Induction of protective immunity against influenza virus in a macaque model: comparison of conventional and iscom vaccines

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Cynomolgus macaque monkeys (Macaca fascicularis) were immunized twice intramuscularly, either with a conventional non-adjuvanted subunit vaccine or with a candidate immune-stimulating complex (iscom) vaccine, each containing 10 µg envelope glycoprotein of a recent human influenza A(H3N2) virus (A/Netherlands/18/94). In contrast to the macaques vaccinated with the classical subunit vaccine, those immunized with the iscom vaccine developed high titres of specific IgM, IgA and IgG serum antibodies, as well as high titres of haemagglutination-inhibiting and virus-neutralizing serum antibodies. Also, specific proliferative T cell responses were only found in the iscom-vaccinated monkeys and their levels were similar to those found in monkeys experimentally infected with the homologous virus. Upon intratracheal challenge with the homologous virus, the iscom-vaccinated monkeys were completely protected from detectable virus replication in lungs, pharynx and nose, whereas those vaccinated with the classical subunit vaccines were not, or were only partially protected. The kinetics of specific serum antibody development in the iscom-vaccinated monkeys after challenge were quite similar to those of monkeys after secondary infection with the same virus. In contrast, the post-challenge kinetics of serum antibody development in the monkeys vaccinated with the classical subunit vaccines resembled those of naive monkeys, confirming that these vaccines only provided limited protection in such animals.

Introduction

Subunit, split and whole inactivated influenza virus vaccines, which are routinely used to protect humans against recurring influenza episodes, are produced with a combination of three influenza virus strains, annually recommended by the World Health Organization based on information about circulating viruses. Although these vaccines have been shown to reduce influenza-related morbidity and mortality considerably, there remains a need for more efficacious vaccines (Strassburg et al., 1986; Keren et al., 1988; Govaert et al., 1994). Besides considering alternative routes of vaccination, much attention is presently being paid to the evaluation of novel adjuvant and antigen presentation systems to increase vaccine efficacy. These include the use of bacterial cell wall components (Gupta et al., 1993), squalene in water emulsions (Ott et al., 1995), liposomes or virosomes (De Haan & Wilschut, 1995; Glück, 1995) and immune-stimulating complex (iscom) vaccines (Rimmelzwaan & Osterhaus, 1995b). Iscoms are of particular interest since they have not only been shown to be potent inducers of antibody and cell-mediated immune responses, but have also proved to induce high levels of protection against infection in many virus systems (for a review see Rimmelzwaan & Osterhaus, 1995b).

Influenza virus iscoms have been used successfully in mouse models, eliciting higher specific antibody levels, T cell responses and mucosal immunity than conventional vaccine preparations, which resulted in better protection (Lövgren, 1988; Lövgren et al., 1990; Ben-Ahmeida et al., 1992, 1994; Ghazi et al., 1995). Here we present the first data on the potential of influenza virus iscoms in a non-human primate model. In naive primates this form of antigen presentation proved not only to be more efficient in inducing antibody and T cell responses than classical subunit vaccines, but also provided absolute protection against homologous challenge infection.
Methods

**Virus preparations.** A virus isolate of the influenza epidemic of 1993/1994 in The Netherlands, A/Netherlands/18/94 (H3N2), was selected. This virus was isolated from a pharyngeal swab collected from a 10-month-old boy with influenza. The virus was passaged in tertiary monkey kidney (MK) cells once and subsequently biologically cloned by passing in MK cells three times under limiting dilution conditions. Finally, a virus stock was produced by collecting culture supernatant of MK cells (passage 8) and stored at −135 °C in the presence of 25% saccharose until use. The identity of the virus stock was confirmed in the haemagglutination inhibition (HI) assay using reference ferret sera directed to this and a panel of other viruses. MK cells were cultured in minimal essential medium (MEM) containing 10% foetal calf serum (Hyclone), penicillin (100 IU/ml), streptomycin (100 μg/ml), amphotericin (2.5 μg/ml), 1% vitamins (ICN Biomedicals) and 1% non-essential amino acids (ICN Biomedicals) and washed with PBS before infection. After infection, MK cells were cultured in MEM containing 4% bovine serum albumin (fraction V, Gibco-BRL), 1% vitamins, 1% non-essential amino acids (ICN Biomedicals), antibiotics and 4 μg/ml trypsin (Gibco-BRL) (infection medium) For the preparation of virus antigen used in ELISA and for T cell proliferation studies, 11-day-old embryonated eggs were inoculated with A/Netherlands/18/94. After clearance of the allantoic fluid by low-speed centrifugation the virus was concentrated 60-fold by centrifugation for 1 h at 100,000 g and resuspended in PBS.

**Vaccine preparations.** Iscoms were prepared from A/Netherlands/18/94 glycoproteins (flu-iscoms), essentially as described previously (Rimmelzwaan et al., 1994). In brief, influenza virus A/Netherlands/18/94 was harvested from embryonated eggs and concentrated by ultracentrifugation as described above. Subsequently, the virus was purified by density centrifugation on sucrose gradients. Purified virus was transferred to a commercial vaccine company, which used the material to formulate a vaccine batch (flu-glycoprotein preparation II) according to routine procedures. The haemagglutinin (HA) content of this preparation was determined by a single radial diffusion assay. In addition, purified virus was lysed in 2% decanoyl-N-methylglucamide (MEGA) (Bachem), and ribonucleaseprotein complexes were removed by centrifugation on 20% sucrose. The supernatant containing the viral membrane glycoproteins was split into two portions. One half was dialysed against PBS and was designated flu-glycoprotein preparation I. Quillaja glycosides (ISCOPREP 703, Isotec), cholesterol and phosphatidyl ethanolamine (5:1:1:1, by weight) were added to the other half of solubilized virus protein and the mixture was dialysed extensively against PBS. The quality of the iscom preparation was controlled by analytical gradient centrifugation which was performed in parallel with iscoms prepared with [3H]cholesterol. In this way it was demonstrated that >90% of the influenza virus proteins co-migrated with the radioactive cholesterol indicating that protein and lipids were complexed in the same structure. In addition, the iscom preparation was analysed by negative contrast electron microscopy, revealing the typical cage-like structure with a diameter of 40 nm. The protein concentration of the respective vaccine preparations was determined by the colorimetric method of Bradford (1976), adapted to a microtitre system. The HA content was also analysed by the haemagglutination assay and by Western blotting in order to confirm the nature of the proteins and their conformational integrity. Control iscoms were prepared with the envelope protein of simian immunodeficiency virus (SIV) essentially as described for the flu-iscoms (Hulsokette et al., 1995).

**Immunization of cynomolgus macaques.** Prior to immunization, young adult cynomolgus macaques bred in captivity were tested for the absence of influenza virus-specific serum antibodies in the HI assay using a panel of influenza viruses which circulated in the last decade. Monkeys were immunized with 10 μg (protein) of the respective vaccine preparations twice with an interval of 4 weeks. Two monkeys were immunized per vaccine preparation.

**Infection of cynomolgus macaques with A/Netherlands/18/94 H3N2.** Six months after the first immunization, two naive control monkeys and two which had experienced a primary infection were inoculated intratracheally (i.t.) with 107 TCID50 of the A/Netherlands/18/94 stock propagated on MK cells. Nasal swabs (NS), pharyngeal swabs (PS), lung lavages and heparinized blood samples were collected at days 2, 4, 6, 8, 11 and 15 post-infection. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood by centrifugation in a gradient containing 4% (w/v) dextran 500 (Sigma) and 32% (v/v) of a solution containing 32.8% (w/v) sodium metrizoate (Nycomed). After collection the cells were washed three times with RPMI 1640 medium containing penicillin (100 IU/ml) and streptomycin (100 μg/ml). Cells were then cryopreserved in medium containing 20% human serum and 10% DMSO at −135 °C. Plasma samples were stored at −20 °C until use. Lung lavages were analysed for the presence of cell-free virus by inoculating MK cells with tenfold serial dilutions of lung lavage fluid (LLF) obtained after centrifuging and separating supernatant and cells which had been cryopreserved. After culturing the MK cells for 7 days, the supernatants were tested for the presence of viral antigen in an HA assay using turkey erythrocytes. NS and PS were submerged in transport medium (infection medium without trypsin, with double concentration antibiotics), shaken before use and tenfold serial dilutions of the medium used to inoculate MK cells as described for LLF.

**Serological assays**

**HI assay.** The HI assay was performed essentially as described previously (Masurel et al., 1981), using turkey erythrocytes for agglutination and four haemagglutinating units of A/Netherlands/18/94.

**Virus neutralization (VN) assay.** Serial dilutions of the respective plasma samples, which had been heat-inactivated for 30 min at 56 °C, were incubated for 1 h at 37 °C with 100 TCID50 of the MK stock of A/Netherlands/18/94. The mixture was then transferred to MK cell monolayers in microtitre plates, which were washed before infection. After incubation for 1 h, the cells were washed with PBS and incubated in infection medium at 37 °C for 7 days. The supernatants of the cultures were then tested for HA activity.

IgG ELISA. ELISA plates (EIA/RIA high binding, Costar) were coated with concentrated virus disrupted in 1% Triton X-100 and diluted in 0.1 M carbonate buffer pH 9.6 containing 0.01% Triton X-100 for 16 h at room temperature. Subsequently, 0.1 ml volumes of the respective plasma samples were added to the wells. The samples were serially diluted in PBS containing 0.2% non-fat milk powder, 0.1% bovine serum albumin, 3% NaCl, 0.5% ovalbumin and 10% normal goat serum (ELISA buffer), starting at a 1:100 dilution. After washing with de-mineralized water containing 0.05% Tween 80, horseradish peroxidase-anti-mouse IgG (Fc) antibody preparation (Nordic) was added and incubated for 1 h at 37 °C. The plates were washed and 0.1 ml volumes of substrate solution (0.1 mg/ml tetramethylbenzidine (TMB) (Sigma) and 0.003 % H2O2 in 0.1 M sodium acetate buffer pH 5.5) was added. After 10 min the colour reaction was stopped by adding 0.1 ml 2 M H2SO4. The absorbance was read at 450 nm. IgG titres were expressed as the reciprocal of the serum dilution still giving Amax values more than three times the background value.

IgM ELISA. ELISA plates (EIA/RIA high binding, Costar) were coated with Fab, goat anti-human IgM antibodies (Organon Teknika-Cappel).
Anti-IgM coated plates were then incubated with 1:100 dilutions of individual monkey serum samples. Dilutions were made in ELISA buffer supplemented with 2% normal rabbit serum. Subsequently the plates were incubated with influenza virus A/Netherlands/18/94, which was disrupted in 1% Triton X-100 prior to dilution in ELISA buffer. The plates were then incubated with 1:25-diluted culture supernatant of hybridoma HB 65, which secretes MAb specific for the influenza virus nucleoprotein (ATCC HB65, H10-L10-4R5, originally established by Yewdell et al., 1981), followed by incubation with horseradish peroxidase-conjugated rabbit anti-mouse Ig antibody preparation (Dako). Between each incubation step the plates were washed with demineralized water containing 0.05% Tween-80. The plates were developed as described above and the absorbance was read at 450 nm. IgM titres were expressed as $A_{450}$ values.

IgA immunofluorescence assay (IFA). Madin Darby canine kidney (MDCK) cells were infected with A/Netherlands/18/94 at an m.o.i. of 0.2 TCID$_{50}$ per cell. After 6 h, the cells were harvested by trypsinization, air-dried onto microscope slides and fixed with ethanol at −70 °C for 30 min. After fixation, the slides were dried and washed twice with distilled water and once in PBS. Subsequently, serial dilutions were added of the respective plasma samples, which had been pretreated with a goat anti-Fc hyperimmune serum (Gullsorb, Gull Laboratories) in order to precipitate the IgG molecules. After washing, FITC-conjugated goat anti-monkey IgA antibody preparation (Nordic) was added and the slides were incubated for 1/2 h. The slides were then washed, embedded in glycerol and analysed using a fluorescence microscope.

T cell proliferation assay. Monkey PBMC were obtained by sedimentation of heparinized blood on dextran–sodium metrizoate gradients (as described above). PBMC were cultured in round-bottom 96-well microtitre plates (Costar) at a density of 10$^5$ cells per well in 150 µl RPMI supplemented with 10% (v/v) pooled human serum, penicillin (100 U/ml), streptomycin (100 µg/ml) and 2 mM L-glutamine. Concentrated A/Netherlands/18/94 was added at a concentration of 100 ng per well, which was found to be optimal for the stimulation of PBMC of human donors with histories of influenza virus infections. PBMC were incubated for 3 days at 37 °C with a pulse of 1 µCi $[^3]H$thymidine for the last 16 h. Cells were harvested and the incorporated $[^3]H$thymidine was measured in a scintillation counter (1205 Betaplate, LKB). The results are expressed as stimulation indices (SI), which represent the ratio of the mean proliferation of triplicate cultures in response to stimulation to that of medium controls. Proliferation of allantoic fluid antigen from non-infected eggs was always less than 15% of the response to viral antigen.

FACS-analysis. PBMC were cultured as described for the T cell proliferation assay. The expanded cell population was incubated with monoclonal antibodies directed against human CD4 (FITC-labelled, Ortho) and CD8 (PE-labelled, Becton Dickinson) for 45 min at 0 °C, washed with PBS and the fluorescence was measured in a FACSscan (Becton Dickinson).

Results

Virus-specific antibody responses

In a series of serological assays the development of virus-specific serum antibodies upon vaccination and infection was monitored in the monkeys (Figs 1–5).

IgM response. Monkeys immunized with flu-iscoms and monkeys infected with influenza virus A/Netherlands/18/94 developed a virus-specific serum IgM antibody response (Fig. 1), which was relatively short-lived (< 70 days and < 56 days, respectively). Immunization with A/Netherlands/18/94 glycoprotein preparations I and II and with SIV iscoms failed to induce a specific serum IgM response. Upon challenge infection 6 months after the primary immunization, the flu-iscom-immunized monkeys and the monkeys which had experienced a primary infection did not show a second influenza virus-specific IgM serum antibody response. However, all monkeys that had failed to develop IgM responses after immunization showed a specific IgM response after the challenge infection.

IgA response. Virus-specific IgA serum antibody responses were observed after infection with influenza virus A/Netherlands/18/94 and after vaccination with flu-iscoms (Fig. 2). The IgA titres declined to undetectable levels within 3 months in both groups of monkeys. In contrast to the IgM responses observed in these animals, IgA responses were again induced
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Fig. 2. Development of IgA plasma antibody responses to influenza virus A/Netherlands/18/94 after immunization or infection and subsequent homologous challenge infection. Two monkeys were immunized per group (○ and ●).

Fig. 3. Development of IgG plasma antibody responses to influenza virus A/Netherlands/18/94 after immunization or infection and subsequent homologous challenge infection. Two monkeys were immunized per group (○ and ●).

after challenge infection with A/Netherlands/18/94. In the monkeys immunized with SIV iscoms or flu-glycoprotein preparations I or II, no specific IgA serum antibody response was induced. After challenge infection, IgA responses similar to those found in naive control monkeys were demonstrated.

**IgG response.** Infection with influenza virus A/Netherlands/18/94 or immunization with flu-iscoms induced strong virus-specific IgG responses (Fig. 3). The highest antibody titres were observed after vaccination with flu-iscoms. The IgG antibody titres persisted for more than 6 months after primary immunization. One of the monkeys immunized with glycoprotein preparation II showed an IgG antibody response in which the titre declined to undetectable levels within 6 months. No IgG antibody response was demonstrated in the monkeys immunized with SIV iscoms or glycoprotein preparation I. Upon challenge infection, a strong booster response was observed in the monkeys with pre-existing IgG titres induced by immunization with iscoms or by infection. In all other monkeys virus-specific IgG responses were first observed after challenge infection. IgG antibodies tended to be detectable sooner in the monkeys immunized with either of the A/Netherlands/18/94 glycoprotein preparations I and II (6–8 days post challenge infection), as compared to naive monkeys or monkeys immunized with the control SIV iscom preparation (from 11 days post infection onwards), indicating that they had responded to vaccination by induction of a memory response.

**HI and VN antibody response.** The kinetics of the HI and VN antibody responses resembled those found for the IgG response (Figs 4 and 5). The HI and VN antibody responses also proved to be most prominent in the flu-iscom-vaccinated monkeys. The monkey that showed an IgG response upon immunization with glycoprotein preparation II also developed HI and VN antibodies, which again declined to undetectable levels within 6 months. The HI and VN antibodies in the flu-iscom-vaccinated monkeys persisted until challenge with minimum titres of 80 and 320, respectively.
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Fig. 4. Development of HI plasma antibody responses to influenza virus A/Netherlands/18/94 after immunization or infection and subsequent homologous challenge infection. Two monkeys were immunized per group (○ and ●).

Fig. 5. Development of VN plasma antibody responses to influenza virus A/Netherlands/18/94 after immunization or infection and subsequent homologous challenge infection. Two monkeys were immunized per group (○ and ●).

Virus-specific T cell responses

The in vitro proliferative responses of PBMC from monkeys immunized with the respective vaccine preparations are shown in Fig. 6. The two monkeys immunized with flu-iscoms showed the strongest in vitro proliferative T cell responses with SI of 3·4 and 9·2, respectively. In contrast, the monkeys immunized with the non-adjuvanted flu-glycoprotein preparations or with SIV iscoms did not show an in vitro proliferative T cell response (SI ranging from 0·8 to 1·8). PBMC from monkeys that were infected twice with A/Netherlands/18/94 did not show any in vitro proliferative T cell response. The specificity of these responses was demonstrated by showing that control antigen obtained from non-infected embryonated eggs failed to stimulate PBMC of the flu-immune monkeys (data not shown). In addition, T cell proliferative responses could not be measured in vitro in the PBMC obtained from any of the animals before vaccination.

Protection from challenge infection

Six months after immunization or primary infection all monkeys were infected i.t. with 10^7 TCID_{50} of the homologous influenza virus A/Netherlands/18/94 challenge stock. The monkeys which had previously experienced a primary infection were included in the challenge experiment as a positive control for protective immunity. They proved to be completely protected from the secondary infection since no virus could be isolated from their respiratory tracts (Fig. 7). Likewise, monkeys immunized with flu-iscoms proved to be completely protected from homologous challenge infection since again no virus could be isolated from their respiratory tracts (Fig. 7). In contrast, in monkeys immunized with the flu-glycoprotein preparation II, a virus isolation pattern was observed similar to that observed in the naive control monkeys. In both groups of monkeys, virus was isolated from LLF, PS and NS; however, clinical symptoms were not observed. From the macaques immunized with flu-glycoprotein preparation I, virus was isolated from LLF and PS, but not from NS. The virus titres
found in PS tended to be lower than those found in the control monkeys. In the monkeys immunized with the SIV-iscom preparation a virus isolation pattern was observed that was again similar to the one observed in the naive control monkeys.

**Discussion**

The use of the iscom antigen delivery system to enhance vaccine-induced immune responses has resulted in potent experimental vaccines against several viruses (for a review see Rimmelzwaan & Osterhaus, 1995b). Their potential as an adjuvant system for influenza vaccines was first shown in mouse models (Lövgren, 1988; Lövgren et al., 1990; Ben-Ahmeida et al., 1992, 1994; Ghazi et al., 1995), which eventually led to the development of the first commercially available iscom vaccine, used to control equine influenza (Sundquist et al., 1988; Mumford et al., 1994). In the present study, we showed for the first time that flu-iscoms can be administered safely to non-human primates without any signs of toxicity, while inducing strong immune responses and complete protection. In the same setting, non-adjuvanted viral proteins of the same origin, which closely resemble current subunit vaccines, were poorly immunogenic and failed to induce protection in naive cynomolgus monkeys. This illustrates that influenza virus iscoms may be considered a serious novel vaccine candidate for human influenza. We considered it necessary to use a primate model since the value of studies carried out in rodents with candidate vaccines intended for human use is limited due to major differences in anatomy and immune function between them. A primate model for influenza offers particular advantages for future studies concerning local vaccine application and induction of direct protection at mucosal surfaces. The reproducibility of the infection procedure in these monkeys was demonstrated by showing that similar virus isolation patterns were obtained from four naive monkeys upon i.t. infection in two independent experiments.

Since the effectiveness of current influenza vaccines for humans varies, largely depending on factors such as age and history of influenza virus infections, future studies with candidate vaccines will also be carried out in monkeys with a history of influenza virus infections, resulting in specific antibodies of different levels and specificities. In this light it is interesting to note that we have recently shown, in a cynomolgus monkey model for measles (Van Binnendijk et al., 1994), that measles virus iscoms efficiently induced measles virus-neutralizing antibodies in the presence of levels of pre-existing specific antibodies that abolished antibody induction by currently used vaccines (R. S. van Binnendijk, M. C. M. Poelen, G. van Amerongen, P. de Vries & A. D. M. E. Osterhaus, unpublished results). In our present study the iscom vaccine preparation induced antibodies of all immunoglobulin classes, with kinetics similar to those observed after infection with the homologous influenza virus.

The IgG, HI and VN antibody titres persisted for a period of more than 6 months (HI titres > 80), illustrating the longevity of the immunity induced by this vaccine preparation. It may therefore be speculated that vaccination with an influenza virus iscom vaccine would provide optimal protective immunity for the duration of the annually recurring influenza season. Although the observed booster response upon challenge of monkeys primed by primary infection or flu-iscom vaccination suggests replication of the challenge virus, this could not be confirmed by virus isolation. Furthermore, the possibility that the antigenic load in the challenge doses gave rise to this booster response cannot be excluded. In the cynomolgus monkeys, serum IgA titres declined to undetectable levels within 3 months post-infection or -vaccination, and upon challenge infection, in contrast to IgM antibodies, an IgA response reoccurred. This suggests that IgA antibodies may be more indicative of acute infection than IgM antibodies and may therefore be suitable as a serological diagnostic marker for acute infection. Although not studied in these experiments, we hypothesize that the iscom vaccine probably also induced secretory IgA (sIgA) antibodies in the nasopharyngeal area, since iscoms have been shown to induce mucosal immunity after parenteral administration (Thapar et al., 1991) and the monkeys proved to be protected from virus replication at this mucosal site after challenge infection. It has been demonstrated in mice that mucosal protection against...
infection with influenza virus is indeed mediated by sIgA at mucosal sites (Renegar & Small, 1991a, b).

We and others have demonstrated that iscoms may efficiently induce T helper cell and cytotoxic T cell (CTL) responses (Mowat et al., 1991; Takahashi et al., 1990; Heeg et al., 1991; Jones et al., 1988; Van Binnendijk et al., 1992), which may also be important for protection against influenza virus infection. In the present study the non-adjuvanted viral glycoprotein preparations failed to induce T cell responses in the macaques, whereas the iscom-based vaccine induced strong proliferative T cell responses. FACS analyses of the in vitro expanded T cell populations obtained from monkeys immunized with flu-iscoms or infected with live virus revealed the expansion of CD8⁺ in addition to CD4⁺ T cell populations. This was concluded because stimulation of PBMC with paraformaldehyde-fixed virus resulted in a lower percentage of CD8⁺ blast cells than in cultures stimulated with infectious virus (data not shown). To what extent CD8⁺ virus-specific
CTL may indeed contribute to vaccine-induced protective immunity or recovery after infection is, however, still a subject of debate (Rimmelzwaan & Osterhaus, 1995).

Taking the results together, the cynomolgus macaques proved to be a suitable non-human primate species to study protective immunity against influenza virus infection induced by experimental vaccines. The flu-iscom preparation induced an influenza virus-specific immune response in these animals that both in a quantitative and qualitative sense was superior to the one elicited by non-adjuvanted glycoprotein preparations and led to complete protection. This also implies that for the induction of a protective immune response with iscoms, less than the usually used 10–15 μg protein per dose will probably be needed. This may be of major importance for the large-scale production of vaccines against newly emerging pandemic strains where limited production capacity is one of the main problems.

Furthermore, the antibody response induced by vaccination with the influenza iscom preparation also proved to be broadly reactive. HI titres of 40 were found in the flu-iscom-immunized monkeys to the 1987 vaccine strain A/Sichuan2/87, which are considered to be protective. Therefore, in the case of epidemics with normally arising drift virus, the iscom vaccine preparations would probably also be less sensitive to mismatches with new virus strains. In view of these advantages over currently used vaccines, it seems justifiable to further evaluate the potential of flu-iscoms in humans.

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