Paper III

The formulation of an influenza vaccine influences the T-helper response –analysis of the cytokine response profile

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The formulation of an influenza vaccine influences the T-helper response –analysis of the cytokine response profile

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Abstract.

We have previously found that whole virus vaccine induced a higher serum IgG2a/IgG1 ratio than split virus vaccine and thus that the formulation of an influenza vaccine can affect the T helper response. In this study we investigated how different influenza vaccine formulations influences the resulting T helper immune responses after vaccination. Spleen cells from vaccinated or infected mice were stimulated *in vitro* to determine the associated cytokine profile. Whole virus vaccine induced more interleukin (IL)-2, interferon (IFN)-γ and IL-12, whereas split virus vaccine elicited higher levels of IL-4 and IL-5. These results confirm and extend our earlier observations that whole virus vaccine induced a Th1 biased response and split virus vaccine elicited more of a Th2 response. Whole virus vaccine also gave rise to higher concentrations of IFN-γ, IL-6 and TNF-α, which are pyrogenic and may explain the increased reactogenicity of whole virus vaccine. Higher concentrations of IL-10 and IL-6 were elicited by whole virus vaccine and these cytokines induce a mucosal IgA response in mice. In conclusion, whole virus vaccine induced a more similar cytokine profile to natural infection; a Th1 response and cytokines inducing IgA and could therefore provide improved vaccine efficacy against influenza.

Keywords: Influenza; vaccine; T helper; cytokines

Introduction

After encounter of an antigen, the immune system may induce specific $CD4^+$ T-helper (Th), $CD8^+$ T cytotoxic (Tc) cells and B-cells. Two distinct Th cell subsets, Th1 and Th2, secrete different cytokines that alters the immune response against the antigen. In the case of a viral infection, a Th 1 response is normally induced with its main signals being interferon (IFN)-γ and interleukin (IL)-2 [1]. A Th1 response leads to the generation of cytotoxic T cells, activation of macrophages cells and increases the expression of FcR [2]. Mice with defects in IFN-γ expression [3] or the IFN-γ receptor [4] have impaired Th1 response and experience delayed viral clearance after influenza virus infection [5]. On the other hand, if the antigen is non-replicating, a Th2 response will be induced and result in a stronger serum antibody response. The cytokines involved in establishing a Th2 response will typically be IL-4, IL-5, IL-6 and IL-10 [1].

Influenza infection induces a Th1 response [6, 7], which is characterised by a predominance of the IgG2a subclass in mice [8-12]. The inactivated influenza vaccines used today elicit an adequate immune response, but the resulting immunity will normally be biased towards a Th2 type, characterised by IgG1 as the main IgG subclass in mice [9]. This indicates that the immune response produced after parenteral immunisation with inactivated influenza virus vaccines may not be optimal to prevent influenza infection. However, the formulation of an influenza vaccine not only influences the immunogenicity [13, 14] but we have found it also affects the resulting immune response in mice [15, 16]. Whole virus vaccine was found to elicit an IgG2a bias, whereas split virus vaccine produced a more mixed IgG1 and IgG2a response [15]. A vaccine that induces a Th1 response may be more appropriate to contain and resolve an influenza virus infection. We therefore compared the Th response by investigating the cytokine profiles elicited after vaccination with split and whole virus

vaccine formulations. The data showed, as already indicated by the concentrations of serum IgG2a and IgG1, that split and whole virus vaccines indeed elicit different cytokine profiles. Split virus vaccine induced higher concentrations of Th2 cytokines, whilst whole virus vaccine elicited a clear Th1 cytokine profile and also primed for an IgA response.

Materials and Methods

Mice

Female mice, six-eight weeks old BALB/c A obtained from Taconic M&B A/S, (Ry, Denmark) were housed as previously described [15]. The animals, 2 groups with 24 mice per group were vaccinated in both hind legs (50µl per leg) into the quadriceps muscles with 1 or 2 doses at 3-week intervals with 15µg HA of monovalent A/Panama /2007/99 (H3N2) split or whole virus vaccine (kindly provided by Sanofi-Aventis, Lyon, France). A third group consisted of twelve unvaccinated mice that were infected with 2000 MID_{50} A/Moscow/10/99 (H3N2) whilst awake, to produce an initial upper respiratory tract infection [17]. Mice (4 animals per group) were sacrificed and the blood and spleen were collected at various time intervals after infection or vaccination with one dose (5, 7, and 21 days), or vaccination with two doses (4, 6 and 21 days).

Spleen cells

Lymphocytes were isolated from the spleen as previously described [18]. Harvested spleen lymphocytes ($1x10^6$) were resuspended in RPMI 1640 with L-glutamine, 0.1mM non-essential amino acids, 10mM Hepes pH 7.4, 1mM sodium pyruvate, 100U/ml penicillin, 100µg/ml streptomycin, 0.25µg/ml fungizone and 10% fetal calf serum. The lymphocytes were stimulated *in vitro* with 5µg/ml A/Panama /2007/99 (H3N2) split virus vaccine for 72 hours at 37°C and 5% CO_2. The supernatant was removed from the lymphocytes and stored at –20°C until assayed in the multiplex assay. ConA (4µg/ml) and medium alone served as positive and negative controls, respectively.

Enzyme linked immun sorbent assay (ELISA)

The ELISA method was used to detect influenza-specific serum IgG2a and IgG1 antibodies as previously described [15, 16] and the IgG2a/IgG1 ratio was calculated for each individual mouse.

The Multiplex enzyme linked immuno sorbent bead assay was performed to detect secreted cytokines in the supernatants from stimulated spleen cells, using a 10-plex kit (cat. no. LMC0001) from Biosource (Camarillo, USA). The kit was used according to the manufacturers instructions with a Luminex 100 System [19]. Cytokines involved in the type 1 response (IL-2, IFN-γ, and IL-12), the type 2 response (IL4, IL-5, IL-6 and IL-10) as well as inflammatory cytokines (IL-1β, TNF-α and Granulocyte macrophage-colony stimulating factor (GM-CSF)) were quantified (pg/ml) by using appropriate standards.

Haemagglutination inhibition (HI) assay

The assay was carried out using 8 haemagglutinating units of virus and 0.7% turkey red blood cells as previously described [20]. HI titres are reported as inverse of the serum dilution needed to inhibit 50% haemagglutination. Due to a low volume of serum available, the serum dilutions started at 1 in 20 and titres < 20 were assigned a value of 10 for calculation purposes.

Statistical analysis

Differences between the IgG2a/IgG1 ratios and median cytokine concentration (multiplex) were analysed by Mann-Whitney using SPSS version 12 for windows. A p value ≤ 0.05 was considered significant.

Results and Discussion

Whole virus vaccine has been shown to be more immunogenic than split virus vaccine in man [13, 14]. However, relatively little information is available on how antigen presented in different vaccine formulations influences the subsequent immune response. We have previously reported that whole virus vaccine induced higher IgG2a/IgG1 ratio than split virus vaccine [15]. A predominance of IgG2a antibodies indicates a Th1 biased response [10-12], which is mainly a cell-mediated immune response. Although we have found that two doses of whole virus vaccine did not give better protection than split virus vaccine against an antigenically closely related virus in mice [16], it is nevertheless important to investigate the cytokine profile associated with the immune response to different vaccine formulations.

Vaccination resulted in a more rapid and higher increase in HI titres than viral infection (Fig. 1 A). One dose of whole virus vaccine induced higher titres 5 and 7 days after vaccination than split virus vaccine. This earlier increase in serum antibody produced after vaccination with whole virus formulation was similar to our previous observations [15] and indicates that whole virus vaccine may elicit protective levels of antibody more quickly after immunisation than split virus vaccine in naïve mice. Mice vaccinated with two doses of split virus vaccine had a more rapid increase an antibody response than two doses of whole virus vaccine. However, the HI titres were similar for both vaccine formulations at day 21.

The distribution of the serum IgG2a and IgG1 subclasses can be indicative of the type of Th response induced by vaccination [9]. There was a clear difference in the IgG2a and IgG1 subclass distribution elicited by the two vaccine formulations. Mice immunised with one dose of whole virus vaccine had a significantly higher IgG2a/IgG1 ratio ($p<0.01$) than split virus vaccine (Fig. 1 B), confirming that the vaccine formulation influences the quality of

the subsequent humoral immune response [15, 16]. Unvaccinated mice that were infected with virus had an IgG2a/IgG1 ratio of approximately 3 (not shown), similar to our previous findings [16].

Whole virus vaccine elicited more IL-12 ($p< 0.01$), which is important in establishing a type 1 response [21], as well as the Th1 cytokines IL-2 and IFN-γ ($p < 0.01$) (Fig. 2 A). This explains the clear difference observed in the IgG subclass distribution between the vaccine formulations after one dose of vaccine as the two formulations elicited two distinct cytokine profiles [15, 16]. The concentration of the Th2 cytokines IL-4 ($p<0.01$) and IL-6 (day 7) was higher after one dose of split virus vaccine than after one dose of whole virus vaccine, however, the latter formulation induced more IL-10 (Fig. 2 B). The concentration of IL-5 was low for both vaccine formulations after one dose of vaccine (Fig. 2 B). Whole virus vaccine induced more GM-CSF ($p<0.05$) and TNF-α (day5), whereas split virus vaccine elicited higher concentration of TNF-α at day 7 (Fig. 3 C). The inflammatory cytokine IL-1β was also detected in higher concentrations after immunisation with one dose of split virus vaccine. Only low concentrations of secreted cytokines were detected 21 days after the last dose of vaccine (both vaccine formulations) or after infection (data not shown). Unvaccinated, infected mice had very low IL-4, IL-5 and IL-2 concentration, but higher concentrations of IL-10 and IFN-γ (Fig. 2 D), similar to mice vaccinated with whole virus vaccine. Similar concentrations of IFN-γ were detected at 5 days after infection and in mice immunised with whole virus vaccine.

After two doses of vaccine the difference in IgG2a/IgG1 ratios between the vaccine formulations was less pronounced, but the ratio was still significantly higher for whole virus vaccine ($p<0.01$). The IgG2a/IgG1 ratio remained constant after two doses of whole virus vaccine, whereas the concentration of IgG1 increased leading to a decline in the IgG2a/IgG1

ratio in mice immunised with two doses split virus vaccine. This coincided at day 4 with a very high concentration of IL-4, which is known to be a potent inducer of a Th2 response [1, 9]. Split virus vaccine also induced higher concentrations of IL-5 ($p<0.05$). Two doses of whole virus vaccine induced higher levels of IL-10 ($p<0.01$) than two doses of split virus vaccine. Lower concentrations of IFN-γ were observed after two doses than after one dose of whole virus vaccine, and this may explain the lowering of the IgG2a/IgG1 ratio observed after two doses. However, the concentration of IL-2 and IL-12 remained high ($p<0.01$). Whole vaccine induced a higher inflammatory response shown by GM-CSF and TNF-α after two doses of vaccine, but split virus induced higher concentration of IL-1β at day 6.

We have previously observed that mice immunised with whole virus vaccine had higher numbers of IgA ASC in the spleen, bone marrow and lungs after viral challenge than mice immunised with split virus vaccine [16]. This could be explained by the higher concentrations of IL-10 (both after one and two doses) and IL-6 (after two doses), as these cytokines, even in the absence of IL-4, can stimulate the production of IgA [22, 23]. This is particularly interesting as one of the activities of IFN-γ is up-regulation of poly immunoglobulin receptor (pIgR) [24], which actively transports secretory IgA and whole virus vaccine may therefore be better suited to mount a mucosal immune response. IgA is believed to be more cross-reactive than IgG [25-27] and thus vaccination with whole virus vaccine may produce a more cross-reactive antibody response, which is more appropriate to combat infection with drifted influenza virus. If two doses of vaccine are needed to elicit protective levels of immunity in man, the broad cross-reacting effect of whole virus vaccine could also be use to prime an response an then combined with one dose of the more serum IgG inducing split virus vaccine.

The use of whole virus vaccine formulation in man is largely abandoned due to a higher level of side reactions [14, 28, 29]. The cytokines IL-1β, TNF-α, IL-6 and IFN-γ have all been identified as pyrogenic [30] and these cytokines are detected in higher concentrations after immunisation with whole virus vaccine. However, the additional benefits of possibly more mucosal and cross-reactive antibody responses and induction of Tc cells by vaccination with whole virus vaccine instead of split virus vaccine, may in a pandemic scenario outweigh the risk of mild side effects. If whole virus vaccine elicits the same immune response in naïve humans as in naïve mice, whole virus vaccine may again become the preferred influenza vaccine formulation for a pandemic vaccine and possibly also for the yearly outbreaks of influenza.

Acknowledgements

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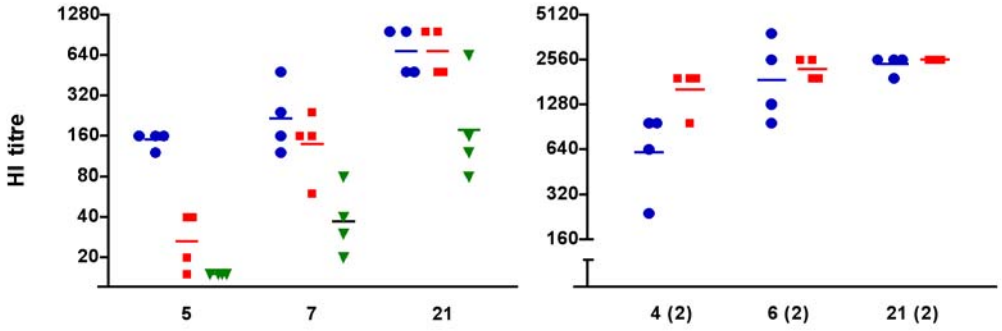
Figure 1

A


HI titre
1280
640
320
160
80
40
20
5
7
21
5120
2560
1280
640
320
160
4 (2)
6 (2)
21 (2)

B

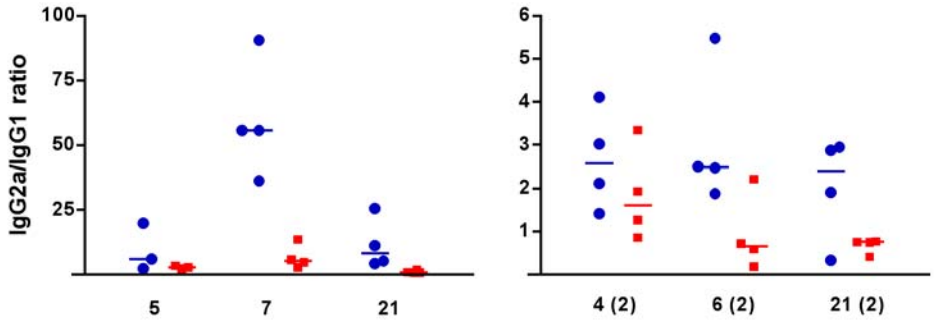
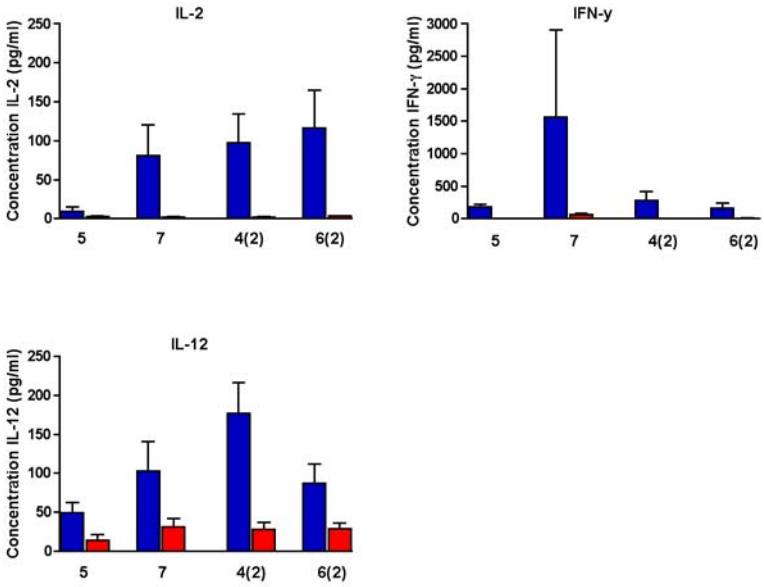

IgG2a/IgG1 ratio
100
75
50
25
5
7
21
6
5
4
3
2
1
4 (2)
6 (2)
21 (2)

Figure 2

A


IL-2
Concentration IL-2 (pg/ml)
250
200
150
100
50
0
5
7
4(2)
6(2)
IFN-y
Concentration IFN-γ (pg/ml)
3000
2500
2000
1500
1000
500
0
5
7
4(2)
6(2)
IL-12
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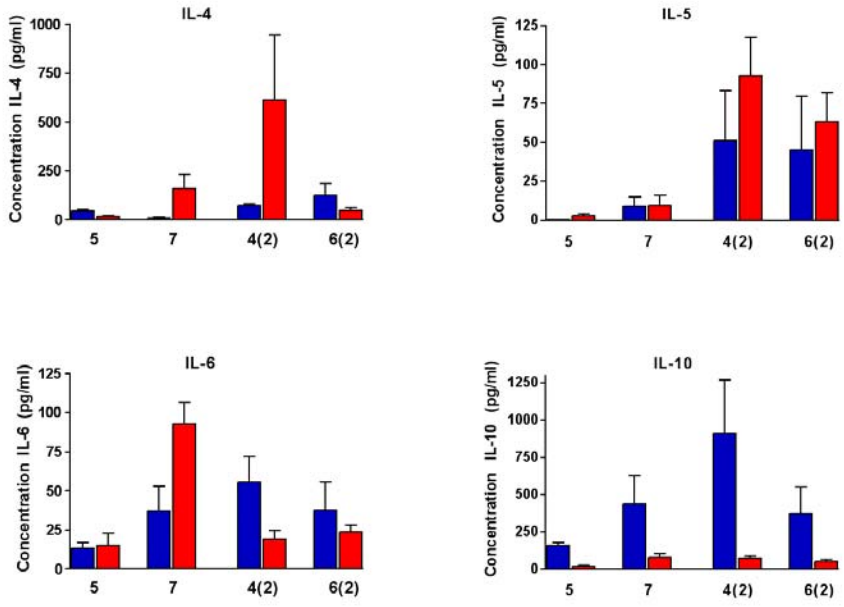
200
150
100
50
0
5
7
4(2)
6(2)

B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Concentration IL-4 (pg/ml)
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750
500
250
0
5
7
4(2)
6(2)
IL-5
Concentration IL-5 (pg/ml)
125
100
75
50
25
0
5
7
4(2)
6(2)
IL-6
Concentration IL-6 (pg/ml)
125
100
75
50
25
0
5
7
4(2)
6(2)
IL-10
Concentration IL-10 (pg/ml)
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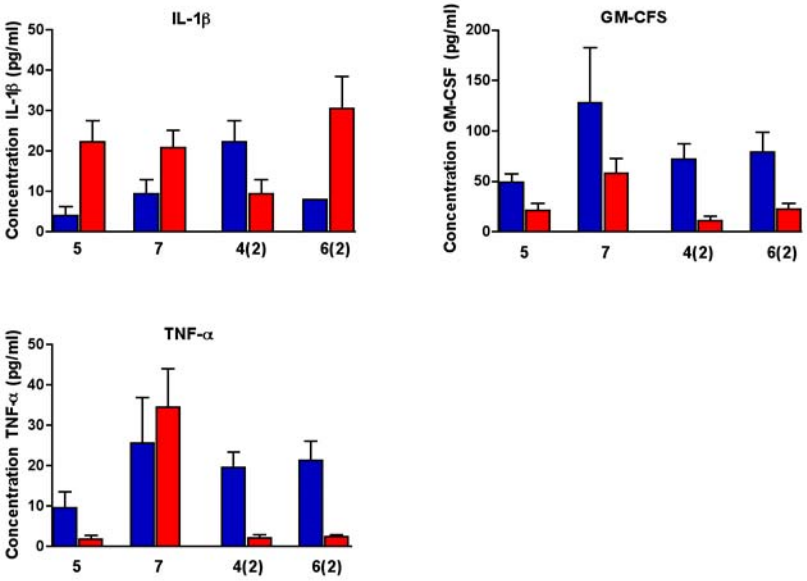
1000
750
500
250
0
5
7
4(2)
6(2)

C


IL-1β
Concentration IL-1β (pg/ml)
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10
20
30
40
50
5
7
4(2)
6(2)
GM-CFS
Concentration GM-CSF (pg/ml)
0
50
100
150
200
5
7
4(2)
6(2)
TNF-α
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20
30
40
50
5
7
4(2)
6(2)

D

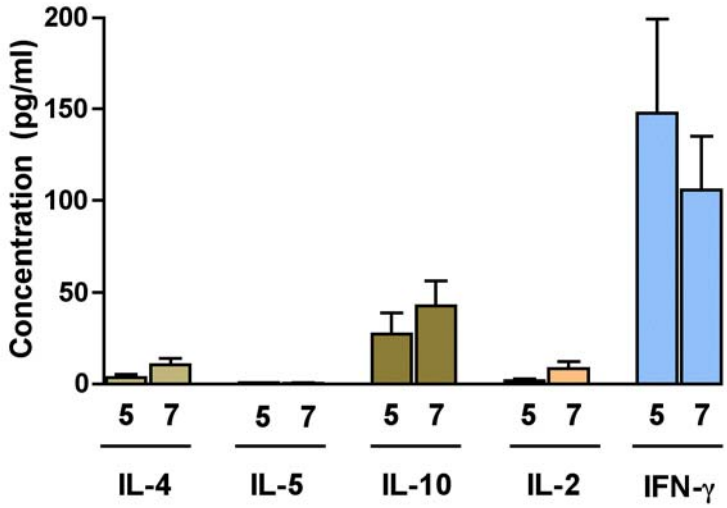
Concentration (pg/ml)
0
50
100
150
200
5 7
IL-4
5 7
IL-5
5 7
IL-10
5 7
IL-2
5 7
IFN-γ

Figure legends

Figure 1. The serum antibody response after vaccination or infection. Serum antibody responses were analysed at days 5, 7 and 21, after one dose of vaccine and at days 4, 6 and 21 after two doses of vaccine after vaccination with 15 μg of either split (red) or whole (blue) virus vaccine. A) Individual HI titres and geometric mean (bar) detected after vaccination or infection (green) with 2000MID_{50} influenza virus. B) Individual IgG2a/IgG1 ratios and median (bar) observed after vaccination.

Figure 2. The cytokine response after vaccination or infection. The mean concentration (± the standard error of mean) of cytokines secreted from *in vitro* stimulated splenic lymphocytes. Lymphocytes were isolated at days 5 and 7 (one dose vaccine and infection) and days 4 and 6 (two doses of vaccine) and stimulated for 72 hours with 5 μg/ml split vaccine. Blue bars represent mice vaccinated with whole virus and red bars represent split virus vaccine. The panels represent the cytokine response after vaccination as follows: A) cytokines involved in a type 1 response, B) cytokines involved in a type 2 response, C) cytokines involved in inflammatory responses, and the cytokines elicited after infection are shown in D).