VIROLOGY

Induction of IL-2 and IFN- γ in BALB/c mice immunised with subunit influenza A vaccine in combination with whole cell or acellular DTP vaccine

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Splenocytes from mice immunised with two doses of subunit influenza A/Beijing/353/89 vaccine mixed with whole cell DTP (wDTP), acellular DTP (aDTP) or PBS were collected 7 and 10 days after the second immunisation, and re-stimulated with subunit influenza vaccine or live virus *in vitro*. Interleukin-2 (IL-2) and interferon- γ (IFN- γ) were assayed in supernates from these cultures by an ELISA procedure. Splenocytes from mice given subunit influenza vaccine in wDTP produced greater than two-fold and greater than five-fold responses of IL-2 and IFN- γ , respectively, compared with splenocytes from mice immunised with subunit vaccine alone. In contrast, the response of splenocytes from mice immunised with subunit vaccine in saline or aDTP was similar, significantly less than for vaccine in wDTP ($p < 0.01$) and only slightly greater than for controls ($p < 0.05$). The production of IL-2 and IFN- γ by these spleen cells was not significantly different on days 7 and 10 post-immunisation. Previous reports have shown that wDTP and aDTP enhance the serum antibody response of mice to influenza vaccine, but wDTP enhanced the response 100-fold greater than aDTP, and induced greater IgG2a and IgG2b subclass antibody responses; this last result indicates a cell-mediated immune response to vaccine. The present studies confirm these earlier findings; furthermore, as the IL-2 and IFN- γ responses of splenocytes are associated with Th-1 subset T-lymphocyte response, the findings indicate a cytotoxic T-cell response to immunisation. The results indicate that influenza vaccine combined with wDTP induced a cell-mediated response in mice, which could confer a more solid immunity to challenge virus infection.

Introduction

Influenza is a major cause of morbidity and mortality, particularly among the elderly and other at risk groups. The current available vaccines confer protection on only 60–90% of vaccinees, with a lower rate in the elderly population [1, 2], but reduce hospitalisation in a higher percentage of persons. The failure of these inactivated vaccines to protect a greater percentage of vaccinees may be due to several factors, including virus variation, vaccine preparation in eggs, poor local antibody production and lack of cell-mediated responses [3]; the importance of these responses in immunity has been reported in numerous studies [4, 5]. In particular, studies in mice and human subjects

suggest that MHC class-I restricted cytotoxic T lymphocytes (CTL) may have a role in immunity to influenza [5, 6].

Inactivated influenza vaccines have been shown to produce serum antibody responses but poor CTL responses [7]. However, a measurable MHC class-I CD8⁺ CTL response has been observed by others [8, 9]. In contrast, a marked CTL response has been observed after live virus vaccine infection and this may be related to the more solid immunity seen following infection [3]. In earlier studies, subunit influenza A virus vaccine in diphtheria-tetanus-pertussis (DTP) vaccine was shown to give an enhanced serum IgG antibody response in mice, a broader IgG subclass antibody response and a more solid immunity against virus infection than that seen following immunisation with saline vaccines [10, 11]. In addition, influenza vaccines in DTP induced serum IgG2a

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and IgG2b antibody responses which indicated a response of Th-1 subset lymphocytes [11, 12], which are mediators of CTL responses. To test this more rigorously, the CTL response of mice to subunit influenza vaccine in saline, whole-cell DTP (wDTP) or acellular DTP (aDTP) was investigated. Lymphocyte cultures were prepared from groups of immunised mice and stimulated with either subunit influenza vaccine or live virus. The supernates were then removed and assayed for IL-2 and IFN- γ , which are secreted by Th-1 lymphocytes, to seek an explanation for the more solid immunity seen in mice immunised with vaccine in DTP, to provide further evidence of a CTL responses and to compare the relative merits of wDTP and aDTP in potentiating immune responses to influenza vaccine.

Materials and methods

Influenza virus and virus vaccine

Seed influenza virus A/Beijing/353/89 (H₃N₂) was kindly supplied by Dr T. Carstairs, Evans Medical, Speke, Liverpool. A virus pool was prepared by inoculating 0.2 ml of a 10³ dilution of seed virus into the allantoic cavity of 10–11-day embryonated chicken eggs; after incubation at 37°C for 72 h, allantoic fluids were harvested, sealed in ampoules and stored at –70°C. Virus infectivity was determined by inoculating 0.1 ml of 10-fold dilutions of virus in phosphate-buffered saline (PBS), pH 7.2, into embryonated eggs; inoculated eggs were incubated for 72 h at 33°C before the allantoic fluids were tested for virus by haemagglutination with fowl erythrocytes. From the results obtained the 50% egg infectivity dose (EID₅₀) was calculated.

The influenza virus used for vaccine preparation was influenza virus A/Beijing X109, a reassortant of influenza viruses A/Beijing/353/89 (H₃N₂) and A/PR/8/34 (H₁N₁). The subunit vaccine prepared from this virus was the influenza A (H₃N₂) component incorporated into the commercial purified influenza surface antigen vaccine 'Flurigen' for 1992/93, and was kindly supplied by Evans Medical. The concentration of haemagglutinin (HA) in the subunit vaccine was calculated by single radial diffusion. Standard reagents were kindly supplied by Dr J. Wood, National Institute for Biological Standards and Control, Potters Bar, London.

Adjuvants

Diphtheria, tetanus and whole cell pertussis vaccine (wDTP) and acellular DTP (aDTP) were kindly supplied by Evans Medical. In both cases these were inoculated into mice at a 1 in 4 dilution of the recommended human dose which was the highest dose tolerated by mice. Each mouse was inoculated with 5.0 μ g of virus HA in wDTP, aDTP or saline into the

thigh; the inoculum of 0.225 ml produced a bleb which disappeared within 1 h of inoculation.

Animals

BALB/c mice were obtained from the closed, randomly bred colony held at the University of Sheffield. Mice were used at age 8–10 weeks when their weight was *c.* 20–25 g.

Experimental protocol

Three groups of BALB/c mice (four animals/group) were immunised with 5 μ g of HA of subunit A/Beijing X109 mixed with wDTP, aDTP or saline; further control groups received wDTP, aDTP or PBS alone. Two doses of each vaccine were given at 11-day intervals. Seven and 10 days after the second immunisation, the spleens of two mice in each group were removed, pooled and disrupted and the cells were further disassociated by gently flushing through a syringe and needle with 10 ml of RPMI medium. To lyse the red blood cells, 5 ml of RBC lysis solution (NH₄ Cl 8.3 mg/ml, KHCO₃ 1 mg/ml and EDTA 37 μ g/ml in distilled water) was added to the splenocyte cell pellet, the cells were lightly centrifuged and the supernate was discarded. For separation of T cells, the cell pellet in 2 ml of RPMI media was layered into 5 ml of separation media (Lymphoprep; Tech Gen International Ltd) in plastic universal tubes, and centrifuged at 1800 rpm; after this procedure, the interface layer containing the T cells was collected, washed and counted in a trypan blue 1% solution to determine the number of viable cells.

For proliferation of T lymphocytes, feeder cells from normal mouse spleens were treated with mitomycin C 50 μ g/ml and incubated for 30 min at 37°C; the cells were then washed three times in RPMI containing fetal calf serum (FCS) 1%, and counted as described above. Feeder cells (2.5×10^6 /ml) and T cells (5×10^6 /ml) were mixed together and dispensed in a volume of 200 μ l/well in a 96-well tissue culture plate and stimulated with 2 μ g HA subunit influenza A/Beijing/353/89 virus/well or 2 μ g of live influenza A/Beijing/353/89 virus protein/well. The plates were incubated for 5–6 days at 37°C in an atmosphere of CO₂ 5%. After this time 100 μ l of supernate was collected aseptically and stored at –70°C before determination of supernatant IL-2 and IFN- γ . Each measurement was carried out in eight wells and the mean value (and SD) was determined.

Cytokine (IL-2, IFN- γ) assay by ELISA

Culture supernates were assayed for cytokines in a sandwich ELISA test, by a modification of a published method [13]. Ninety-six well plates were coated by inoculating each well with purified rat monoclonal anti-cytokine antibody against mouse IL-2 or IFN- γ and

incubated overnight at 4°C; these monoclonal antibodies were used at a concentration of 2 µg/well in a volume of 50 µl. Sites that bind protein non-specifically were blocked by adding PBS containing FCS 10%, 200 µl/well, for 2 h at room temperature. The plates were then washed three times with PBS-Tween 20 0.05%; after this, 100 µl of supernate samples or control or medium, were added to each well and the plates were incubated for 2 h at 37°C. The plates were again washed with PBS-Tween 0.5%, and 100 µl of biotinylated anti-cytokine antibody, 0.5 µg/ml, were added to each well. After incubating the plates at 37°C for 1 h, 100 µl of HRP-strep (horseradish peroxidase-streptavidin) at a 1 in 4000 dilution were added to each well, and the plates were incubated at 37°C for 30–45 min. After three further washes with PBS-Tween 0.05%, 100 µl of substrate (3, 3', 5, 5'-tetramethyl benzidine 6 mg in 1.0 ml of dimethyl sulphoxide dissolved in 10 ml of acetate buffer and 5 µl of H₂O₂) were added to each well, and the plates were incubated for 30 min at 37°C. The reaction was stopped with 50 µl of sulphuric acid 10% and the absorbance value was determined with an Antos Labtec ELISA Reader 2001 at a wavelength of 450 nm. A standard curve was constructed for each plate and for each cytokine from assays of eight double dilutions of recombinant cytokine in triplicate. The sensitivity of the assay was taken as 3 SDs above the mean value from six blank wells; this was <0.3 units/ml for IL-2 and <0.11 units/ml for IFN-γ. The ELISA values of negative controls wDTP, aDTP, PBS were similar to the reading given by blanks.

Statistical analysis

Comparisons between cytokine production from cultures of splenocytes taken from groups of mice immunised with influenza subunit vaccine in wDTP, aDTP and saline with respect to the incidence and

levels of IL-2 or IFN-γ were made by the Mann-Whitney U test.

Results

The IL-2 response of spleen cell cultures after immunisation

The IL-2 response of spleen cells from mice given two doses of subunit A/Beijing X109 influenza vaccine in wDTP and re-stimulated *in vitro* with subunit vaccine was 1.20 SD 0.09 on day 7 and 1.10 SD 0.07 units/ml on day 10 after immunisation, as measured by ELISA (Table 1); the results were essentially the same ($p = >0.05$). In contrast, the IL-2 responses of re-stimulated spleen cells taken 7 and 10 days after immunisation with vaccine in aDTP at 7 and 10 days after immunisation were 0.69 SD 0.01 and 0.66 SD 0.01 units/ml, respectively (Table 1). This result was not significantly different from that produced by cells from animals immunised with subunit vaccine in saline which gave readings of 0.65 SD 0.04 and 0.60 SD 0.04 units/ml. When spleen cell cultures were stimulated with live virus, the IL-2 response of cells taken from mice given vaccine in wDTP was 2.20 SD 0.02 and 2.00 SD 0.04 units/ml at 7 and 10 days after immunisation; these figures were significantly greater than the response to stimulation with inactivated vaccine ($p = <0.01$). Small increases were also observed after live virus stimulation of spleen cells from animals immunised with subunit vaccine in aDTP or saline, which were significantly greater than for controls ($p = <0.05$) and less than for cells from animals immunised with vaccine in wDTP ($p = <0.01$). These results, shown graphically in Fig. 1, indicate that the only marked response in IL-2 production was seen in spleen cells from animals given vaccine in wDTP, whilst the response from animals given vaccine in aDTP or saline was similar and relatively small.

Table 1. IL-2 induction in cultures of immune spleen cells re-stimulated *in vitro* with influenza A/Beijing X109 subunit vaccine or A/Beijing/353/89 virus

Vaccine given	IL-2 units (SD)/ml from spleen cells stimulated with			
	SV		Virus	
	7 days	10 days	7 days	10 days
5.0 µg HA + wDTP	1.20 (0.09)	1.10 (0.07)	2.20 (0.03)	2.00 (0.04)
5.0 µg HA + aDTP	0.69 (0.01)	0.66 (0.01)	0.85 (0.01)	0.79 (0.04)
5.0 µg HA + PBS	0.65 (0.04)	0.60 (0.04)	0.78 (0.04)	0.70 (0.01)
wDTP	0.54 (0.01)	0.51 (0.01)	0.60 (0.01)	0.51 (0.01)
aDTP	0.39 (0.01)	0.38 (0.01)	0.49 (0.01)	0.40 (0.01)
PBS	0.34 (0.07)	0.51 (0.01)	0.47 (0.02)	0.49 (0.05)

SV, subunit A/Beijing X109 vaccine.

Virus, influenza A/Beijing/353/89 virus.

Fig. 1. IL-2 levels in cell supernates after re-stimulation of spleen cells from BALB/c mice immunised twice with 5 μ g of HA + wDTP (▨); 5 μ g of HA + aDTP (■); 5 μ g of HA + PBS (▤); wDTP (□); aDTP (◐) and PBS alone (◑). The cells were re-stimulated with (a) subunit influenza A/Beijing X109 vaccine 2 μ g/ml or (b) influenza A/Beijing/353/89 virus (2 μ g/ml HA protein).

IFN- γ production in spleen cell cultures from immunised animals

The IFN- γ response in spleen cells collected from mice 7 and 10 days after immunisation and re-stimulated with subunit A/Beijing X109 vaccine or influenza A/Beijing/353/89 virus are shown in Fig. 2. For mice given subunit vaccine in wDTP, the IFN- γ response to in-vitro stimulation with subunit vaccine was 0.60 SD 0.01 at 7 days and 0.50 SD 0.03 units/ml at 10 days after the second immunisation, respectively; this result was significantly greater than controls ($p = <0.01$). In contrast, the IFN- γ response of spleen cells from mice immunised with subunit vaccine in aDTP was 0.40 SD 0.01 and 0.35 SD 0.03 units/ml (Fig. 2), which was significantly greater than controls ($p = <0.05$), but significantly less than the IFN- γ levels observed in mice given vaccine in wDTP ($p = <0.01$). Furthermore, the IFN- γ response to spleen cells from animals given subunit vaccine in saline was 0.20 SD 0.07 and 0.20 SD 0.06 units/ml at 7 and 10 days post-inoculation, respectively; this result was significantly lower than the response to vaccine in aDTP ($p = <0.05$) and to vaccine in wDTP ($p = <0.01$).

The IFN- γ response to spleen cells from immunised mice stimulated with live influenza vaccine is shown in Fig. 2. For spleen cells from animals immunised with influenza vaccine in wDTP the response was 1.01 SD 0.03 and 0.82 SD 0.03 units/ml; this was significantly greater than the response to cells from other vaccinated groups or controls ($p = <0.01$). A significant IFN- γ response was seen for spleen cells from mice given subunit vaccine in aDTP; the readings were 0.41 SD 0.03 and 0.40 SD 0.02 units/ml at 7 and 10 days, respectively. However, no significant increase was seen for animals given subunit vaccine in saline alone. Thus, the most marked IFN- γ responses were

seen for animals given vaccine in wDTP; a significant but smaller response was seen for animals given vaccine in aDTP and no significant response was seen in animals given saline alone, compared to controls (Fig. 2).

Discussion

The currently available inactivated vaccines against influenza confer protection in 60–90% of vaccinees; the lower rates pertain to the elderly population which is most at risk [1, 2]. A more solid immunity has been reported for live influenza virus vaccine [3]. The reasons for these differences are not known; however, the disappointing protection conferred by inactivated vaccine may be due to the relatively poor local antibody production and cell-mediated immune responses which are induced, compared to the responses elicited by live virus infection or attenuated virus vaccines. The cell-mediated immune response to inactivated vaccine is controversial. Some authors have demonstrated induction of an MHC class-I cytotoxic T-cell (CTL) response to inactivated influenza vaccine in both mice and human subjects [14], while others have found this response to be poor and ephemeral [15]. Cell-mediated responses to killed vaccine can be enhanced by immunisation with a suitable adjuvant or carrier; thus, QS-21-, muramyl dipeptide- and ISCOM-formulated vaccines are reported to stimulate CTL responses [16–18]. Freund's complete adjuvant is an efficient stimulator of cellular immunity [19, 20]. None of the above adjuvants is licensed for use in man; the only licensed adjuvants for human use are the aluminium salts which are not efficient mediators of cellular immunity.

Previous studies have shown that inactivated influenza

Fig. 2. Concentration of IFN- γ in cell supernates after re-stimulation of spleen cells from BALB/c mice immunised twice with 5 μ g of HA + wDTP (▨); 5 μ g of HA + aDTP (■); 5 μ g of HA + PBS (▤); wDTP (□); aDTP (▧) and PBS alone (▩). The cells were re-stimulated with (a) subunit influenza A/Beijing X109 vaccine 2 μ g/ml or (b) of influenza A/Beijing/353/89 virus (2 μ g/ml HA protein).

virus vaccine mixed with cellular DTP (wDTP) induced a marked serum antibody response, and broad IgG subclass antibody responses of IgG2a and IgG2b, which suggests induction of a cell-mediated immune response [21]. In addition, vaccine incorporated into wDTP gave a more solid immunity to challenge virus infection, and was >250-fold more effective in inducing immunity to challenge virus infection than saline vaccine [10]. An enhanced immune response and immunity to challenge were seen in mice immunised with vaccine plus acellular DTP (aDTP), compared to saline vaccine. However, the humoral antibody response was less, the IgG2a and IgG2b antibody responses were reduced, and protection against challenge infection was found to be significantly less than for vaccine in wDTP [11]. The adjuvant effect was due to the LPS component of DTP [10], and the concentration of this in wDTP was 100-fold greater than for aDTP [11]; it was suggested that this difference could explain the better adjuvant effect of wDTP. The adjuvant effect of LPS has been reported by others [22]. The present studies were undertaken to investigate further the cell-mediated immune responses of mice to subunit influenza vaccines administered with wDTP, aDTP or saline. It is known that CD8⁺ CTL induction is dependent on IL-2 and IFN- γ from an activated Th-1 subset of T-lymphocytes; thus, the induction of these two cytokine responses is a measure of a CTL response [21, 23, 24]. These cytokine responses were measured in supernates of T-lymphocyte cultures from immunised mice stimulated *in vitro* with influenza vaccine or live influenza virus. These results indicate that spleen cells from mice given vaccine in wDTP produced a significant IL-2 and IFN- γ response following stimulation by subunit vaccine or live virus. The effect was markedly less for spleen cells from mice immunised with vaccine incorporated into aDTP or saline where the

response was small and similar; thus, the findings indicate that immunisation with vaccine in wDTP can induce an MHC class-I CD8⁺ CTL response. This confirms the conclusion of this response based on the observation of an IgG2a and IgG2b serum antibody response.

It is suggested that the above cell-mediated immune responses may be responsible for the enhanced immunity to challenge virus infection induced by vaccine in wDTP, which is 100-fold more effective than that seen in mice given vaccine in aDTP or saline where the cell-mediated immune response was significantly less or undetectable. The enhanced cell-mediated immune responses to vaccine in wDTP did not require adjuvant complexed to vaccine, as the effects are observed by simple mixing [10]. Moreover, the results suggest that as wDTP is licensed, it or its components could provide an adjuvant that would enhance the CTL responses against other micro-organisms where this response is important in determining immunity, and suggest a strategy whereby influenza vaccines could be given to young children or the elderly, in whom immune responses to saline vaccine are relatively poor and morbidity and mortality are higher than in other age groups.

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