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Research Article

Studies on the IgA-independent immunological responses in mice to influenza virus challenge after oral vaccination with irradiated whole virus and an erythrocyte complex

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Summary Previous studies have described an oral influenza vaccine comprising whole irradiated virus and an erythrocyte complex (IV-EC), which gave broad-based protection against influenza virus challenge in mice. The present study examined the immune responses generated after live virus challenge of vaccinated mice, particularly to determine whether mice vaccinated with IV-EC had enhanced CTL activity to compensate for the previously reported diminution in lung IgA response. Oral vaccine groups examined were IV-EC, live virus alone (LV) or live virus-erythrocyte complex (LV-EC), compared with irradiated virus and erythrocyte alone controls. The antibody responses of IV-EC and LV-EC vaccinated mice showed significantly elevated lung and serum IgG2a levels post live virus challenge, with no comparable increases in IgG1 levels compared to controls. Spleen cells from IV-EC mice showed an enhanced post-challenge proliferative response to antigen compared with mice that had received live oral vaccines, indicating enhanced cellular activity post IV-EC immunization. However, CTL activity was not enhanced for IV-EC mice, and live virus-vaccinated mice had reduced CTL activity compared with controls, indicating that CTL were not important for post-vaccine protection. Cytokine analysis revealed a predominant IFN-γ response in spleen cells from orally vaccinated mice, whereas IL-4 was not detected in any lung or spleen culture analysed. The results suggest, therefore, that protection from live influenza challenge after IV-EC or LV-EC vaccination was due to an IFN-mediated IgG2a response. Definitive confirmation of the role of these factors in post-vaccine protection can now be tested in IgG2a-depleted or IFN-γ gene knockout mouse models.

Key words: erythrocyte complex, immunoglobulin A response, influenza virus, irradiated virus, oral vaccination.

Introduction

Immunity to influenza virus infection in humans and rodents has been shown to involve both humoral and cell-mediated pathways. There has been substantial ongoing interest in determining whether humoral or cell-mediated immunity is the most important, or if they are both of equivalent importance to protection and recovery from infection.[1,2] Such fundamental knowledge on the immune response to influenza infection is crucial to the development of vaccines and the special vaccination challenges presented by this virus. The development of mucosal vaccines for influenza virus[3] offers an alternative strategy to traditional parenteral vaccine strategies as well as the advantages of the common mucosal immune system, which allows for vaccine-mediated priming of an immune response in distal mucosal sites post oral administration. Oral vaccines would also offer practical advantages, particularly in terms of administration to the patient.

Previous studies have highlighted the effectiveness of an orally administered, γ-irradiated influenza virus and an erythrocyte complex to protect against both homologous and heterologous influenza challenges.[4,5] It has been demonstrated that the dose of irradiation used to inactivate the virus is critical to the generation of a lung antibody response, with IgA particularly sensitive to this factor.[5] When the 30 kGy dose of γ-irradiation was used to inactivate vaccinating virus, the IgA response was ablated in the lungs while the lung IgG response was still detectable; serum IgG and the capacity of the vaccine to protect from live virus challenge was unaffected.[5] In this investigation, we describe further studies on mechanisms that underpin the ability of orally delivered irradiated virus–erythrocyte complexes to protect mice from live virus challenge. The relative importance of humoral and cell-mediated immunity in oral vaccine-primed protection from influenza infection is also discussed.

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Materials and Methods

Virus

Influenza virus strain A/Queensland/6/72 (H3N2) stocks were grown in embryonated chicken eggs, concentrated, purified and the titre of the virus stocks determined exactly as reported previously.[5]

Virus inactivation

Concentrated virus stocks (8.7 $\log_{10}$ plaque forming units (PFU)/mL, HA:2560 units) were inactivated by 30 kGy gamma irradiation from a ^{60}Cobalt source as previously reported.[5] At this γ-irradiation dose, viral haemagglutinin (HA) activity was retained at 100% compared to live virus controls.

Mice

Six- to eight-week-old male Swiss albino (outbred) and CBA/H (inbred) mice were used in this study (supplied by the University of Newcastle Animal Facility).

Mice vaccination and live virus challenge protocols

The protocols for the preparation and administration of the whole influenza/erythrocyte oral vaccines, as well as the protocol for the intranasal infection of mice with live A/Qld/6/72 virus, were followed exactly as reported previously.[5] Oral vaccines were given on days 1, 3, 5, 12 and 19; protection has also been found when irradiated virus–erythrocyte complex (IV-EC) oral vaccines were administered on days 1, 3 and 5, with live challenge delayed for 2 weeks after the last dose.[4]

The final vaccine formulations were live virus–erythrocyte complex (LV-EC), IV-EC, live virus alone (LV), irradiated virus alone (IV) and chicken red blood cells alone (cRBC).

Live challenge virus was detected in the lungs of control mice, indicating that the anaesthesia had not inactivated the virus inoculum.[4,5]

Spleen cell proliferation

CBA/H mice were orally vaccinated, challenged and killed following the previously reported protocols.[5] Spleens were removed, pooled and passed through sterile grids to produce a single cell suspension. These cells were plated into flat-bottomed 96-well trays at a concentration of 10^6 cells/well in DMEM plus 5% heat-inactivated FCS (CSL, Melbourne, Vic, Australia). To these cells, media alone (control), irradiated (30 kGy) A/Qld alone, 10^7/mL cRBC alone or irradiated A/Qld + 10^7/mL cRBC were added. Tritiated [^{3}H]-thymidine (Amersham, Buckinghamshire, UK, 37 MBq) was added to these cultures at a concentration of 37 kBq/well and the cultures incubated for 24 h at 37°C, after which the cells were harvested onto glass fibre mats (ICN, Sydney, NSW, Australia), scintillant added and the samples counted on a liquid scintillation counter.

Enzyme-linked immunosorbent assay analyses of IgG subclasses

Analyses of sera and lung samples by ELISA for specific anti-A/Qld/6/72 antibodies were performed exactly as previously reported.[5] The detection of IgG subclasses was achieved by treating whole A/Qld/6/72 virus-coated wells with a 10^{-3} dilution of either anti-IgG1 or anti-IgG2a biotinylated monoclonal antibodies (PharMingen, San Diego, CA, USA) after the addition of the diluted serum and lung samples. Total IgG levels were detected using a 10^{-3} dilution of a biotinylated antimouse IgG γ-chain antibody.

Cytotoxic T lymphocyte assays

Groups of CBA/H or Swiss mice were vaccinated orally and challenged exactly as reported earlier. At day 4 post-infection, mice were killed and spleens and lungs collected. Spleens from individual mice were passed through sterile grids to produce a single cell suspension. Lungs were individually homogenized on ice in 10 mL PBS (containing 0.25% gelatin with antibiotics), after which the tissue debris was allowed to settle and the cells were collected from the supernatant before being transferred to a fresh tube. Erythrocytes were removed from the lung and spleen cell preparations by water lysis (10 × HBSS was added to restore osmotic conditions). The effector cells were counted, washed three times and then dispensed into 96-well round-bottomed tissue culture trays in DMEM-10% FCS at E:T cell ratios of 60:1 for spleen cells and 20:1 for lung cells.

L929 (H-2^k, fibroblasts) or P815 (H-2^d, DBA mastocytoma) target cells were infected with A/Qld/6/72 at a multiplicity of infection (MOI) of 25 (2×10^6 target cells in 200 μL DMEM-10% FCS). After incubation for 1.5 h at 37°C, the infected cells and non-infected control cells were washed three times, resuspended in DMEM-10% FCS, and added to the effector cells at a final concentration of 2×10^4 cells/well.

The plates were incubated at 37°C (5% CO_2/humidified) for 6 h. Cytotoxicity was measured by a non-radioactive methodology, which relied on the detection of lactate dehydrogenase (LDH) released from lysed cells into culture supernatants (CytoTox 96, Promega, Madison, WI, USA). For this assay, the spontaneous release of LDH from the effector cell population also needed to be taken into account for the final calculation of percentage cytotoxicity; this was subtracted from the experimental release value. The final estimate of cytotoxicity was calculated by the following formula:

$$\%\text{ Specific lysis} = \frac{\text{Experimental release} - \text{spontaneous release}}{\text{Maximum release} - \text{spontaneous release}} \times 100$$

Maximum release was determined by incubating control cells with 1.0% Triton-X detergent. Cytotoxicity for non-infected target cells was found to be less than 10%. Cells from non-infected mice were found to have less than 10% cytotoxicity against infected target cells.

Cytokine ELISA measurements

Single cell suspensions from spleens and lungs were plated at 10^6 cells/well in DMEM-5% FCS. The appropriate wells were infected with 10^5 PFU A/Qld or treated with media alone (controls). After 3 days of incubation at 37°C (5% CO_2/humidified), supernatants were collected and assayed for IFN-γ or IL-4 using ELISA. Biotinylated antibodies against these cytokines were purchased from PharMingen and the assays were performed following the manufacturer's instructions. Blocking was done using PBS containing 3% BSA and the streptavidin-peroxidase conjugate was purchased from Amersham and was used at a dilution of 10^{-3}. A positive result was taken as a reading greater than two standard deviations above the mean background absorbance at 450 nm. The final concentration of each cytokine was calculated from a standard curve using the appropriate recombinant cytokine (supplied with the antibodies).

Statistical analysis

Groups of three to five mice were used in each experiment, with each study performed at least three times. Unpaired *t*-test analyses were performed to compare the means of results

Table 1 Detection of IgG1 and IgG2a antibody responses to A/Qld/6/72 virus in serum and lung samples from orally vaccinated and live A/Qld challenged (intranasal) mice using ELISA

Vaccine regimen	Lung Total IgG	Lung IgG1	Lung IgG2a	Sera Total IgG	Sera IgG1	Sera IgG2a
cRBC	0.064 ± 0.002	0.019 ± 0.008	0.032 ± 0.005	0.109 ± 0.01	0	0
IV	0.081 ± 0.008	0.018 ± 0.008	0.028 ± 0.006	0.127 ± 0.01	0	0
LV	0.924 ± 0.016	0.026 ± 0.005	0.111 ± 0.003	0.890 ± 0.033	0	0.102 ± 0.005
IV-EC	0.828 ± 0.019	0.035 ± 0.005	0.070 ± 0.004	0.852 ± 0.023	0	0.053 ± 0.003
LV-EC	0.894 ± 0.028	0.068 ± 0.01	0.120 ± 0.01	0.910 ± 0.035	0.050 ± 0	0.088 ± 0.01

Data are the mean optical density – background (450 nm) ± SD. Chicken red blood cells (cRBC) were administered orally at a dose of 10^8/mL for both controls and virus complexes. Total IgG was detected by a biotinylated mAb against IgG-γ chain. CBA/H mice (n = 5) were orally vaccinated on days 1, 3, 5, 12, 19, challenged with live A/Qld on day 22 and organs and blood collected on day 25. No IgA was detected in the lungs of irradiated virus (IV)–erythrocyte complex (EC)-vaccinated mice, nor was IgA detected in the serum of any vaccine regimen examined. LV, live virus.

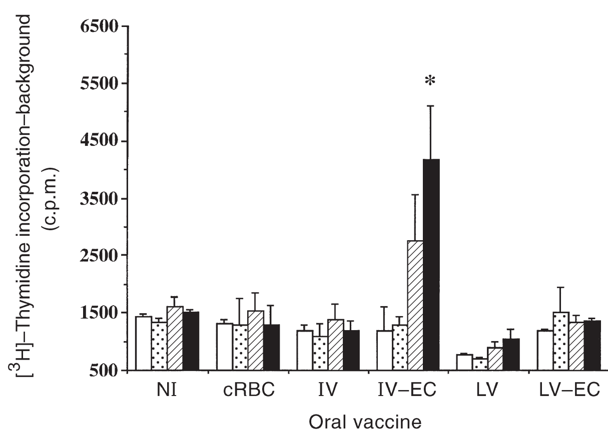

Figure 1 *In vitro* proliferation of spleen cells from orally vaccinated and live A/Qld-challenged (intranasal) CBA/H mice (n = 3). (□), Media; (⚃), chicken red blood cells (cRBC); (▨), 30 kGy A/Qld; (■), 30 kGy A/Qld plus cRBC. *$P < 0.05$ compared with the same stimulation conditions for irradiated virus (IV)-vaccinated mice. NI, not immunized; EC, erythrocyte complex; LV, live virus.

obtained from experimental and control groups. Comparisons were considered to be statistically significant at a probability level of less than or equal to 5% ($P \leq 0.05$).

Results

Immunoglobulin G subclass detection in serum and lung homogenates

Enzyme-linked immunosorbent assays for the detection of specific IgG1 and IgG2a responses to A/Qld were performed for serum and lung samples from groups of orally vaccinated mice (Table 1). These studies showed that for mice vaccinated with LV-EC, both IgG1 and IgG2a were detected in serum and lung samples. Both subclasses of IgG were detected in the lungs of LV and IV-EC vaccinated mice, whereas only IgG2a was found in the serum of mice receiving these oral vaccines. In the serum of the control IV vaccinated mice, neither IgG1 nor IgG2a were found. Both antibody subclasses were detected in the IV lung samples, but at levels less than those found for the cRBC control vaccine. For LV, LV-EC and IV-EC vaccines, IgG2a was detected at higher levels in the lung compared with IgG1.

Antibodies from IV-EC and LV-EC vaccinated mice were isolated from lung and serum samples by protein A purification. It was found that IgG2a was present in serum at high concentrations (LV-EC = 105 μg/mL, IV-EC = 70 μg/mL), with IgG1 also detectable, but at lower concentrations (LV-EC = 34 μg/mL, IV-EC = 29 μg/mL). The dominance of IgG2a suggests a Th1 response to challenge virus, but cytokine studies were required for confirmation.

Spleen cell proliferation responses in orally vaccinated mice to in vitro *influenza stimulation*

Figure 1 shows that mice that had been immunized orally with IV-EC had the highest spleen cell proliferative response to *in vitro* stimulation by 30 kGy virus and cRBC. The proliferative response by IV-EC spleen cells was also significantly increased ($P < 0.05$) by 30 kGy virus alone stimulation, compared with the cRBC alone and media alone stimulated controls. The comparative proliferation of IV-EC spleen cells after either 30 kGy virus alone or 30 kGy virus and cRBC *in vitro* stimulation was not significantly different ($P > 0.1$). Spleen cells from IV vaccinated mice showed no increased proliferation to *in vitro* stimulation compared with cells from non-treated and cRBC-vaccinated control animals.

The spleen cell proliferation results for IV-EC mice were in contrast with the responses found for LV- and LV-EC-vaccinated mice. Cell proliferation responses to *in vitro* stimulation by 30 kGy virus (± cRBC) for both live vaccines were lower than for mice vaccinated with IV-EC and did not exceed the proliferative responses observed for identically stimulated cells from non-immunized mice.

Cytotoxic T lymphocyte responses in the lungs and spleens of orally vaccinated mice

CBA/H mice orally vaccinated with cRBC alone showed a detectable primary CTL response in the spleen 4 days after intranasal live influenza challenge (Fig. 2a), similar to that found for infected, non-immunized mice (data not shown).

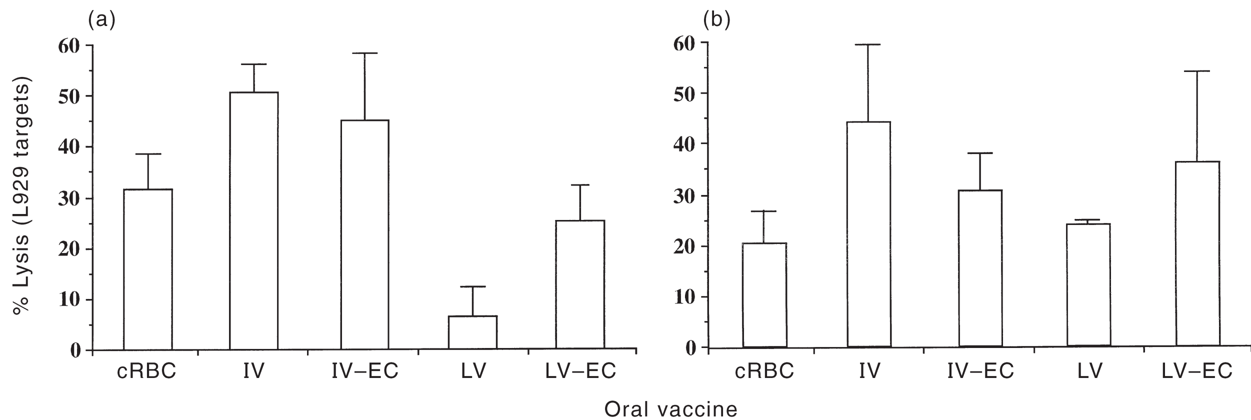

Figure 2 Activity of CTL in the spleens (a) and lungs (b) of orally vaccinated and live A/Qld-challenged (intranasal) CBA/H mice ($n = 3$). Spleen data represent the mean 60:1 effector:target lysis ± SD. Lung data represent the mean 20:1 effector:target lysis ± SD. Activity of CTL in chicken RBC (cRBC)-vaccinated and intranasally challenged mice was identical to that found for mice that had not been orally vaccinated prior to intranasal infection. IV, irradiated virus; EC, erythrocyte complex; LV, live virus.

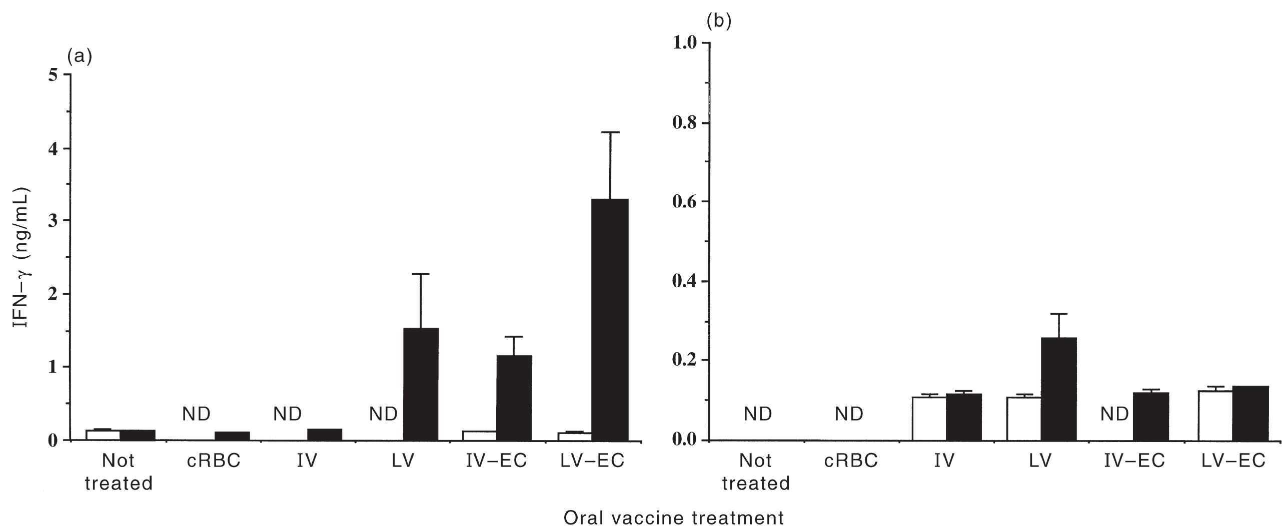

Figure 3 Interferon-γ production in whole spleen (a) and lung (b) *ex vivo* cell cultures from orally vaccinated and intranasally A/Qld-challenged CBA/H mice ($n = 3$). Cells were stimulated *in vitro* with live A/Qld virus (■; multiplicity of infection = 0.1) in DMEM-FCS or treated with DMEM-FCS alone (□; control cultures). ND, not detected.

Compared with cRBC vaccination, the mean CTL response found 4 days after challenge was significantly increased for IV vaccination ($P < 0.025$), but not for the IV-EC vaccination group ($P > 0.15$). The LV oral vaccination resulted in a significant inhibition of CTL response compared with both the cRBC and IV vaccine controls and the LV group was also found to have significantly lower CTL activity in comparison with the LV-EC vaccination group ($P < 0.025$). Studies with outbred Swiss mice showed similar results, with both IV-EC and LV-EC oral vaccine groups showing significantly decreased spleen CTL activity compared with the cRBC control ($P < 0.03$, $P < 0.01$, respectively; data not shown).

Lung CTL responses (Fig. 2b) were found to be reduced for LV-vaccinated mice in comparison with the IV, IV-EC and LV-C vaccine groups, but this was not as marked as that found in the spleen. However, due to large variations of CTL activity in the lung samples from these vaccine groups, the results were not significant compared to the controls ($P > 0.05$).

For both inbred and outbred mouse strains, the LV-EC and IV-EC oral vaccine regimens tested did not stimulate enhanced secondary CTL activity compared with the primary CTL responses to live intranasal challenge observed for the cRBC alone and IV controls. Augmented CTL function was not, therefore, a factor in the clearance of live challenge virus from IV-EC or LV-EC vaccinated mice.

Cytokine analysis

To investigate the nature of the T cell involvement in orally vaccinated mice, spleen and lung cells were cultured for 3 days (± A/Qld) and supernatants collected for IFN-γ and IL-4 analyses. Figure 3a shows elevated IFN-γ concentrations detected in virus-stimulated spleen cell cultures from both LV and LV-EC vaccinated mice, with a mean enhancement of IFN-γ levels found for LV-EC cultures compared with LV cultures that was not statistically significant ($P = 0.20$). An IFN-γ response was also detected in virus-stimulated cells from IV-EC vaccinated mice, which was significantly increased ($P = 0.02$) compared with virus-stimulated spleen cells cultured from IV-vaccinated animals. The IFN-γ response was not significantly higher for LV-EC-vaccinated mice compared with IV-EC-vaccinated mice ($P = 0.09$).

For lung cell cultures (Fig. 3b), only virus-stimulated cells from LV-vaccinated animals exhibited a mean IFN-γ increase compared with non-infected control cultures from the same vaccine group, but this increase was not statistically significant ($P = 0.06$).

Interleukin-4 protein was not detected in lung or spleen cultures from any control or oral vaccine group.

Discussion

Previous reported studies have shown that whole influenza virus–erythrocyte complex oral vaccines prime for specific local and systemic antibody responses, as well as protection from live heterologous and homologous virus challenge as determined by significantly decreased lung virus titres (2.5–4.3 $\log_{10}$ PFU/mL reduction post homotypic or heterotypic challenge).[4,5] Live virus oral vaccination was effective with and without the erythrocyte carrier, but IV was only capable of inducing protection if in complex with an erythrocyte carrier. In vaccines consisting of IV-EC, clearance of challenge virus was found in the absence of a local lung IgA response,[5] suggesting an enhanced role for CTL post IV-EC vaccination. A recent study,[6] using gene knockout technology to suppress the expression of IgA, found that IgA production was not essential for the protection of mice from influenza infection and disease, giving support to our previous studies[5] as well as impetus to further explore immunity in an IgA-deficient model of influenza infection.

Cytotoxic T lymphocytes generated during a primary response to influenza infection in mice have been known for some time to be effective against influenza-infected cells *in vitro*.[7] The post-vaccination importance of cell-mediated immunity is of interest as a possible mechanism to target conserved influenza proteins and, therefore, provide broad-based protection. Studies suggest that such cell-mediated activity is found in mouse models. For example, a study has shown that T cell-mediated protection is possible in mice devoid of antibody and B cells,[8] while another study has found that $CD8^+$ T cells are independently effective against low doses of challenge virus.[9] Furthermore, gamma-irradiated influenza virus has been found to prime for memory CTL responses, which were protective for mice exposed to lethal heterologous influenza virus challenge.[10] The immunogenicity of internal proteins for CTL has been shown by work studying the priming efficacy of influenza virus nucleoprotein, which has found that significant *in vitro* CTL activity is induced and enhanced protection conferred for virus-challenged mice.[11,12] Enhanced cellular proliferation was demonstrated for IV-EC mice in the present study, compared with LV-, LV-EC- and control-vaccinated mice. This result suggests a difference between the IV-EC versus LV/LV-EC vaccination approaches in terms of the timing of cellular responses to live virus challenge. Whether this results in CTL activity differences was the focus of further experiments.

While secondary *in vitro* stimulation has been shown to enhance CTL activity for *in vitro* assays,[7] secondary stimulation *in vivo* via repeated oral vaccinations with influenza antigens did not enhance CTL responses to challenge virus in the present study. When considering the value of CTL responses in broad-based vaccines, as discussed earlier, it appears superficially from the CTL data presented here that the oral vaccines may be at a significant disadvantage. However, oral vaccination provides significant cross-protection, as measured by challenge virus clearance from the lung;[4] these results therefore suggest that protection is antibody mediated. The importance of antibody responses to broad-based protection from influenza infection have been supported by previous studies. Cross-reactive IgA, raised against whole virus, has been found to correlate with protection against heterologous challenge, whereas CTL activity did not.[13] Another study has found that HA-reactive IgA is cross-protective for mice, provided that the optimum concentration is present in the respiratory tract at the time of infection.[14] The role of IgA in cross-protection following oral vaccination is questionable for our model, because lung IgA was undetectable in mice vaccinated orally with IV-EC, but significant clearance of challenge virus was still found;[5] IgG-based cross-protection post IV-EC oral vaccination is therefore implicated. It has been found in anti-influenza responses that IgA has greater cross-reactive activity than IgG,[14] but another study on boosting and priming via a mucosal route (intranasal) with inactivated influenza virus plus cholera toxin has found that both cross-protective IgA and IgG were generated.[15] From this evidence, our results suggest that if the IgA response was deficient, compensatory cross-reactive IgG can provide protection from live virus challenge. Also of interest to antibody cross-protection has been the identification of a conserved transmembrane influenza protein (M2), which is an effective target for antibody-mediated heterologous protection in BALB/c mice.[16] The possibility therefore exists that anti-M2 responses primed for by the whole virus-based oral vaccines used in the present study also contributed to the broad-based protection previously observed,[4] particularly because we have found that both total serum IgG antibodies and protein A fractionated IgG2a antibodies isolated from IV-EC and LV-EC vaccinated mice had *in vitro* reactivity to influenza core antigens (data not shown).

The identification of the immune responses observed to live virus challenge after oral vaccination as either the Th1 or Th2 phenotypes was pursued. The finding that the antibody response prominently featured serum IgG2a (Table 1) immediately suggested that Th1 mechanisms were involved. This suggestion was confirmed by cytokine analyses, where IFN-γ was detected in virus-stimulated spleen cell cultures from LV-, LV-EC- and IV-EC-vaccinated mice. Interleukin-4

was also examined, but was not detected in spleen or lung cell cultures from any vaccine or control group, supporting the conclusion that Th1-driven IgG2a responses were of greatest importance to challenge virus clearance. A previous report on the IV-EC vaccine used reverse transcriptase polymerase chain reaction techniques to semiquantify the transcription of the IFN-γ and IL-4 genes in Peyer's patch T cells.[17] This study found strong transcription of IFN-γ genes, but also detected message for IL-4. The present study confirms these observations for IFN-γ, but suggests that IL-4 gene transcription does not result in the secretion of IL-4 protein by influenza-immune cells. A recent study compared the functions of IFN-γ during an influenza infection in mice and found that IFN-γ production at immunologically relevant sites did not lead to an accumulation of $CD8^+$ cells, nor was the cytolytic activity of these cells impaired when IFN-γ was neutralized.[18] This study proposed that the crucial activities of IFN-γ for influenza infection are the regulation of cell migration and cell retention in the lung parenchyma, as well as the generation of antiviral IgG2a responses. These findings support the conclusion that for the oral vaccine model reported here, the mechanism of protection from A/Qld virus challenge was an IFN-γ-stimulated IgG2a response.

The study of oral vaccines in the mouse model and the proposition of the importance of particular IgG subclass antibodies to protection post-vaccination has direct relevance to the development of human vaccines, particularly because the generation of protective IgG serum antibodies is considered fundamental to the success of human influenza vaccines. The IgG subclass response has been found to depend on whether the influenza vaccine is live or inactivated, but generally the IgG1 subclass has been favoured as the mediator of post-vaccine protection, particularly as human IgG1 can activate the complement cascade and participate in antiviral antibody-dependent cell-mediated cytotoxicity mechanisms.[19–21] An oral vaccine study has also been performed in humans;[22] this investigation has shown the dissemination of antigenically stimulated immunocompetent cells to distal mucosal sites while also detecting the capacity of these cells to generate a local immune response. This led the authors to conclude that the common mucosal immune system is functional in the human and therefore available for utilization in the delivery oral vaccines, provided that effective delivery systems (e.g. liposomes) are available.

Investigation of immune mechanisms underpinning challenge virus clearance in orally vaccinated mice found that the IgG subclass response was similar for IV-EC and LV-EC oral vaccines, with IgG2a found to be predominant. The CTL activity relative to cRBC alone and virus alone controls was not enhanced for either LV-EC or IV-EC oral vaccines, but this did not impact on the effectiveness of challenge virus clearance post-vaccination. It was hypothesized that the deficiency in lung IgA response for IV-EC vaccinated mice[5] may result in some compensatory $CD8^+$ T cell activity, but this was not found. The production of IgG2a, stimulated by IFN-γ activity, was suggested by our findings to be the mechanism responsible for challenge virus clearance post oral vaccination for both LV-EC and IV-EC vaccines.

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