% of the animals (data not shown).

Differences were also observed in the abilities of ISCOMs and vaccine to protect mice against morbidity as determined by weight loss. Four days after challenge, no virus could be detected in the lungs of mice immunized twice with influenza vaccine or flu-ISCOMs containing 10 or 1 µg of HA, respectively (figure 2A). In four separate experiments, mice immunized with 1 µg of HA as flu-ISCOMs consistently showed no significant weight loss during 14 days following challenge. Mice given a 10-fold higher dose of HA as subunit vaccine lost a mean of 9.7% (range, 3%–15%) of their starting weights by day 6; however, they recovered the weight by day 14. Figure 2B shows the results of a typical experiment. Nonimmunized mice showed 50% mortality by day 14; however, no deaths occurred in either of the immunized groups. Collectively, these data show that compared with subvirion vaccine, flu-ISCOMs induce significantly higher HAI titers and offer improved protection after challenge with homologous virus, as measured both by infection (lung titers) and morbidity (weight loss).

Induction and specificity of CTL. CTL capable of specifically lysing virus-infected target cells were detected in restimulated spleen cells from mice immunized with live virus or flu-ISCOMs but not subvirion monovalent vaccine. Representative data from two separate experiments are shown in table 1 and figure 3. One immunization with flu-ISCOMs (1 µg of HA) generated CTL that lysed target cells infected with the homologous A/Taiwan influenza virus but not those infected with the H3N2 virus A/Philippines, suggesting that the dominant CTL response was directed against proteins other than the highly conserved type-specific M1 protein and NP. This was consistent with the finding that spleen cells from BALB/c mice given flu-ISCOMs failed to lyse target cells pulsed with a synthetic peptide representing known CTL epitopes, or infected with a synthetic peptide (HA2 189–199) representing a known CTL epitope. Of interest, for mice immunized twice with either H1 or H3 subtype viruses, reflecting the dominance of CTL that recognized the conserved NP epitope. Sufficient
Figure 1. Hemagglutination inhibition (HAI) and virus lung titers in BALB/c mice immunized with influenza immunostimulating complexes (flu-ISCOMs) or subvirion vaccine and challenged with live homologous virus. Groups of 6 mice were given 1 (A, B) or 2 (C, D) immunizations with A/Taiwan/1/86 (A/Taiwan) flu-ISCOMs or subvirion vaccine containing 0.05–5 μg of influenza hemagglutinin (HA) per dose on days 0 and 21. Control mice were given PBS. Serum HAI titers (A, C) were determined 13 days after each immunization. Mice were challenged with 5 LD50 of mouse-adapted A/Taiwan virus 14 days after first or second immunization. Virus lung titers were measured 4 days later (B, D) as described in Materials and Methods. Data are mean ± SE.

Homology exists within the amino acid sequence HA2 189–199 of influenza viruses of the H1 and H2 strains to enable recognition by HA-specific CTL generated from either virus subtype [26]. Thus, the CTL line derived from BALB/c mice immunized with A/Taiwan (H1) flu-ISCOMs also lysed target cells infected with A/Japan (H2N2) virus (figure 3), further supporting the conclusion that flu-ISCOMs induce CTL that preferentially recognize the amino acid sequence HA2 189–199 within the HA molecule.

Protection against challenge with heterotypic virus. Class I MHC–restricted CTL have been implicated in the recovery phase of influenza virus infection [6]. Since in this study, flu-ISCOMs induced CTL capable of recognizing epitopes from both H1 and H2 influenza virus subtypes, experiments were done to determine whether mice immunized with A/Taiwan H1 ISCOMs were protected against challenge with an H2 virus, A/Japan/56 (H2N2).

In this case, mice inoculated twice with flu-ISCOMs containing 1 μg of A/Taiwan (H1N1) HA, subvirion A/Taiwan vaccine with 10 μg of HA, or live A/Taiwan virus (400 HAU) were fully protected against mortality and morbidity associated with challenge by homologous mouse-adapted A/Taiwan virus
Figure 2. Comparison between virus lung titers and weight loss following virus challenge for mice immunized with influenza immunostimulating complexes (flu-ISCOMs) or subvirion vaccine. Groups of 6 BALB/c mice were immunized twice with A/Taiwan/1/86 (A/Taiwan) flu-ISCOMs, subvirion vaccine containing 0.1–10 μg of influenza hemagglutinin (HA), or PBS and then challenged with 5 LD₅₀ of mouse-adapted A/Taiwan virus as described in Materials and Methods. After infection, mice were weighed daily for up to 14 days (B) or sacrificed 4 days after challenge for determination of virus lung titers (A). Mortality in PBS control and immunized groups was 50% and 0%, respectively, 14 days after challenge. Data are mean ± SE.

Table 1. Specificity of CTL induced by influenza immunostimulating complexes (flu-ISCOMs), subvirion vaccine, or virus.

<table>
<thead>
<tr>
<th>Immunization of CTL line</th>
<th>E:T</th>
<th>Medium</th>
<th>A/Taiwan (H1N1)</th>
<th>A/Philippines (H3N2)</th>
<th>HA peptide (189–199)</th>
<th>NP peptide (147–158)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/Taiwan ISCOMs (1 μg HA)</td>
<td>30</td>
<td>1.6</td>
<td>24.0</td>
<td>3.0</td>
<td>40.6</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>2.6</td>
<td>31.8</td>
<td>5.7</td>
<td>46.2</td>
<td>0.6</td>
</tr>
<tr>
<td>A/Taiwan virus (400 HAU)</td>
<td>24</td>
<td>1.8</td>
<td>32.3</td>
<td>60.5</td>
<td>11.0</td>
<td>53.8</td>
</tr>
<tr>
<td>A/Taiwan vaccine (10 μg HA)</td>
<td>30</td>
<td>11.1</td>
<td>13.7</td>
<td>ND</td>
<td>9.7</td>
<td>12.4</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>17.9</td>
<td>12.0</td>
<td>ND</td>
<td>16.7</td>
<td>18.0</td>
</tr>
</tbody>
</table>

NOTE. T cell cultures were tested for killing of ³¹Cr-labeled P815 target cells incubated with tissue culture medium alone, infected with H1N1 (A/Taiwan/1/86 [A/Taiwan]) or H3N2 (A/Philippines/1/82 [A/Philippines]) viruses, or pulsed with synthetic peptides representing known CTL epitopes from H1 hemagglutinin (HA) (aa HA2 189–199) or nucleoprotein (NP) (aa 147–158[R–]) recognized by major histocompatibility complex class I (H-2Kᵈ)–restricted CD₈⁺ CTL. Data represent mean % lysis from triplicate determinations of representative experiment on spleen cell cultures pooled from 2 mice, tested at various effector-to-target ratios (E:T). ND = not determined; HAU = hemagglutination units.
Figure 3. Induction of cytotoxic T lymphocytes (CTL) cross-reactive for influenza H1 and H2 subtypes by H1 influenza immunostimulating complexes (flu-ISCOMs). CTL cultures were generated and examined for lytic activity as described in table 1. T cell cultures were tested for killing of $^{51}$Cr-labeled P815 target cells infected with H1N1 (A/Taiwan/1/86) or H2N2 (A/Japan/305/57) viruses or pulsed with synthetic influenza hemagglutinin peptides (HA2 189–199) or nuclear protein peptides (NP147–158[R]). Data points represent mean % lysis from representative experiment for triplicate assays on spleen cell cultures pooled from 2 mice and tested at effector-to-target ratios (E:T) of 30:1 and 60:1.

Figure 4. Mortality and weight loss following heterotypic (H2) virus challenge of mice immunized with H1 influenza immunostimulating complexes (flu-ISCOMs), subvirion vaccine, or live virus. Groups of 6 BALB/c mice were immunized twice with A/Taiwan/1/86 (A/Taiwan) flu-ISCOMs or subvirion vaccine containing 1 and 10 μg of HA, respectively. Other groups of mice were given live virus (400 hemagglutination units) or PBS. All animals were subsequently challenged with 5 LD$_{50}$ of mouse-adapted homologous A/Taiwan virus (A, B) or heterotypic (H2) virus, A/Japan/305/57 (C, D) as described in Materials and Methods. After infection, mice were observed for mortality (A, C); survivors were weighed daily for up to 14 days (B, D). Each data point in B and D represents mean ± SE weight of surviving mice.
ity, since prechallenge sera of immunized animals failed to inhibit agglutination of red blood cells by A/Japan influenza H2 virus (table 2).

In additional studies, A/Taiwan H1 flu-ISCOMs failed to protect mice against challenge with an H3 virus (data not shown). The ability of flu-ISCOMs to protect mice against H1 and H2 but not H3 influenza subtype viruses parallels the differential recognition of these subtypes by CTL elicited by flu-ISCOMS (figure 3).

Discussion

In the present study, we have demonstrated the ability of flu-ISCOMs to protect mice against challenge with both homologous and serologically distinct type A influenza viruses. The protective efficacy of flu-ISCOMs was significantly better than that seen for commercial subvirion vaccine tested at equivalent doses of HA. The superiority of flu-ISCOMs over vaccine was demonstrated when protection was assessed in various ways: virus load in the lung, mortality, morbidity, and rate of recovery from infection. Immunologic correlates associated with protection and recovery from influenza virus infection have been suggested by studies performed in humans and animals. Preexisting local secretory IgA and serum IgG antibodies specific for HA correlate with prevention of infection by homologous virus [4, 5]. Recovery from infection with homotypic virus involves both serum IgG and class I and class II MHC-restricted CTL [27].

CTL also appear to enhance the elimination of virus following heterotypic challenge, thereby reducing morbidity and mortality [11]. In this study, we demonstrated that the ability of parenterally administered flu-ISCOMs to confer improved protection in mice against infection with homologous virus correlated with the induction of higher serum HAI titers, reflecting the presence of antibodies specific for HA. At equivalent doses of HA, the serum HAI titers induced by ISCOMs containing proteins from a recent vaccine strain of influenza were at least 10-fold higher than those induced by vaccine. Similar findings have been shown for detergent-extracted antigens from laboratory strains of influenza virus and for other proteins formulated as ISCOMs tested in various species [14].

A characteristic feature of ISCOMs is their ability to induce antigen-specific, CD8\(^+\)/class I MHC-restricted T cell responses against incorporated antigens, such as the human immunodeficiency virus envelope protein [28] and ovalbumin [29]. In an earlier study, Jones et al. [30] demonstrated the induction of influenza-specific CTL in mouse lungs following intranasal inoculation with flu-ISCOMs. However, the antigen specificity of these CTL was not determined. The present study is the first to report on the antigen specificity and virus cross-reactivity of CTL elicited by flu-ISCOMs in mice. flu-ISCOMs containing influenza HA of the subtype H1 induced CTL that lysed target cells infected with H1 and H2 but not H3 strains of virus. In contrast, CTL from mice immunized with live H1 virus killed target cells infected with influenza viruses of subtypes H1, H3 (present study), or H2 (unpublished data). No CTL were generated in mice immunized with the subvirion vaccine, presumably due to the inability of flu proteins in that context to be correctly processed and expressed with MHC class I molecules for priming of CD8\(^+\) T cells [31].

The differential virus subtype recognition by CTL elicited by flu-ISCOMs and live virus correlated with their abilities to lyse target cells pulsed with known MHC class I H-2K\(^d\)–restricted peptides from influenza NP (NP147–158[R]) and HA (HA2 189–199), previously shown to be recognized by CD8\(^+\) CTL arising from influenza virus infection [20, 21]. The immunodominant CTL response induced by live virus was against NP147–158[R\(–\)], a sequence that is conserved among H1, H2, and H3 virus subtypes [26]. In contrast, flu-ISCOMs elicited CTL that recognized HA2 189–199, which is conserved among H1 and H2 but not H3 influenza viruses, but did not generate CTL against NP. This finding was unexpected since the amount of NP associated with the flu-ISCOM preparations was similar to the levels found in the subvirion vaccine as determined by polyacrylamide gel electrophoresis (data not shown).

Table 2. Serum hemagglutination inhibition (HAI) titers against H1 and H2 subtype influenza viruses from BALB/c mice immunized with A/Taiwan/1/86 (A/Taiwan) influenza immunostimulating complexes (flu-ISCOMs), subvirion vaccine, or virus.

<table>
<thead>
<tr>
<th>Immunization</th>
<th>Against A/Taiwan</th>
<th>Against A/Japan</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HA dose (µg)</td>
<td>Day 13</td>
</tr>
<tr>
<td>A/Taiwan ISCOMs</td>
<td>1</td>
<td>40</td>
</tr>
<tr>
<td>A/Taiwan vaccine</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>A/Taiwan virus</td>
<td>400 HAU</td>
<td>≤10</td>
</tr>
<tr>
<td>PBS</td>
<td>—</td>
<td>≤10</td>
</tr>
</tbody>
</table>

NOTE: Groups of BALB/c mice were immunized twice with A/Taiwan ISCOMs, subvirion vaccine, or live virus at days 0 and 21. Blood samples were obtained at days 13 and 34, and sera were tested for HAI activity against homologous A/Taiwan (H1N1) and heterotypic A/Japan (H2N2) viruses as described in Materials and Methods. Values represent mean titers for pooled sera. HA = hemagglutinin.
Differences in the efficiency of processing of ISCOM-associated NP and HA by antigen-presenting cells may explain the inability of flu-ISCOMs to induce CTL specific for NP in BALB/c mice. NP may not be associated with ISCOMs in a manner that allows efficient cytoplasmic processing of the protein for production of peptides that bind to MHC class I molecules. Viral membrane proteins, such as HA, that can associate with phospholipid on the surface of the ISCOM particle may be more accessible to cytoplasmic proteases involved in processing for class I presentation. Of interest, Weiss et al. [32] reported that lipitated but not native NP could be efficiently incorporated into ISCOMs, although such ISCOMs did not induce CTL in C3H (H-2^b) mice. However, in recent studies, we showed that NP-specific, class I MHC–restricted CTL are induced by H1 flu-ISCOMs in C57BL/6 (H-2^b) mice (unpublished data). Since there is no H-2^b binding motif within HA2 189–199, this result suggests that in the appropriate mouse strain that is unable to mount a class I-MHC–restricted CTL response against HA, correct processing and presentation of ISCOM-associated NP for induction of CD8^+ T cells can occur.

We have shown that the ability of H1 flu-ISCOMs to induce cross-reactive CTL against an epitope common to the transmembrane domains of the H1 and H2 influenza virus subtype hemagglutinins (not present in H3 viruses) correlated with protection against mortality and morbidity due to infection with H1 and H2 but not H3 influenza viruses. However, since in the present study we have only classified the CTL responses on the basis of recognition of the immunodominant class I MHC–restricted CTL epitopes NP147–158(R^0) and HA2 189–199, we cannot rule out the possibility that CTL specific for other epitopes within HA, NP, or other flu proteins may also be contributing to the cross-protection observed. Studies are in progress at the PMC Canada laboratory to identify additional CTL epitopes recognized by CTL induced by flu-ISCOMs.

Mice given H1N1 influenza virus were poorly protected against challenge with an H2N2 virus. This result is surprising since live virus induced CTL specific for a known H-2K^d–restricted epitope of NP (NP147–158[R^0]) that is conserved among H1 and H2 viruses. One explanation for this finding is that the observed cross-protection may be mediated by immunologic effectors other than CD8^+ T cells. This possibility may also explain the finding that 25% of mice immunized with subvirion vaccine of the H1 subtype were protected against lethal challenge with the H2 influenza virus despite failing to induce a measurable CTL response. For example, mice transgenic for β2-microglobulin deletion, which lack functional MHC class I molecules, can still clear virus after infection [33] and are protected against lethal virus challenge by immunization with vaccinia expressing NP [7]. In studies [7] and [33], CD4^+ CTL were implicated in the protection. In this mouse model, there may also be qualitative and quantitative differences in the T lymphocyte subsets and their secreted cytokines following immunization with flu-ISCOMs compared with those for live virus (or subvirion vaccine) that could contribute to the enhanced clearance of virus after infection. However, no significant differences have been seen in the amount and type of Th1 and Th2 cytokines induced by virus and flu-ISCOMs (unpublished data) [18].

Another possible explanation for the differences in cross-protection induced by influenza virus and flu-ISCOMs is that the functional activities of CTL induced by flu-ISCOMs and live virus differ and may depend on their specificities for the HA and NP proteins, respectively. Of interest, data from a recent report suggests that the resistance of certain mouse strains to Leishmania species may be dependent not only on the ability of the host to induce a Th1 response against the pathogen but also on the antigen specificity of the T cells generated [34]. CTL specific for HA have been implicated in mediating cross-protection in mice given a recombinant HA2 fragment from influenza HA expressed as a fusion protein. Immunization with the HA2 protein of the H1 strain protected BALB/c mice against H1 and H2 but not H3 strains of influenza virus [35].

Evidence for a protective role for NP-specific CTL has been less conclusive and may depend on the form of the antigen and the ability to induce long-term memory T cells [36]. For example, while cross-protection has been clearly observed in mice immunized with cDNA encoding NP [11], conflicting results have been reported for soluble NP [37, 38] and vaccinia-NP recombinants [39, 40]. Further studies are being done in the PMC Canada laboratory to elucidate the effector mechanisms responsible for mediating cross-protection induced by flu-ISCOMs.

The ability of flu-ISCOMs, compared with live virus or subvirion vaccine, to induce higher serum HAI titers and enhanced cross-protection against H2 influenza virus suggests that flu-ISCOMs may offer an advantage as an improved influenza vaccine in humans. ISCOMs have now been prepared with more defined fractions of Quil A suitable for use in humans [41]. Furthermore, flu-ISCOMs protect mice against virus challenge when given intranasally [42], offering the possibility of mucosal immunization strategies for future influenza vaccines.

Acknowledgments

We thank P. Chong and Y. Kwok for synthesis and characterization of peptides and S. Cockle, S. Loosemore, and U. McGuiness (Pasteur Mériex Connaught Research, Toronto) for reviewing the manuscript.

References