doi: 10.1111/j.1365-3083.2005.01633.x

Whole influenza virus vaccine is more immunogenic than split influenza virus vaccine and induces primarily an IgG2a response in BALB/*c* mice

A.-O. Hovden*, R. J. Cox* & L. R. Haaheim

Influenza Centre, Section for Microbiology and Immunology, The Gade Institute, University of Bergen, Haukeland University Hospital, Bergen, Norway

Received 18 March 2005; Accepted in revised form 2 May 2005

Correspondence to: Dr A.-O. Hovden, Influenza Centre, Section for Microbiology and Immunology, The Gade Institute, University of Bergen, Armauer Hansens Building, Haukeland University Hospital, N-5021 Bergen, Norway. E-mail: arnt-ove.hovden@gades.uib.no

Abstract

The aim of this study was to compare the kinetics and the magnitude of the humoral immune response to two different influenza vaccine formulations, whole and split virus vaccines. BALB/*c* mice were immunized intramuscularly with one or two doses (3 weeks apart) of 7.5, 15 or 30 μg of haemagglutinin of monovalent A/Panama/2007/99 (H3N2) split or whole virus vaccine. The two vaccine formulations induced similar kinetics of the antibody-secreting cells response; however, differences in the magnitude were observed in the spleen and bone marrow. Vaccination with whole virus vaccine generally elicited a quicker and higher neutralizing antibody response, particularly after the first dose of vaccine. The two vaccine formulations gave different immunoglobulin G (IgG) subclass profiles. Split virus vaccine stimulated both IgG1 and IgG2a antibodies suggestive of mixed T-helper 1 (Th1) and Th2 response, whereas whole virus vaccine induced mainly an IgG2a antibody response, which is indicative of a dominant Th1 response. The increased immunogenicity of whole virus vaccine in a naïve population could reduce the vaccine concentration needed to provide protective immunity.

Introduction

Influenza remains one of the most common infections worldwide, claiming up to one million lives each year [1, 2]. The virus undergoes frequent mutations in the surface glycoproteins, haemagglutinin (HA) and neuraminidase, allowing it to escape the host's acquired immunity. This process is called antigenic drift and results in the need for yearly updating of vaccine strains. Antigenic shift occurs when an influenza A virus with a completely novel HA is introduced into the human population, e.g. from an avian reservoir and may result in a pandemic with unprecedented levels of worldwide morbidity and mortality. Influenza pandemics have occurred three times during the twentieth century. The 'Spanish flu' in 1918–1919 was the most serious pandemic, with a death toll between 20 and 40 million people [3, 4]. Even today an influenza pandemic could have a significant impact on global health, requiring a quick and determined response to reduce death and serious illness and ease the burden on society's infrastructure.

Influenza vaccines remain the cornerstone of influenza prophylaxis, but the efficacy of the vaccine largely depends on the antigenic match between the vaccine strains and the strains of virus circulating in the community. Currently, inactivated vaccines are the most utilized influenza vaccines and are available in three formulations: whole virus, split virus (detergent disrupted virion) and purified subunit vaccines. The immunogenicity of different influenza virus vaccine formulations were studied in the 1970s and although whole virus vaccine was found to be more immunogenic, its use was largely discontinued due to more frequent side reactions [5, 6]. Today, split virus vaccine is the most commonly used influenza vaccine. After vaccination with inactivated influenza vaccine, immunity consists mainly of a serum antibody response directed to the HA and less mucosal and cell-mediated responses. In a pandemic situation, time is a limiting factor for the preparation of vaccine and the ability to produce enough vaccine demands strategies for use of limited vaccine supplies. Evaluation of new influenza vaccines requires comprehensive information on the humoral response to conventional vaccines and extensive

*Both authors contributed equally to this work.

knowledge of the dose–response to different influenza subtypes. There is thus a need for investigation of different vaccine formulations, especially to re-evaluate the use of whole virus formulation [7–9].

The aim of this study was to compare the detailed kinetics of the immune responses of split and whole virus vaccine (one or two doses and three vaccine concentrations) in a mouse model. Furthermore, we investigated the time course of the systemic antibody-secreting cells (ASC) response in the spleen and the bone marrow and provide a detailed analysis of the serum antibody response using haemagglutination inhibition (HI), virus neutralization (VN) and enzyme-linked immunosorbent assay (ELISA) assays. We found that whole virus vaccine induced a predominantly immunoglobulin G2a (IgG2a) response [T-helper 1 (Th1)-like response], whereas split virus vaccine produced a mixed IgG2a and IgG1 response (Th2-like response).

Materials and methods

Mice. Six-week-old female BALB/*c* A mice were purchased from Taconic M&B A/S (Ry, Denmark). The animals were housed according to Norwegian law on the use of experimental animals. Animals were kept at 21 °C with 12 h light–dark cycles and food and water *ad libitum*. The animals were divided into a control group (eight unvaccinated mice) and six additional groups of 48 mice that were vaccinated intramuscularly into the quadriceps muscles with one or two doses (7.5, 15 or 30 μg HA) of monovalent A/Panama/2007/99 (H3N2) split or whole virus vaccine at 3-week intervals (vaccine kindly provided by Sanofi-Aventis, Lyon, France). All doses were administrated into both hind legs (50 μl per leg), except 30 μg split virus vaccine, which was immunized in two sites per hind leg (80 μl per leg) to deliver the correct amount of antigen. Mice (four animals per group) were sacrificed and the blood, spleen, femur and tibia bones of the hind leg were collected at various time intervals after vaccination (days 3, 5, 7, 9, 14 and 21 after each immunization). Additionally, eight control mice (unvaccinated) were sacrificed before vaccination. Mice vaccinated with two doses of 15 μg split virus vaccine and sacrificed 3 days after second vaccination were excluded due to an error in vaccination.

ELISPOT. The ELISPOT assay was used to detect influenza-specific ASC from the spleen and bone marrow as previously described [10, 11]. Briefly, ELISPOT plates were coated with 100 μl/well of 10 μg/ml of split virus vaccine [A/Panama/2007/99 (H3N2)] overnight at 4 °C. An appropriate number of lymphocytes (100,000–400,000 lymphocytes/well) were added to the plate and incubated in a humidified CO_2 incubator at 37 °C overnight. After incubation, the influenza specific ASC were detected using 2 μg/ml biotinylated class (IgG; 1030-08, IgA; 1040-08, IgM; 1020-08, Southern Biotechnology, Birmingham, AL, USA) and IgG subclass (IgG1; 1070-08, IgG2a; 1080-08, IgG2b; 1090-08, IgG3; 1100-08, Southern Biotechnology)-specific antibodies. The spots were enumerated using a stereo microscope and the mean number of class and IgG subclass influenza-specific ASC per 500,000 lymphocytes was calculated for each individual mouse.

Haemagglutination-inhibition assay. The HI test was carried out as earlier described, using eight haemagglutinating units of virus (50 μl) and 0.7% turkey red blood cells [11]. Non-specific inhibitors were removed by treating the serum overnight at 37 °C with receptor-destroying enzyme (Denka Seiken, Tokyo, Japan) and then subsequently inactivated at 56 °C. HI titres are reported as the reciprocal of the highest dilution of serum needed to inhibit 50% haemagglutination. Titres less than 10 were assigned a value of 5 for calculation purposes.

ELISA. Influenza-specific serum antibodies were detected by ELISA as previously described [12]. Briefly, 96-well ELISA plates (Greiner, Frickenhausen, Germany) were coated with 10 μg/ml of split A/Panama/2007/99 (H3N2) virus overnight at 4 °C (100 μl/well). Influenza-specific serum antibodies were detected using class and IgG subclass biotinylated conjugates as detailed under ELISPOT. The antibody concentration was determined by using appropriate immunoglobulin standards (Sigma, St Louis, MO, USA, IgG, I-5381; IgA, M-1421; IgM, M-3795). The background absorbance was subtracted from the sample absorbance, and the class and IgG subclass antibody concentrations were calculated (using linear regression of the log-transformed readings).

Virus-neutralization assay. The VN assay was carried out as described earlier [10]. Briefly, quadruplicate twofold dilutions of serum were incubated with 500$TCID_{50}$ A/Panama/2007/99 (H3N2) influenza virus at room temperature for 1 h before transfer to confluent MDCK monolayers in 96-well tissue culture plates (Nunc, Roskilde, Denmark). After 30 min incubation at 35 °C, the serum–virus mixture was removed and replaced with new medium and incubated for 72 h in a humidified incubator at 35 °C with 5% CO_2. Virus-containing wells were detected by an HA assay with 0.7% turkey red blood cells. The VN titres are reported as the reciprocal of the highest serum dilution needed to neutralize 50% of infectious virus calculated by the method of Reed and Muench [13].

Statistical analysis. The two-sided Student's t-test was used to analyse differences in the data, mean antibody concentrations (ELISA), geometric mean titres (VN and HI, log-normalized data) and mean numbers of ASC (ELISPOT) using SPSS version 12 for windows. A *P*-value ≤ 0.05 was considered significant.

Results

Six groups of 48 mice were vaccinated once or twice intramuscularly with either split or whole virus vaccine at

three different concentrations (7.5, 15 or 30 μg HA) of monovalent A/Panama/2007/99 (H3N2) virus vaccine. All mice received two injections of the appropriate vaccine concentration per dose, except the 30 μg split group that needed four injections to deliver the appropriate amount of vaccine. At selected days, after vaccination, the animals were sacrificed and the blood, spleen and bone marrow were collected. Both vaccines were well tolerated, none of the mice showed any clinical signs of illness and no deaths were recorded. A control group of mice were sacrificed prior to the first dose of vaccine.

The antibody-secreting cell response

Lymphocytes were harvested from the spleen and the bone marrow at the time of sacrifice and the influenza-specific class and IgG subclass of ASC were enumerated in an ELISPOT assay.

ASC in the spleen

The main influenza-specific antibody classes of ASCs were IgG and IgM (Table 1) with low numbers of IgA ASC detected (IgA data not shown). Prior to vaccination, very low IgM (three specific ASC per 5×10^5 lymphocytes) or no IgG background ASC were detected. Generally, peak numbers of influenza-specific IgM ASC were detected earlier than peak numbers of IgG ASC. After the second dose of vaccine, influenza-specific ASC were detected at an earlier time point than after the first dose of vaccine. After one dose of vaccine, a significantly higher number of ASC ($P < 0.05$) was observed after the highest vaccine concentration (30 μg) compared to the lowest (7.5 μg) for both vaccine formulations. The number of influenza-specific IgG ASC peaked between days 5–14 after the first dose of vaccine for both vaccine formulations, whereas the influenza-specific IgM ASC peaked earlier at days 5–7. No significant differences were found between the peak numbers of influenza-specific ASC (IgG and IgM) after whole and split virus vaccination.

After two doses of vaccine, no significant differences were detected in the numbers of IgG ASC after split virus and whole virus vaccination, with the exception of the 30 μg group. In contrast, higher numbers of IgM ASC were detected after two doses of 7.5 and 15 μg whole virus vaccine. The peak numbers of IgG ASC were observed 5 days after the second vaccination for both vaccines, whereas an earlier peak was found for IgM at day 3 for the whole virus vaccine group and days 3–9 for the split virus vaccine group. The two vaccines elicited a different IgG subclass profile following vaccination, irrespective of vaccine concentration or number of injections. Mice that were immunized with split virus vaccine produced both IgG1 and IgG2a ASC, whilst whole virus vaccine induced a predominantly IgG2a response. Whole vaccine also had a

Table 1 Splenic influenza specific immunoglobulin G (IgG) and IgM antibody-secreting cells (ASC) elicited after vaccination with one or two doses of A/Panama/2007/99 (H3N2)

Antibody class	Vaccine formulation	Vaccine concentration (μg)	Days after first vaccination					Days after second vaccination					
			3	5	7	9	14	21	3	5	7	9	14	21
IgG	Split	7.5	0 ± 0	8 ± 6	14 ± 10	9 ± 5	22 ± 12	10 ± 54	2 ± 15	174 ± 88	51 ± 12	10 ± 2	3 ± 1	5 ± 1
		15	0 ± 0	4 ± 2	56 ± 28	4 ± 2	15 ± 3	0 ± 0	Not available	78 ± 15	19 ± 8	24 ± 10	11 ± 8	13 ± 3
		30	2 ± 1	87 ± 50	60 ± 24	23 ± 2	31 ± 13	25 ± 9	614 ± 153	736 ± 309	87 ± 12	33 ± 8	12 ± 6	6 ± 2
	Whole	7.5	0 ± 0	6 ± 3	31 ± 9	19 ± 11	22 ± 9	13 ± 5	39 ± 21	46 ± 14	29 ± 4	21 ± 4	8 ± 1	12 ± 2
		15	0 ± 0	69 ± 38	27 ± 9	24 ± 15	28 ± 13	15 ± 7	37 ± 10	39 ± 5	41 ± 5	27 ± 5	11 ± 3	13 ± 2
		30	0 ± 0	48 ± 23	82 ± 26	71 ± 24	90 ± 48	20 ± 3	40 ± 10	37 ± 1	27 ± 4	22 ± 3	18 ± 1	10 ± 2
IgM	Split	7.5	16 ± 6	47 ± 27	27 ± 18	22 ± 8	20 ± 8	8 ± 2	12 ± 3	28 ± 6	33 ± 5	5 ± 2	3 ± 1	5 ± 2
		15	0 ± 0	37 ± 24	56 ± 32	5 ± 2	9 ± 1	0 ± 0	10 ± 10	7 ± 2	1 ± 0	17 ± 9	14 ± 6	20 ± 6
		30	62 ± 27	129 ± 65	88 ± 37	27 ± 6	15 ± 4	11 ± 3	187 ± 46	65 ± 26	37 ± 10	16 ± 7	9 ± 1	17 ± 7
	Whole	7.5	6 ± 3	26 ± 13	28 ± 7	13 ± 4	7 ± 1	10 ± 1	49 ± 34	31 ± 11	23 ± 5	17 ± 3	17 ± 2	14 ± 4
		15	60 ± 26	145 ± 68	27 ± 14	22 ± 7	8 ± 2	10 ± 3	70 ± 29	29 ± 7	21 ± 5	23 ± 4	17 ± 3	19 ± 6
		30	56 ± 21	123 ± 63	58 ± 28	24 ± 6	14 ± 5	12 ± 2	69 ± 19	16 ± 5	17 ± 3	19 ± 3	29 ± 7	12 ± 4

Data are presented as mean ASC per 5×10^5 lymphocytes ± standard error of the mean.

tendency to induce more IgG2b and IgG3 ASC, although the numbers of ASC were generally low (data not shown). The ratio of IgG2a/IgG1 was always significantly higher ($P < 0.05$) after whole virus than split virus vaccination for all vaccine concentrations and both one and two doses (data not shown).

ASC in the bone marrow

The main antibody class detected in the bone marrow was IgG, predominantly of the IgG1 and IgG2a subclasses (Fig. 1), with lower numbers of IgM and IgA ASC detected (IgM and IgA, results not shown). The increase in the number of influenza-specific IgG ASC in the bone marrow after the first vaccination was gradual up to day 14 and the numbers remained constant up to 21 days. After the second vaccination, the numbers of IgG ASC were higher and peaked at days 5–9, but remained elevated after 21 days (50% of peak IgG ASC numbers). No clear differences in the numbers of IgG ASC induced after split and whole virus vaccination were observed. The IgG2a/IgG1 ratio was significantly higher ($P < 0.05$) after whole virus vaccination for both doses and all vaccine concentrations (except after one dose of 7.5 μg HA, data not shown).

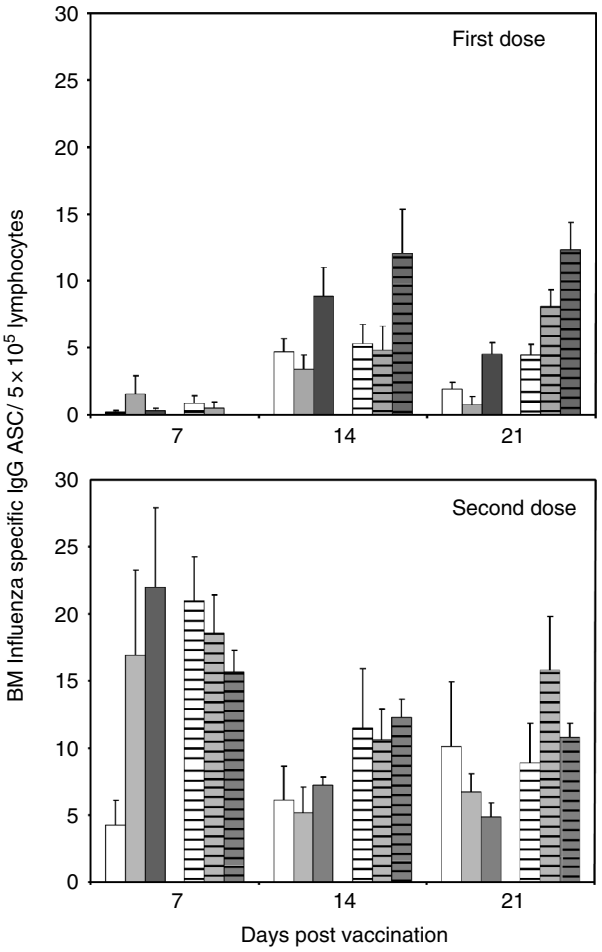

Figure 1 Influenza-specific IgG ASC per 5×10^5 lymphocytes in the bone marrow after first dose and second dose of split or whole virus vaccine. Legends (from left to right), 7.5 μg (white), 15 μg (light grey), 30 μg (dark grey). Open bars represent mice vaccinated with split virus vaccine and striped bars represent mice vaccinated with whole virus vaccine.

Serum antibody response measured by ELISA

The serum concentrations of the influenza-specific antibody class (IgG, IgA and IgM) and the IgG subclass were determined by ELISA. IgG was the predominant antibody class detected, whereas IgM was detected at lower concentrations, but appeared earlier after vaccination (Table 2). IgA was not detected until after the second vaccination and then detected only at very low concentrations (data not shown). Generally, after the first vaccination, the influenza-specific serum IgG concentrations gradually increased up to day 21, whereas the IgM concentrations peaked at days 5–9 and remained two to five times above background levels for the rest of the observation period. After the second dose of vaccine, both IgG and IgM levels were boosted and reached peak concentrations at 7–9 days after vaccination.

After the first vaccination, the IgG concentration was significantly higher ($P < 0.05$) for all concentrations of whole virus vaccine compared to split virus vaccine, although there were no differences in the maximum IgG concentration between the 7.5 μg groups. Whole virus vaccine induced a two- to 10-fold higher serum IgM concentrations than the split virus vaccine. An earlier increase in antibody concentrations was observed after whole virus vaccination, with up to a 40-fold higher influenza-specific IgG at 7 days after the first vaccination, compared to mice immunized with split virus vaccine.

After the second dose of vaccine, peak IgG concentrations were higher after whole virus vaccination ($P < 0.05$) than split virus vaccination, with the exception of 30 μg vaccine. Similar serum IgM concentrations were detected after vaccination with 30 μg of both vaccines, but at least threefold higher IgM concentrations were detected after whole virus vaccination with the two lowest vaccine concentrations compared with split virus vaccination. The highest concentrations of influenza-specific IgG and IgM were generally observed between days 7–9, with the exception of 15 μg whole virus vaccine group which had the highest concentration on day 5. After the second dose of vaccine (both split and whole virus vaccines), there were significantly ($P < 0.05$) higher serum antibody concentrations in the 30 μg group than the two lowest doses.

A different distribution in IgG subclasses was observed, with significantly more IgG2a and IgG3 found after whole virus vaccination and more IgG1 and IgG2b detected after split virus vaccination (IgG2b and IgG3, data not shown). The IgG2a/IgG1 ratio was approximately 1 after split virus vaccination, in contrast to the ratio of whole virus vaccine

Table 2 Influenza-specific serum immunoglobulin G (IgG) and IgM antibody concentrations induced after one or two doses of A/Panama/2007/99 (H3N2) vaccine

Antibody class	Vaccine formulation	Vaccine concentration (μg)	Days after first vaccination						Days after second vaccination					
			3	5	7	9	14	21	3	5	7	9	14	21
IgG	Split	7.5	0 ± 0	1 ± 0	2 ± 1	13 ± 4	28 ± 4	83 ± 22	171 ± 36	190 ± 33	287 ± 46	211 ± 30	162 ± 31	141 ± 30
		15	0 ± 0	1 ± 0	6 ± 0	11 ± 3	43 ± 10	51 ± 18	Not available	134 ± 31	270 ± 11	245 ± 35	148 ± 16	136 ± 27
		30	0 ± 0	2 ± 0	5 ± 1	45 ± 8	135 ± 24	220 ± 108	101 ± 4	445 ± 78	637 ± 61	1002 ± 217	1009 ± 122	504 ± 118
	Whole	7.5	0 ± 0	10 ± 2	53 ± 12	48 ± 2	84 ± 10	87 ± 15	116 ± 8	159 ± 15	445 ± 59	627 ± 40	311 ± 15	154 ± 10
		15	0 ± 0	4 ± 0	46 ± 11	65 ± 8	177 ± 31	214 ± 36	229 ± 30	182 ± 13	541 ± 86	803 ± 125	590 ± 135	234 ± 28
		30	0 ± 0	8 ± 1	190 ± 34	221 ± 20	570 ± 82	728 ± 42	659 ± 55	656 ± 99	700 ± 128	851 ± 33	619 ± 7	733 ± 97
IgM	Split	7.5	1 ± 0	4 ± 0	11 ± 3	9 ± 2	8 ± 3	2 ± 1	7 ± 1	15 ± 5	18 ± 2	13 ± 2	14 ± 5	18 ± 1
		15	0 ± 0	4 ± 0	7 ± 1	2 ± 0	1 ± 0	1 ± 0	2 ± 0	5 ± 1	6 ± 1	8 ± 2	4 ± 1	3 ± 1
		30	3 ± 1	22 ± 10	20 ± 4	13 ± 1	8 ± 4	6 ± 1	50 ± 9	78 ± 44	31 ± 11	111 ± 12	26 ± 3	31 ± 10
	Whole	7.5	1 ± 0	65 ± 5	56 ± 7	34 ± 15	5 ± 0	4 ± 1	6 ± 1	33 ± 1	23 ± 5	57 ± 6	42 ± 3	38 ± 3
		15	8 ± 1	67 ± 8	56 ± 9	69 ± 12	9 ± 1	9 ± 1	48 ± 5	63 ± 7	39 ± 5	49 ± 2	46 ± 2	28 ± 1
		30	9 ± 2	60 ± 12	68 ± 8	55 ± 6	9 ± 0	8 ± 0	38 ± 2	62 ± 5	73 ± 7	46 ± 7	48 ± 8	10 ± 1

Serum antibody was measured by enzyme-linked immunosorbent assay and data are presented as the mean concentration (μg/ml) ± standard error of mean.

group that ranged from 6 to 496 (Fig. 2). After the second dose of whole virus vaccine, the ratio was lower, but still higher than mice vaccinated with split virus vaccine. The IgG2a/IgG1 ratio was always significantly higher ($P < 0.0001$) after whole virus vaccination for all vaccine concentrations, irrespective of the number of doses.

HI antibodies

Generally, after the first dose of split or whole virus vaccine, the HI titres increased up to day 21 (Table 3). After the first dose of vaccine, whole virus vaccine induced twofold higher HI titres at day 21 than split virus vaccine for all vaccine concentrations. Whole virus immunization also induced a significantly ($P < 0.05$) earlier increase in titres with up to a 13-fold difference by day 5. After the second vaccination, antibody titres were boosted for both vaccines and reached peak titres between days 7–14. Whole virus vaccine induced significantly higher

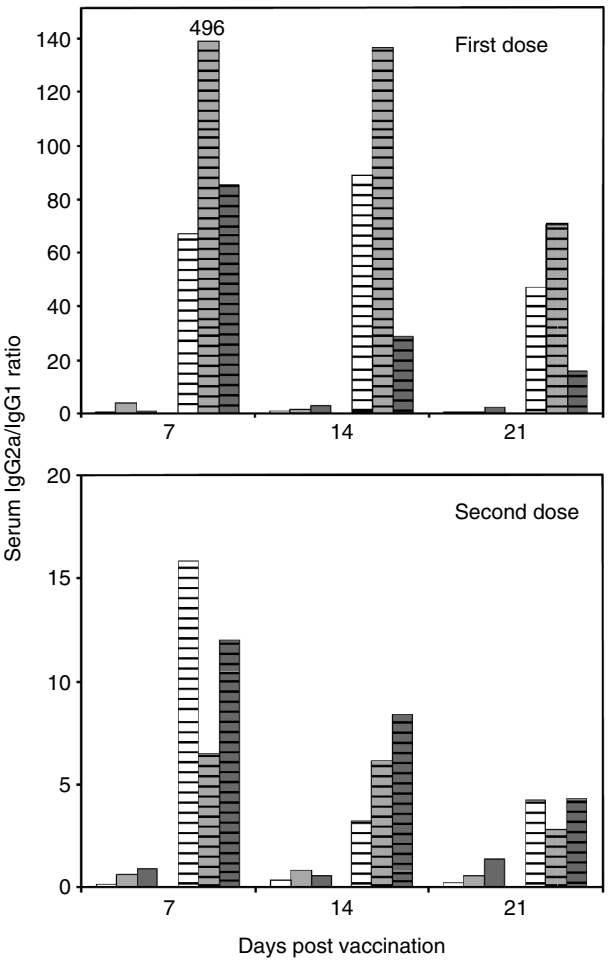

Figure 2 The serum IgG2a/IgG1 ratio observed after vaccination with first dose and second dose of split and whole virus vaccination. Legends (from left to right), 7.5 μg (white), 15 μg (light grey), 30 μg (dark grey). Open bars represent mice vaccinated with split virus vaccine and striped bars represent mice vaccinated with whole virus vaccine.

Table 3 Haemagglutination inhibition (HI) titres induced after vaccination with one or two doses of A/Panama/2007/99 (H3N2)

		Days after first vaccination						Days after second vaccination					
Vaccine formulation	Vaccine concentration (μg)	3	5	7	9	14	21	3	5	7	9	14	21
Split	7.5	5	11	58	53	332*	659	1459	1280	1846	1677	554	842
	15	5	20	129	58	466*	501	Not available	516	867	1615	1568	1032
	30	5	19	257*	202	867	1142	434	1864	3135	3369	4434	2833
Whole	7.5	5	74*	538	1191	784	1417	1417	2063	3135	1280	1568	2454
	15	5	195*	421	842	1142	1417	2003	2283	1109	2637	2217	640
	30	7	250*	932	1002	1280	2217	2063	2637	1920	2217	2918	1568

HI titres are presented as geometric mean of four mice per sampling point. In man, an HI titre of 40 is considered to indicate protective levels of immunity.
*All mice had an HI >40.

($P < 0.05$) HI titres than the split virus vaccine for the two lowest vaccine concentrations (7.5 μg and 15 μg) after both one and two doses. Increasing HI titres were observed with increasing vaccine concentrations after the second dose of split virus vaccine ($P < 0.05$).

VN antibodies

The serum VN titres increased gradually up to 21 days after the first dose of both vaccines (Table 4). One dose of whole virus vaccine induced significantly higher VN titres ($P < 0.05$) than split virus vaccine, with at least threefold higher titres at day 21. VN titres also increased significantly ($P < 0.05$) earlier after whole virus vaccination, with five- to sevenfold higher titres observed at day 7 compared with split virus vaccine group. After the second vaccination, the VN titres reached a peak at days 9–14. Whole virus vaccine (all concentrations) elicited significantly higher titres ($P < 0.05$) than split virus vaccine after the second dose of vaccine at all time points. Significant differences ($P < 0.05$) in VN titres between the vaccine concentrations were observed after the second dose of vaccine between 7.5 and 30 μg (both split and whole virus groups), while only between 15 and 30 μg after whole virus vaccination.

Discussion

The most utilized influenza vaccine today is the split virus vaccine formulation. However, whole virus vaccine formulation is considered more immunogenic in a naïve population and may be needed in a pandemic situation to elicit an adequate immune response. Vaccination will remain a very important measure to limit the consequences of a new pandemic, but there will be a limited vaccine supply. Therefore, further knowledge of the vaccine (formulation, concentration and the number of doses) and the kinetics of the immune response induced after vaccination are required. This study compared the humoral immune response after vaccination of mice with one or two doses of monovalent split or whole influenza virus vaccines, characterizing both the class and IgG subclass of systemic ASC and serum antibody responses, in addition to VN and HI antibodies.

B cells differentiate into memory B cells and plasma cells after activation by a foreign antigen, but they can also secrete antibody before becoming fully differentiated plasma cells [14]. Such ASC are present in large numbers in the spleen within the first week of activation [14]. The influenza-specific IgG class dominated the systemic ASC response after vaccination, similar to our earlier observations in the peripheral blood of man [11]. A comparable

Table 4 Virus-neutralisation antibody titres detected after immunisation with one or two doses of A/Panama/2007/99 (H3N2)

		Days after first vaccination						Days after second vaccination					
Vaccine formulation	Vaccine concentration (μg)	3	5	7	9	14	21	3	5	7	9	14	21
Split	7.5	17	19	36	24	85	319	640	613	1457	1522	795	668
	15	30	18	22	27	217	562	Not available	1226	1337	1457	698	1076
	30	20	40	52	30	217	668	508	1396	1660	1589	1810	1124
Whole	7.5	8	42	167	80	359	945	905	2451	2915	3620	3044	1918
	15	8	53	160	118	761	1733	2915	1031	2873	4305	3780	2061
	30	8	60	380	153	987	4886	3780	3780	5346	5583	5583	5120

Viral neutralization titres are presented as geometric mean titre.

time course of the splenic ASC response was observed after vaccination with both split and whole virus vaccines, but differences were found in the magnitude of the response. Peak numbers of splenic IgG ASC were often detected at 7 days after the first vaccination and earlier at 5 days after the second vaccination. The time course observed in the bone marrow, however, was different to that observed in the spleen. IgG ASC increased up to 14 days after the first vaccination, whereas it peaked 5–9 days after the second dose of vaccine. Memory B cells and long-lived plasma cells home to the bone marrow 2–3 weeks after a primary response [15, 16]. These plasma cells contribute to the systemic antibody pool, providing one mechanism for maintaining the long lasting humoral immunity.

The HI test is the most utilized and recognized standard for testing influenza-specific serum antibody. An HI titre $\geq$40 is considered to indicate protective levels of antibody against influenza infection in man [17, 18] and is used as one of the evaluation criteria for annual updating of influenza vaccines defined by the Committee for Medicinal Products for Human Use (CHMP) in the EU [19]. The mouse model has been extensively used for studying immune responses to influenza vaccination and infection, but no such HI correlate of immunity has been identified in mice. In this study, whole virus vaccine induced statistically significantly higher VN and HI titres 7 days after the first vaccination in addition to higher serum IgG and IgM at 21 days after the first dose. This earlier and stronger humoral immune response induced by whole virus than split virus vaccine may provide earlier protection after vaccination in naïve individuals. Previously, whole virus vaccine has been shown to be more immunogenic after one dose of vaccine in children [20]. After the second dose of vaccine, both split (all strengths) and whole virus (particularly 7.5 μg) vaccine further boosted the serum IgG antibody response, and increased VN and HI titres were detected. Several studies with avian derived influenza vaccines in man have concluded that two doses of vaccine may be needed to achieve protective levels of immunity [8, 21, 22]. Our results show an additional benefit of two doses of whole virus vaccine and the use of appropriate adjuvants may further increase the immunogenicity of influenza vaccines [21, 23–26].

Two doses of 30 μg split virus vaccine elicited higher numbers of ASC and serum antibody concentrations than 30 μg whole virus vaccine. This may be explained by the four injections needed to deliver the correct amount of antigen and thus allowing a more effective presentation of the antigen to the immune system. Vaccination with an inactivated viral vaccine is generally thought to mainly induce more of a humoral response with very little cellular immunity [23], although some studies have found this to be dependent upon the route of administration and the method of inactivation of influenza virus [27, 28]. Influenza-specific neutralizing antibodies are important in protection against a lethal outcome after challenge with the same strain (homosubtypic immunity) and may also have a role to play in protection between different subtypes (heterosubtypic immunity) [29]. The IgG subclass distribution is indicative of differences in the Th cell response and the IgG2a subclass is believed to signal a Th1 profile [23, 30] often associated with interferon (IFN)-γ [30] in addition to other Th1 cytokines [31]. This is characterized by recruitment of cytotoxic T lymphocytes (CTL), macrophages and NK cells and is the normal response to a viral infection [32], whereas a Th2 profile represents more of a humoral response [30]. There are considerable differences in the Th response to foreign antigens in different mouse strains, and BALB/*c* mice have been reported as the prototypical Th2 mouse strain [33–35]. Despite this, we have found that the IgG2a/IgG1 ratio was significantly shifted towards the IgG2a subclass after whole virus vaccination for both the serum antibody and ASC responses, whereas split virus vaccine produced a more equal ratio. After two doses of whole virus vaccine, the IgG1 concentration increased, lowering the IgG2a/IgG1 ratio, but the ratio still remained fivefold higher than in mice vaccinated with two doses of split virus vaccine. If BALB/*c* mice are prone to a Th2 response, this further strengthens the finding that whole virus vaccine induces a Th1 profile. Our preliminary analysis of Th1 and Th2 cytokines by cytokine ELISPOT and ELISA confirm the IgG subclass results (unpublished results) and further support our finding of a Th1 bias after whole virus vaccination, particularly in naïve mice. Nevertheless, there is a need to investigate the cytokine profile in more detail, after both vaccination and natural influenza infection, and to study the effect of vaccination upon viral challenge.

If indeed a whole virus vaccine stimulates a Th1 profile, it may more efficiently generate cytotoxic T cells and thus provide faster recovery from infection, more similar to the immune response observed after viral infection [36]. The IgG2a antibody subclass plays a major role in complement activation and antibody-dependent cell-mediated cytotoxicity (ADCC) and is found as the most abundant serum IgG subclass antibody class after influenza infection [25, 37, 38]. Our finding that whole virus vaccine in general favours more of a Th1 response in mice has not directly been addressed previously, but Takada *et al.* have reported that a formalin-inactivated influenza vaccine induced more CTL than split virus vaccine [27]. Similarly, a CTL response was observed after influenza vaccination with glycoproteins in virosomes (HA and NA or HA alone) [39–41] or immunstimulating complexes (ISCOM) [42], in addition to heat-treated whole influenza virus [43]. The reason for this remains to be fully elucidated. Whole virus vaccine has been reported to stimulate human cytokine production of interleukin (IL)-12 and tumour necrosis factor-α in dendritic cells and IL-2 and IFN-γ in peripheral blood mononuclear cells *in vitro*, and thus

generating a cytokine milieu that supports endocytosis by antigen-presenting cells and recruitment of CTL [44]. This, together with our data, suggests a different processing of antigen when it is presented in an intact lipid membrane, resulting in major histocompatibility complex I presentation and a Th1 response.

Our results in a mouse model suggest ways to possibly enhance the efficacy of an inactivated influenza vaccine by using a whole virus formulation. The increased immunogenicity of whole virus vaccine may make it possible to reduce the HA concentration administered and thus increase the use of a limited supply of vaccine, particularly in a pandemic situation. The use of a whole virus vaccine in an unprimed population, which induces an earlier neutralizing antibody response, may reduce the time between vaccination and protection.

Acknowledgments

This work has been supported by the European Union (Flupan QLK2-CT-2001-01786) and the Norwegian Department of Health. We thank the staff at the animal house (Vivarium, University of Bergen, Norway) for excellent animal care and Karoline Bragstad for technical assistance. The authors are grateful for the critical reading of the manuscript by Abdullah Madhun and Kate Brakstad.

References

1 WHO. Draft WHO guidelines on the use of vaccines and antivirals during influenza pandemics. Wkly Epidemiol Rec 2002;77:394–404.
2 Yewdell J, Garcia-Sastre A. Influenza virus still surprises. Curr Opin Microbiol 2002;5:414–18.
3 Reid AH, Janczewski TA, Lourens RM. *et al.* 1918 influenza pandemic caused by highly conserved viruses with two receptor-binding variants. Emerg Infect Dis 2003;9:1249–53.
4 Reid AH, Taubenberger JK, Fanning TG. The 1918 Spanish influenza: integrating history and biology. Microbes Infect 2001;3:81–7.
5 Barry DW, Mayner RE, Staton E. *et al.* Comparative trial of influenza vaccines. I. Immunogenicity of whole virus and split product vaccines in man. Am J Epidemiol 1976;104:34–46.
6 Gross PA, Ennis FA, Gaerlan PF, Denson LJ, Denning CR, Schiffman D. A controlled double-blind comparison of reactogenicity, immunogenicity, and protective efficacy of whole-virus and split-product influenza vaccines in children. J Infect Dis 1977;136:623–32.
7 Wood JM. Developing vaccines against pandemic influenza. Philos Trans R Soc Lond B Biol Sci 2001;356:1953–60.
8 Nicholson KG, Colegate AE, Podda A *et al.* Safety and antigenicity of non-adjuvanted and MF59-adjuvanted influenza A/Duck/Singapore/97 (H5N3) vaccine: a randomised trial of two potential vaccines against H5N1 influenza. Lancet 2001;357:1937–43.
9 Stephenson I, Nicholson KG, Wood JM, Zambon MC, Katz JM. Confronting the avian influenza threat: vaccine development for a potential pandemic. Lancet Infect Dis 2004;4:499–509.
10 Cox RJ, Mykkeltvedt E, Robertson J, Haaheim LR. Non-lethal viral challenge of influenza haemagglutinin and nucleoprotein DNA vaccinated mice results in reduced viral replication. Scand J Immunol 2002;55:14–23.
11 Cox RJ, Brokstad KA, Zuckerman MA, Wood JM, Haaheim LR, Oxford JS. An early humoral immune response in peripheral blood following parenteral inactivated influenza vaccination. Vaccine 1994;12:993–99.
12 Brokstad KA, Cox RJ, Olofsson J, Jonsson R, Haaheim LR. Parenteral influenza vaccination induces a rapid systemic and local immune response. J Infect Dis 1995;171:198–203.
13 Reed LJ, Muench H. A simple method of estimating fifty per cent endpoints. Am J Hyg 1938;27:493–97.
14 Hasbold J, Corcoran LM, Tarlinton DM, Tangye SG, Hodgkin PD. Evidence from the generation of immunoglobulin G-secreting cells that stochastic mechanisms regulate lymphocyte differentiation. Nat Immunol 2004;5:55–63.
15 O'Connor BP, Gleeson MW, Noelle RJ, Erickson LD. The rise and fall of long-lived humoral immunity: terminal differentiation of plasma cells in health and disease. Immunol Rev 2003;194:61–76.
16 Cyster JG. Homing of antibody secreting cells. Immunol Rev 2003;194:48–60.
17 Kendal AP, Pereira MS, Skehel J. Concepts and procedures for laboratory-based influenza surveillance. Publication No. B17-35. Centers for Disease Control, Atlanta, GA, 1982.
18 Hobson D, Curry RL, Beare AS, Ward-Gardner A. The role of serum haemagglutination-inhibiting antibody in protection against challenge infection with influenza A2 and B viruses. J Hyg (Lond) 1972;70:767–77.
19 Committee for Proprietary Medicinal Products. Note for guidance on harmonization of requirements for influenza vaccines. CPMP/BWP/214/96, Circular No. 96-0666: 1-22, 1997.
20 Boyer KM, Cherry JD, Welliver RC *et al.* IgM, IgG antibody responses after immunization of children with inactivated monovalent (A/New Jersey/76) and bivalent (A/New Jersey/76-A/Victoria/75) influenza virus vaccines. J Infect Dis 1977;136 (Suppl.):S665–71.
21 Stephenson I, Nicholson KG, Colegate A *et al.* Boosting immunity to influenza H5N1 with MF59-adjuvanted H5N3 A/Duck/Singapore/97 vaccine in a primed human population. Vaccine 2003;21:1687–93.
22 Hehme N, Engelmann H, Kunzel W, Neumeier E, Sanger R. Pandemic preparedness: lessons learnt from H2N2 and H9N2 candidate vaccines. Med Microbiol Immunol (Berl) 2002;191:203–8.
23 Moran TM, Park H, Fernandez-Sesma A, Schulman JL. Th2 responses to inactivated influenza virus can be converted to Th1 responses and facilitate recovery from heterosubtypic virus infection. J Infect Dis 1999;180:579–85.
24 Hehme N, Engelmann H, Kuenzel W, Neumeier E, Saenger R. Immunogenicity of a monovalent, aluminum-adjuvanted influenza whole virus vaccine for pandemic use. Virus Res 2004;103:163–71.
25 Charoenvit Y, Goel N, Whelan M, Rosenthal KS, Zimmerman DH. CEL-1000-a peptide with adjuvant activity for Th1 immune responses. Vaccine 2004;22:2368–73.
26 Chattergoon MA, Saulino V, Shames JP, Stein J, Montaner LJ, Weiner DB. Co-immunization with plasmid IL-12 generates a strong T-cell memory response in mice. Vaccine 2004;22:1744–50.
27 Takada A, Matsushita S, Ninomiya A, Kawaoka Y, Kida H. Intranasal immunization with formalin-inactivated virus vaccine induces a broad spectrum of heterosubtypic immunity against influenza A virus infection in mice. Vaccine 2003;21:3212–18.
28 Armerding D, Rossiter H, Ghazzouli I, Liehl E. Evaluation of live and inactivated influenza A virus vaccines in a mouse model. J Infect Dis 1982;145:320–30.
29 Nguyen HH, van Ginkel FW, Vu HL, McGhee JR, Mestecky J. Heterosubtypic immunity to influenza A virus infection requires B cells but not $CD8^+$ cytotoxic T lymphocytes. J Infect Dis 2001;183:368–76.
30 Stevens TL, Bossie A, Sanders VM *et al.* Regulation of antibody isotype secretion by subsets of antigen-specific helper T cells. Nature 1988;334:255–58.

31 Markine-Goriaynoff D, van der Logt JT, Truyens C *et al.* IFN-gamma-independent IgG2a production in mice infected with viruses and parasites. Int Immunol 2000;12:223–30.
32 Tamura S, Kurata T. Defense mechanisms against influenza virus infection in the respiratory tract mucosa. Jpn J Infect Dis 2004;57:236–47.
33 Peltoniemi J, Setala N, Broberg E. *et al.* Semliki Forest virus infection is enhanced in Th1-prone SJL mice but not in Th2-prone BALB/c mice during Linomide-induced immunomodulation. J Neuroimmunol 2002;132:83–92.
34 Guinazu N, Pellegrini A, Giordanengo L. *et al.* Immune response to a major Trypanosoma cruzi antigen, cruzipain, is differentially modulated in C57BL/6 and BALB/c mice. Microbes Infect 2004;6:1250–58.
35 Ramakrishna C, Ravi V, Desai A, Subbakrishna DK, Shankar SK, Chandramuki A. T helper responses to Japanese encephalitis virus infection are dependent on the route of inoculation and the strain of mouse used. J Gen Virol 2003;84:1559–67.
36 Liang S, Mozdzanowska K, Palladino G, Gerhard W. Heterosubtypic immunity to influenza type A virus in mice. Effector mechanisms and their longevity. J Immunol 1994;152:1653–61.
37 Fazekas G, Rosenwirth B, Dukor P, Gergely J, Rajnavolgyi E. IgG isotype distribution of local and systemic immune responses induced by influenza virus infection. Eur J Immunol 1994;24:3063–67.
38 Huber VC, Lynch JM, Bucher DJ, Le J, Metzger DW. Fc receptor-mediated phagocytosis makes a significant contribution to clearance of influenza virus infections. J Immunol 2001;166:7381–88.
39 Arkema A, Huckriede A, Schoen P, Wilschut J, Daemen T. Induction of cytotoxic T lymphocyte activity by fusion-active peptide-containing virosomes. Vaccine 2000;18:1327–33.
40 Bungener L, Serre K, Bijl L *et al.* Virosome-mediated delivery of protein antigens to dendritic cells. Vaccine 2002;20:2287–95.
41 Schumacher R, Adamina M, Zurbriggen R. *et al.* Influenza virosomes enhance class I restricted CTL induction through $CD4^+$ T cell activation. Vaccine 2004;22:714–23.
42 Sambhara S, Kurichh A, Miranda R *et al.* Heterosubtypic immunity against human influenza A viruses, including recently emerged avian H5 and H9 viruses, induced by FLU-ISCOM vaccine in mice requires both cytotoxic T-lymphocyte and macrophage function. Cell Immunol 2001;211:143–53.
43 Speidel K, Osen W, Faath S *et al.* Priming of cytotoxic T lymphocytes by five heat-aggregated antigens *in vivo*: conditions, efficiency, and relation to antibody responses. Eur J Immunol 1997;27:2391–99.
44 Saurwein-Teissl M, Zisterer K, Schmitt TL, Gluck R, Cryz S, Grubeck-Loebenstein B. Whole virus influenza vaccine activates dendritic cells (DC) and stimulates cytokine production by peripheral blood mononuclear cells (PBMC) while subunit vaccines support T cell proliferation. Clin Exp Immunol 1998;114:271–76.