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Two Doses of Parenterally Administered Split Influenza Virus Vaccine Elicited High Serum IgG Concentrations which Effectively Limited Viral Shedding upon Challenge in Mice

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Abstract

We have previously found that whole influenza virus vaccine induced a more rapid and stronger humoral response, particularly after the first dose of vaccine, than split virus vaccine in mice. In this study, we have evaluated the protective efficacy of whole and split influenza virus vaccines in mice using a nonlethal upper respiratory tract challenge model. We have also investigated the immunological correlates associated with no or very little viral shedding after viral challenge. Vaccination resulted in reduced viral shedding and shortened the duration of infection by at least 2 days. After one dose of vaccine, whole virus vaccine generally resulted in less viral shedding than split virus vaccine. In contrast, two doses of split virus vaccine, particularly the highest vaccine strengths of 15 and 30 μg HA, most effectively limited viral replication and these mice had high concentrations of prechallenge influenza-specific serum IgG. The vaccine formulation influenced the IgG2a/IgG1 ratio, and this IgG subclass profile was maintained upon challenge to some extent, although it did not influence the level of viral shedding. The concentration of postvaccination serum IgG showed an inverse relationship with the level of viral shedding after viral challenge. Therefore, serum IgG is an important factor in limiting viral replication in the upper respiratory tract upon challenge of an antigenically similar virus.

Introduction

Human influenza is a respiratory tract pathogen, and viral replication occurs predominantly in the epithelial cells of the respiratory tract. Immunity at this site usually involves pre-existing influenza-specific immunoglobulin G (IgG) and secretory IgA (S-IgA) antibodies, which promote viral elimination by forming antibody–virus complexes shortly after infection. Locally produced S-IgA is important in protecting the upper respiratory tract [1], whilst IgG plays an important role in preventing lethal influenza pneumonia [2, 3]. Influenza infection also induces a cytotoxic T lymphocyte (CTL) memory response that has proven to be, at least in mice, important for viral clearance [4–8].

Vaccination is the main method of influenza prophylaxis. There are two types of vaccine, namely parenterally administered inactivated vaccine and intranasal live, attenuated virus vaccine (reviewed in [9]). Split virus is the most utilized inactivated influenza vaccine formulation and induces protective levels of antibody and causes few side reactions [10, 11]. Whole virus vaccine is considered more immunogenic than split virus vaccine [11, 12] but is also more reactogenic and is therefore not widely used. Recently, the urgency for developing pandemic influenza vaccines has been highlighted by a number of avian subtypes crossing the species barrier into man [13, 14]. Vaccine containing avian influenza subtypes (H5 and H9) has proven to be poorly immunogenic in man [15–17], and this has led to the re-evaluation of the use of whole influenza virus vaccine.

We have recently found that whole and split influenza virus vaccines appear to elicit different distribution of IgG

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subclasses in mice [18], which may indicate different T helper (Th) responses [19]. We also found that whole virus vaccine induced an earlier and stronger humoral response, which may provide earlier protection after vaccination in unprimed individuals. In this study, we have therefore extended our work to investigate the efficacy of these vaccines in a nonlethal murine challenge model. We found that one dose of whole virus vaccine resulted in a more limited viral shedding than one dose of split virus vaccine. In contrast, two doses of split virus vaccine were most effective in reducing viral shedding and this was associated with high concentrations of prechallenge vaccine-induced serum IgG. Furthermore, we found that irrespective of the IgG subclass, high concentrations of serum IgG induced by vaccination were an important indicator of the efficacy of the vaccine.

Materials and methods

Mice. Female BALB/*c* A mice (6 to 8 weeks old purchased from Taconic M&B A/S, Ry, Denmark) were housed according to the Norwegian Animal Welfare Act, at a temperature of 21 °C, with 12 h light/dark cycles and food and water *ad libitum*. The animals, six groups with 24 mice per group, were vaccinated intramuscularly into the quadriceps muscles with one or two doses at 3-week intervals of 7.5, 15 or 30 μg HA of monovalent A/Panama/2007/99 (H3N2) split or whole virus vaccine (kindly provided by Sanofi-Aventis, Lyon, France). All mice were vaccinated intramuscularly in both hind legs (50 μl per leg), except mice immunized with 30 mg of split virus vaccine, which required two injections per hind leg (2×40 μl per leg) to deliver the correct amount of antigen. Nineteen unvaccinated mice were infected and used as a control group. Three mice died 3–9 weeks after vaccination and before viral challenge, presumably of natural causes. Nine weeks after the last dose of vaccine, a baseline serum sample was collected, and the mice were then intranasally challenged with 200 MID_{50} of the antigenically similar A/Moscow/10/99 (H3N2) influenza virus (kindly provided by Diane Major, NIBSC, UK). The WHO recommended inclusion of A/Moscow/10/99 (H3N2)-like strain as the H3N2 vaccine component from 2000 to 2003, and the A/Panama/2007/99 (H3N2) strain was most commonly used [20]. The mice were administered with a small volume of inoculum (25 μl) whilst awake to achieve an initial upper respiratory infection [21]. The mice were monitored daily for signs of clinical illness, e.g. loss of activity and ruffled fur, and no clear signs of illness or any significant loss of weight were recorded. Nasal wash (NW) samples were collected daily after viral challenge (up to day 4 or 6 in vaccinated or control mice, respectively) and stored at −80 °C until assayed for the presence of replicative virus [22]. Mice (four animals per group) were killed, and the blood, spleen, lungs, femur and tibia bones of the hind legs were collected at various time points after challenge (days 5, 7 and 21).

Presence of replicating virus in NW samples. NW samples were titrated in 10-fold serial dilutions in maintenance medium (DMEM) containing 2.5 μg/ml of TPCK trypsin (LS003665, Worthington Biomedical, Lakewood, NJ, USA), 100 IU/ml of penicillin, 100 μg/ml of streptomycin and 0.25 μg of fungizone (17–745E, Cambrex Biowhittaker, East Rutherford, NJ, USA) and 0.14% bovine serum albumin (A-6793, Sigma-Aldrich, St Louis, MO, USA) [22]. The diluted samples (10 parallels of each dilution at 100 μl/well) were then incubated on confluent MDCK cell monolayers in 96-well plates at 35 °C for 30 min, before the removal and addition of 100 μl of fresh maintenance medium. After a 72-h incubation period, the presence of replicative virus was detected by using an HA assay with 0.7% turkey red blood cells. Wells were scored for the presence of virus (100% haemagglutination) and the 50% tissue-culture infectious dose ($TCID_{50}$) was calculated by the method of Reed and Muench [23]. All NW samples from the same mouse were tested at the same time.

Antibody-secreting cell (ASC) response. Lymphocytes were isolated from the spleen, bone marrow and lungs, and the number of influenza class-specific ASC was detected in an ELISPOT assay as previously described [18, 22]. Briefly, ELISPOT plates were coated with 10 μg/ml of A/Panama/2007/99 (H3N2) (100 μl/well) of split virus vaccine overnight at 4 °C [24]. After incubation of the appropriate number of lymphocytes at 37 °C, the influenza-specific ASC were detected using 2 μg/ml of goat anti-mouse biotinylated class (IgG; 1030–08, IgA; 1040–08, IgM; 1020–08, Southern Biotechnology, Birmingham, AL, USA) specific antibodies. The number of influenza-specific ASC per 500,000 lymphocytes was calculated for each individual animal.

ELISA. An indirect ELISA was used to detect the class and IgG subclass (IgG1 and IgG2a) of influenza-specific serum antibodies [18]. Briefly, 96-well ELISA plates were coated with 10 μg/ml of A/Panama/2007/99 (H3N2) split virus (100 μl/well) overnight at 4 °C. The influenza-specific antibodies were detected using goat anti-mouse biotinylated class (see ELISPOT) or IgG subclass (IgG1; 1070–08, IgG2a; 1080–08, Southern Biotechnology) specific antibodies. Dilutions of appropriate immunoglobulin standards were added to the plates (IgG, I-5381; IgA, M-1421; IgM, M-3795, Sigma-Aldrich). The concentration of influenza-specific antibody (μg/ml) was calculated for each antibody class and each IgG subclass.

Haemagglutination inhibition (HI) assay. Nonspecific inhibitors were removed by treating the serum overnight at 37 °C with receptor destroying enzyme (Denka Seiken, Tokyo, Japan) and then subsequent inactivation at 56 °C [25]. The assay was carried out using 8 haemagglutinating

units of virus and 0.7% turkey red blood cells as previously described [24]. Titres are reported as the reciprocal of the dilution of serum needed to inhibit 50% haemagglutination. Negative samples (titres <10) were assigned a titre of 5 for calculation purposes.

Virus neutralization (VN) assay. The assay was carried out as previously described [18, 24]. Briefly, heat-treated sera were diluted twofold and incubated in quadruplicate with A/Panama/2007/99 (H3N2) at room temperature for 1 h. The serum/virus mixture was then added to confluent MDCK monolayers in 96-well tissue-culture plates for 30 min at 35 °C and subsequently replaced with medium for 72 h. The presence of virus was recorded by an HA assay with 0.7% turkey red blood cells, and the neutralization antibody titres were calculated [23].

Statistical analysis. Differences between the mean concentration (ELISA) and geometric mean (VN and HI) and mean numbers of ASC (ELISPOT) were analysed by a two-sided Student's *t*-test. The NW titres were analysed by the Mann–Whitney *U*-test using SPSS version 12 for windows. A *P*-value ≤ 0.05 was considered statistically significant.

Results

Mice were vaccinated with one or two doses of monovalent split or whole A/Panama/2007/99 (H3N2) virus vaccine at three different vaccine strengths (7.5, 15 or 30 μg HA). The normal human dose in the current trivalent influenza vaccines is 15 μg HA of each strain. Subsequently, mice (including an unvaccinated control group) were challenged with 200 MID_{50} of the antigenically closely related A/Moscow/10/99 (H3N2) virus 9 weeks after the last dose of vaccine. A low-challenge volume (25 μl) of a nonlethal virus was administrated whilst the mice were awake, aiming to produce a self-limiting upper respiratory tract infection [21, 22, 26]. A prechallenge serum sample and daily NW samples were collected from all mice, before groups of mice were killed at days 5, 7 and 21 after challenge.

Nasal wash viral titres after challenge

Nasal wash samples were collected daily from vaccinated mice for the initial 4 days after viral challenge. Low viral titres were detected 1 day after challenge followed by a sharp increase in viral shedding with the peak titres observed at day 2 in both the vaccinated and the unvaccinated control mice (Fig. 1). Vaccinated mice cleared the infection more quickly than controls. Little or no virus was detected at 4 days after challenge in vaccinated animals, whereas the control mice had up to 24-fold higher titres at day 4 and still low levels of viral shedding (<10 $TCID_{50}$) at day 6 (data not shown). One dose of vaccine (both formulations) significantly reduced peak viral titres ($P < 0.05$) compared with unvaccinated controls (three-

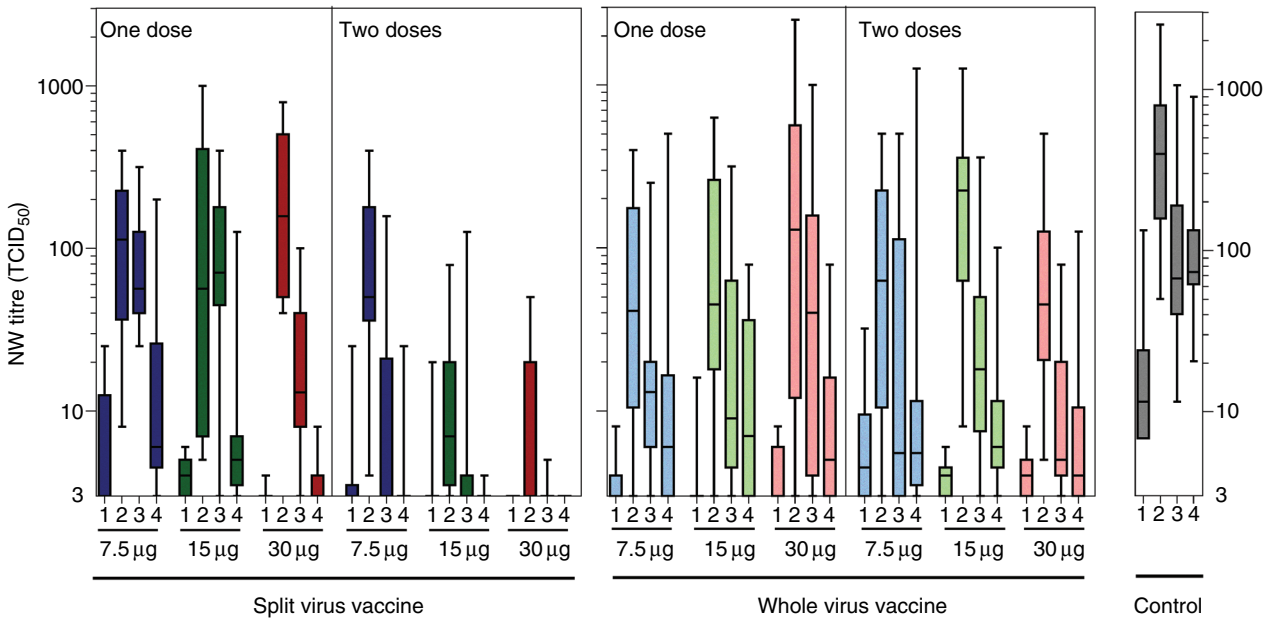

Figure 1 Viral replication after viral challenge of mice previously vaccinated with split or whole virus vaccine. Mice were immunized with one or two doses of split (7.5 μg dark blue, 15 μg dark green or 30 μg dark red) or whole (7.5 μg pale blue, 15 μg pale green or 30 μg pale red) virus vaccine or remained unvaccinated (Control group, grey) and were challenged by intranasal infection 9 weeks after the last dose of vaccine. Nasal wash (NW) samples were collected from all mice at days 1, 2, 3 or 4 after challenge. Bars indicate maximum and minimum NW titres; boxes indicate 75 quartiles, median and 25 quartiles. Each group consisted of 12 mice, with the exception of one dose of 30 μg split and two doses split of 7.5 and 30 μg which all had 11 mice in each group. The control group consisted of 19 unvaccinated mice. NW samples with no detectable virus were assigned a titre of 3 (the lowest detection limit was 4 $TCID_{50}$).

to ninefold). Generally, mice vaccinated with one dose of whole virus vaccine had lower peak viral NW titres than mice vaccinated with one dose of split virus vaccine. Mice immunized with one dose of whole virus vaccine or 30 μg of split virus vaccine had a shorter duration of viral shedding, with lower NW titres observed 3 days after challenge than in mice vaccinated with 7.5 and 15 μg of split virus vaccine. After one dose of vaccine, four of 71 mice had NW < 10 $TCID_{50}$, of which three mice were vaccinated with whole virus vaccine.

Immunization with two doses of either vaccine formulation resulted in significantly lower NW titres than in the control mice. Viral shedding was much lower in mice vaccinated with two doses of split virus vaccine than in those with one dose. In contrast, no clear difference in the viral replication was observed after one or two doses of whole virus vaccine. Mice immunized with two doses of split virus vaccine had eight- to 130-fold lower NW titres on the peak day than the unvaccinated controls, whereas the group vaccinated with whole virus vaccine had only two- to eightfold lower peak NW titres. The lowest NW titres were observed in mice vaccinated with two doses of the two highest strengths of split virus vaccine (15 and 30 μg), which generally had very low levels of viral shedding on the peak day (2 days after challenge). Five mice vaccinated with 30 μg of split virus vaccine had no detectable virus in the nasal cavity at any time point. After two doses of vaccine, 20 mice had a peak NW titre < 10 $TCID_{50}$, of these 13 were vaccinated with split virus vaccine (12 mice from the 15 and 30 μg vaccine groups) and six mice vaccinated with whole virus vaccine (3, 1 or 2 mice vaccinated with 7.5, 15 or 30 μg, respectively). Importantly, mice were also divided into two groups based on the peak viral NW titre with no or low NW titres (<10 $TCID_{50}$) or higher NW titres, to allow correlation with humoral immunological parameters. Due to the low number of mice with low NW titres (<10 $TCID_{50}$) after one dose of vaccine, only mice vaccinated with two doses of vaccine were analysed for the immunological correlates (Table 1).

Table 1 Relationship between no or very low nasal wash (NW) titres and immunological parameters after viral challenge of mice previously vaccinated with two doses of split or whole virus vaccine

			Pre-challenge		Days after challenge: 5		7		21	
			NW < 10 (n = 20)	NW ≥ 10 (n = 50)	NW < 10 (n = 9)	NW ≥ 10 (n = 15)	NW < 10 (n = 5)	NW ≥ 10 (n = 18)	NW < 10 (n = 6)	NW ≥ 10 (n = 17)
Serum*		IgG	933	552	785	423	1267	909	726	947
	VN		ND		538	323	452	293	1974	1065
	HI		960	640	966	823	1311	647	2793	2183
ASC†	Spleen	IgG			10	5	60	98	43	32
	IgA				7	8	39	86	21	35
	Bone marrow	IgG			28	21	62	60	94	56
	IgA				6	6	16	31	42	49
	Lung	IgG			2	5	51	160	5	3
	IgA				6	13	78	98	11	4

HI, haemagglutination inhibition; ND, not done; VN, virus neutralization. The mice were divided into two groups based on peak NW titres <10 or ≥10, irrespective of the vaccine formulation.
*Serum IgG is presented in μg/ml, and VN and HI are presented as geometric mean titres.
†Mean number of influenza-specific ASC per 5×10^5 lymphocytes, no prechallenge samples.

ASC response after challenge

Higher numbers of influenza-specific IgG and IgA ASC and low numbers of IgM ASC were detected after viral challenge of vaccinated mice in the spleen, bone marrow and lungs. Generally, the numbers of influenza-specific IgG and IgA ASC in the spleen and the lungs were highest 7 days after challenge with lower numbers of ASC detected at days 5 (data not shown) and 21 (Fig. 2). In the bone marrow, mice vaccinated with whole virus vaccine had peak numbers of ASC at 7 days after challenge, whereas the highest numbers of ASC were observed at day 21 in the split virus vaccine group. The number of IgM ASC remained essentially stable after viral challenge of

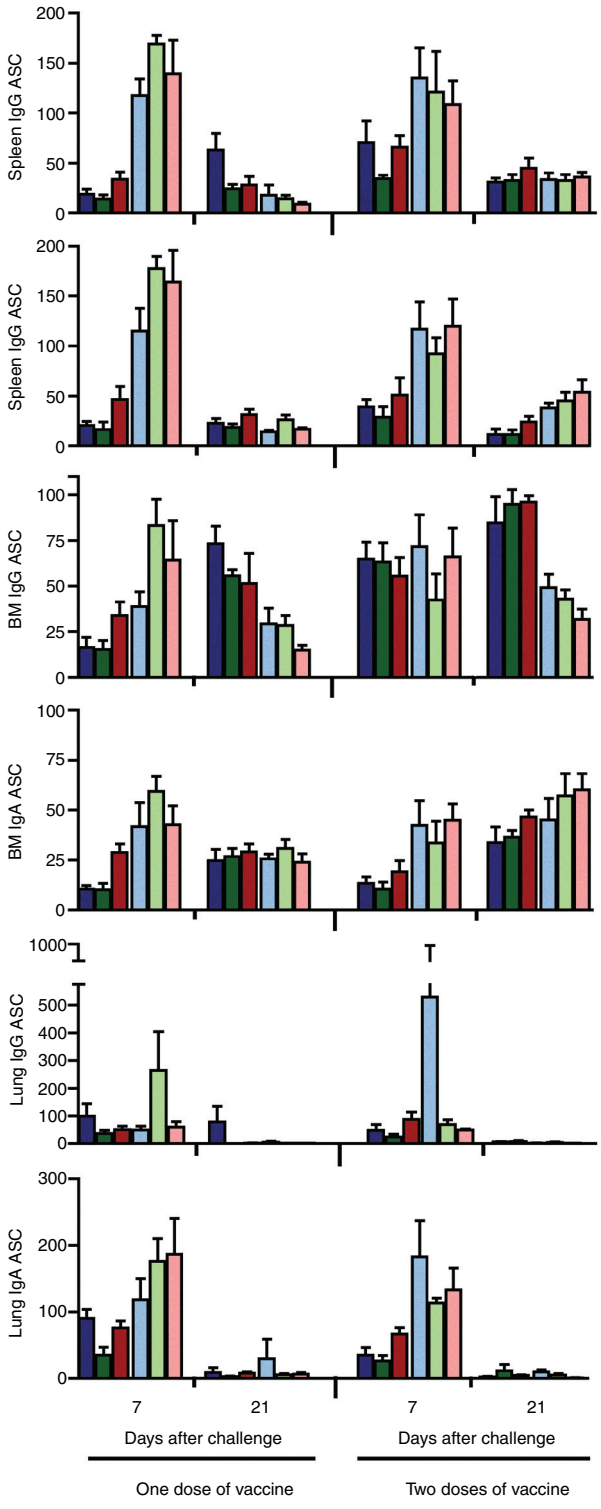

Figure 2 Influenza-specific IgG and IgA antibody-secreting cells (ASC) in the spleen, bone marrow and lungs elicited after viral challenge of mice previously vaccinated with split or whole virus vaccine. Mice were immunized with one or two doses of split (7.5 μg dark blue, 15 μg dark green or 30 μg dark red) or whole (7.5 μg pale blue, 15 μg pale green or 30 μg pale red) virus vaccine and were challenged by intranasal infection 9 weeks after the last dose of vaccine. The data are presented as the mean number of influenza-specific ASC per 500,000 lymphocytes ± SEM.

vaccinated mice. The control mice had very low numbers of influenza-specific (IgM, IgG and IgA) ASC after challenge, usually less than 10 per 5×10^5 lymphocytes, in all tissues (data not shown). The low numbers of lung ASC observed after infection of control mice suggest that the challenge dose of virus resulted in an upper respiratory tract infection.

ASC response in the spleen

Higher numbers of IgA (two- to 10-fold) and IgG ASC (two- to 12-fold) were observed 7 days after viral challenge in mice previously immunized with one or two doses of whole virus vaccine than split virus vaccine. Whereas similar numbers of ASC were detected by 21 days after challenge, except IgG ASC after one dose of split and IgA ASC after two doses of whole virus vaccine. The number of IgM ASC was higher (approximately twofold) after challenge of mice vaccinated with whole virus vaccine at all sampling days (data not shown). When the mice were grouped according to no or low (<10) or higher (≥10) NW titres, the number of splenic ASC at 7 days after challenge was dependent on the level of viral shedding (Table 1). Mice with no or low NW titres (<10) had 40–50% less IgG and IgA ASC in the spleen on the peak day, 7 days after challenge, but similar numbers of ASC on the other sampling days.

ASC response in the bone marrow

Mice immunized with one dose of whole virus vaccine generally had the highest numbers of IgG ASC 7 days after challenge, whereas the IgG ASC numbers increased up to day 21 after challenge in mice immunized with split virus vaccine (Fig. 2). Comparable numbers of IgG ASC were observed 7 days after challenge in mice immunized with two doses of split or whole virus vaccine but were twofold higher at day 21 in mice previously immunized with split virus vaccine. Higher numbers of IgA ASC were detected at 7 days after viral challenge in mice immunized with whole virus vaccine, but similar numbers of IgA ASC were found at day 21 compared with mice vaccinated with split virus vaccine. Similar numbers of IgG ASC were detected at days 5 and 7, but at 21 days after challenge mice with no or low NW titres (<10) had 70% higher numbers of IgG ASC than mice with higher NW titres (≥10) (Table 1).

ASC response in the lungs

Viral challenge of mice immunized with one and two doses of whole virus vaccine resulted in higher numbers of IgA ASC at days 5 (data not shown) and 7 than in mice vaccinated with split virus vaccine, with comparable numbers of IgA ASC detected at day 21 (Fig. 2). Similar numbers of IgG ASC were detected in the lungs after challenge of mice vaccinated with one or two doses of vaccine (both formulations). However, three mice

vaccinated with one dose of 15 μg whole virus vaccine had high numbers of IgG ASC after viral challenge, but this was not associated with higher levels of viral shedding in these mice. In contrast, two mice immunized with two doses of 7.5 μg whole virus vaccine had high numbers of IgG and IgA ASC at 7 days after challenge, and also high peak NW titres (200–501 $TCID_{50}$). Mice with no or low NW titres (<10) had lower numbers of IgG and IgA ASC (up to threefold) at 5 and 7 days after viral challenge than mice with higher NW titres (≥10) (Table 1).

Serum antibody response after challenge

Antibody class and IgG subclasses

IgG dominated the serum response both before and after viral challenge (Table 2) with 1000-fold lower IgA than IgG concentrations detected (results not shown). The prechallenge serum IgM concentrations were similar after one dose of both vaccine formulations but were 10-fold higher after two doses of split virus vaccine than whole virus vaccine (data not shown). The control mice had lower serum IgG antibody concentrations after challenge than vaccinated mice, five- to 22-fold after one dose and four- to 15-fold after two doses of vaccine (Table 2).

Similar concentrations of serum IgG were observed before and up to 7 days after challenge in mice previously immunized with one dose of either vaccine formulation. However, at 21 days after challenge, the IgG concentration was two- to threefold higher in mice vaccinated with whole virus vaccine than split virus vaccine. Mice immunized with two doses of vaccine had higher prechallenge serum IgG concentrations (up to 10-fold) compared with mice vaccinated once (Table 2). After vaccination with the two highest strengths of split virus vaccine (15 and 30 μg), significantly higher ($P < 0.05$) before and after (up to day 7) challenge serum IgG concentrations were detected than in the corresponding whole virus vaccine group. However, 21 days after viral challenge, mice immunized with two doses of split virus vaccine had lower serum IgG concentrations than the comparable whole virus vaccine mice, probably reflecting the lower level of viral replication observed in these animals. Two doses of split virus vaccine formulation were effective at inducing high levels of IgG, and similar IgG concentrations were observed after both immunization with 30 μg, which required four injections to deliver the correct amount of antigen instead of the usual two injections, and 15 mg split virus vaccine. Mice vaccinated with two doses of vaccine and with no or low NW titres (<10) had higher serum IgG concentrations before (70%) and 5 days (85%) after challenge than mice with higher peak NW titres (Table 1).

After viral challenge, the IgG2a subclass dominated the serum antibody response both in mice vaccinated with whole virus vaccine and in unvaccinated controls (Table 2). In contrast, mice immunized with split virus

Table 2 Influenza-specific serum IgG and the IgG2a/IgG1 ratio elicited after viral challenge of mice previously vaccinated with split or whole virus vaccine

		Days after challenge					
		One dose of vaccine			Two doses of vaccine		
Vaccine formulation	Vaccine strength (μg)	Pre-challenge	7	21	Pre-challenge	7	21
Split	7.5	108 ± 12 (0.8)	577 ± 110 (0.9)	774 ± 208 (1.3)	136 ± 18 (1.0)	894 ± 183 (0.6)	361 ± 38 (1.7)
	15	86 ± 7 (0.8)	585 ± 139 (0.9)	773 ± 118 (2.1)	822 ± 70 (1.3)	1401 ± 63 (1.0)	680 ± 253 (2.4)
	30	124 ± 9 (1.3)	878 ± 172 (1.1)	447 ± 11 (3.2)	1295 ± 206 (2.2)	1542 ± 372 (1.3)	455 ± 44 (3.8)
Whole	7.5	119 ± 16 (10.5)	403 ± 10 (4.0)	2085 ± 278 (6.5)	577 ± 72 (6.0)	819 ± 178 (6.1)	1015 ± 22 (9.0)
	15	127 ± 8 (10.6)	532 ± 146 (8.8)	1733 ± 277 (5.5)	489 ± 92 (4.6)	474 ± 100 (3.6)	1385 ± 30 (4.4)
	30	126 ± 10 (6.5)	653 ± 202 (4.6)	1463 ± 283 (4.4)	655 ± 76 (5.1)	929 ± 136 (3.0)	1310 ± 93 (7.1)
Control*		0	3 ± 1 (†)	92 ± 11 (4.5)	Not done		

The mean serum IgG concentration (μg/ml) ± SEM and in brackets the serum IgG2a/IgG1 ratio. In the vaccinated mice, prechallenge concentrations are the mean of 12 mice, whereas data from 7 and 21 days after challenge are the mean of four mice.
*Challenge of 19 unvaccinated controls.
†No IgG1 detected.

vaccine had a more mixed IgG2a and IgG1 antibody response. In these mice, the concentration of IgG2a increased with increasing vaccine strength and was also higher at 21 days after challenge. The concentration of prechallenge serum IgG1 and IgG2a was higher in mice with no or low NW titres (<10) than in mice with higher NW titres, 343 μg/ml of IgG1 compared with 144 μg/ml and 562 μg/ml of IgG2a compared with 332 μg/ml, respectively.

HI and VN antibodies

Generally, the HI and VN titres increased up to 21 days after challenge (Table 3). The HI and VN titres were much lower in the control group than in mice vaccinated with one or two doses of vaccine. Higher VN titres were observed 21 days after challenge in mice vaccinated once with split virus vaccine than mice immunized with whole virus vaccine. Generally, mice vaccinated with two doses of split virus vaccine had higher HI and VN titres than mice vaccinated with whole virus vaccine at all time points, with up to twofold higher HI and VN titres at 21 days after challenge. Mice with no or low NW titres (<10) had higher HI (20–200%) and VN (50–80%) titres compared with mice with higher NW titres at all time points after challenge (Table 1).

Discussion

Mice are widely used as a model for studying the immune response and protective efficacy of influenza vaccines. We have previously observed that the formulation of an influenza vaccine can influence the outcome of the humoral immune response in mice [18]. We found that whole virus vaccine, particularly after the first dose, more rapidly induced higher serum antibody titres (especially neutralizing antibody) and elicited higher numbers of IgG ASC in the spleen and bone marrow. In this study, we have extended our work to investigate the protective efficacy of these two vaccine formulations by using a nonlethal upper respiratory tract challenge model. We have also examined the humoral immunological correlates that were associated with no or very limited viral shedding (NW titres < 10 $TCID_{50}$).

In this study, mice were challenged intranasally 9 weeks after the last dose of vaccine, a period of time more similar to the situation normally observed in man, where several weeks or months may pass before the vaccinee is exposed to epidemic influenza. We found that viral challenge of animals immunized with one dose of either vaccine formulation resulted in less viral shedding (three- to ninefold) and shortened the duration of infection by at least 2 days compared with unvaccinated control mice. One dose of whole virus vaccine generally resulted in less viral replication after viral challenge than one dose of split virus vaccine, and the time course of infection was modified resulting in a sharp reduction in viral shedding from day 3. Although mice immunized with one dose of 30 μg of split virus vaccine had higher level of viral replication on the peak day, they also had a similar sharp decrease in viral shedding from day 3. Mice immunized with two doses of split virus vaccine (15 and 30 μg) had very high prechallenge serum IgG concentrations, in addition to IgM and HI antibodies, which appears to have significantly modified the course of infection resulting in no or very limited

Table 3 Serum haemagglutination inhibition (HI) and virus neutralization (VN) antibody titres induced after viral challenge of mice previously vaccinated with split or whole virus vaccine

			Days after challenge					
			One dose of vaccine			Two doses of vaccine		
	Vaccine formulation	Vaccine strength (μg)	Pre-challenge	7	21	Pre-challenge	7	21
HI	Split	7.5	320	538	3044	640	659	3355
		15	480	640	6089	960	1685	2637
		30	320	640	2918	960	1057	2918
	Whole	7.5	240	659	3729	640	554	1685
		15	240	729	4434	480	466	2637
		30	240	466	4007	960	659	1522
	Control*			6	98			
VN	Split	7.5	ND	153	2347	ND	364	1612
		15		254	3620		472	2560
		30		320	1810		508	1733
	Whole	7.5		236	2153		216	613
		15		226	1810		208	1336
		30		174	1589		320	698
	Control			21	182			

ND, not done. The data are presented as the geometric mean HI and VN titres at 7 and 21 days after challenge. Prechallenge HI titres were obtained from pooled sera from 12 mice.
*Challenge of 19 unvaccinated controls.

viral replication. Others have also shown that parenteral influenza vaccination results in a shorter duration of viral shedding in both man [27] and mice [28]. In man, lower levels of viral shedding are associated with lower grade fever and a reduction in the severity of illness [29].

The common mucosal system allows specific IgA ASC precursor populations induced at one site to migrate and home to other mucosal surfaces. Importantly, cells generated systemically by parenteral vaccination also home to the mucosa although less efficiently than mucosally generated cells [30]. Earlier we have observed that parenteral influenza vaccination of mice resulted in high numbers of IgG ASC and low numbers of IgM in the spleen and bone marrow, whereas there were little or no IgA-secreting cells [18]. In this study, we found only very low numbers of splenic IgG and IgA ASC after infection of unvaccinated control mice, which is probably due to the mild upper respiratory tract infection observed in our experimental animals. Furthermore, we found that upon viral challenge of vaccinated mice, the ASC response in the spleen, bone marrow and lungs consisted mainly of IgG and IgA ASC, with low numbers of IgM-secreting cells. This indicates a class switch to IgA as a result of respiratory tract infection [31].

The nasal associated lymphoid tissue (NALT) is the inductive site of mucosal responses in the upper respiratory tract and is also important in lymphocyte recirculation [32, 33]. In naïve mice, S-IgA and CTLs generated in the NALT are the major effectors involved in recovery from primary influenza infection. In the lower respiratory tract, the bronchus-associated lymphoid tissue (BALT) is probably the inductive site [34]. In a primary influenza infection, IgM ASC are first detected in the lungs at day 5, whereas IgA and IgG ASC appear later and are present for longer periods of time [35]. This contrasts with the earlier and transient detection of IgG and IgA ASC in the lungs found in this study upon viral challenge of vaccinated mice. In mice, influenza infection elicits immunological memory which upon subsequent viral encounter results in an earlier increase in the number of ASC and a concomitant increase in antibody production leading to reduced viral shedding from 2 to 3 days after infection [36, 37]. Further studies are needed to evaluate the role of the ASC response in the NALT to provide a clearer picture of the B-cell response in the upper respiratory tract after viral challenge of parenterally vaccinated mice.

We have previously found that vaccination of mice with whole virus vaccine, particularly after the first dose, produced higher numbers of IgG ASC in the spleen and bone marrow than after vaccination with split virus vaccine [18]. In addition, whole virus vaccine elicited very low numbers of IgA ASC in the bone marrow, whereas no IgA ASC was detected after split virus vaccination (unpublished observations). Upon viral challenge of whole virus vaccinated mice, earlier and higher numbers of IgG and IgA ASC were detected in the spleen and bone marrow than in split virus-vaccinated mice. This may be correlated with more potent IgG and IgA memory responses induced by whole virus vaccine, which was reactivated upon subsequent viral challenge. Mice that had significantly reduced viral replication, and therefore presumably lower levels of antigenic stimulation, had lower numbers of ASC in the spleen and lungs, but higher numbers of IgG ASC in the bone marrow. B cells differentiate into short-lived or long-lived ASC after activation by antigen in the secondary lymphoid organs and the long-lived ASC home to the bone marrow [38–41] where they secrete antibody for long periods of time, contributing to the serum antibody level. We have found that the number of bone marrow IgG ASC detected after vaccination [18] appears to be an important indicator of the concentration of serum IgG found after challenge and thus of the protective efficacy of the vaccine.

Secretory IgA functions mainly in the prevention of influenza infection in the upper respiratory tract, whereas serum IgG is thought to be important in limiting viral replication and therefore severity of illness, e.g. viral pneumonia (reviewed in [36]). Secretory IgA is locally produced (reviewed in [42]) and is actively transported across the epithelial cells of the mucosa [1]. Parenterally administered inactivated influenza vaccine does not effectively induce mucosal IgA in an unprimed host [43]. No IgA was detected in the NW immediately after challenge of our vaccinated mice (data not shown), and thus IgA was probably not involved in the reduction in viral shedding observed in this study. IgM antibodies also have the ability to be actively secreted across the mucosal surfaces in man [44], but this is not unequivocally demonstrated in rodents [45, 46]. We found only very low concentrations of vaccine-induced serum IgM before challenge, and thus it was probably not essential in reducing viral shedding.

Serum IgG enters mucosal secretions by passive diffusion, which occurs more effectively in the lungs than in the nasal cavity [47]. Therefore, in the upper respiratory tract damage to the epithelial layer during viral replication may be important for passage of IgG across the mucosal barrier [48]. Yoshikawa *et al.* [37] have in an elegant study demonstrated that viral neutralization by IgG antibodies alters the kinetics of influenza infection. We have shown here that high concentrations of vaccine-induced influenza-specific serum IgG were important in significantly reducing viral shedding but did not completely prevent infection. This has been confirmed by passive immunization where IgG antibodies have been shown to substantially reduce viral shedding in an upper respiratory tract challenge model but not inhibit infection [3, 26, 49, 50]. It was also concluded in a recent study that following vaccination with as little as 0.07 μg of influenza HA subcutaneously, mice were protected against illness and that this was correlated with systemic IgG antibody [51].

In man, parenteral vaccination generally induces high concentrations of serum IgG and is effective at reducing

morbidity and mortality in years of close antigenic match between vaccine viruses and circulating strains. In contrast, S-IgA has been shown to be more cross-reactive with drifted strains [52] and an ideal influenza vaccine would also benefit from the induction of S-IgA.

Influenza infection induces primarily an IgG2a response as observed in this study and previous study [53], and higher prechallenge serum IgG2a concentrations were found in mice with no or very low levels of viral shedding. The IgG2a subclass is an efficient activator of complement [54], and it participates in the antibody-dependent cytotoxic response [55]. These two functions of IgG antibodies have previously been shown to be important in preventing and clearing influenza virus infection [55]. Mice with limited viral replication also had higher concentrations of IgG1 antibodies, and this antibody class has been shown to be effective in neutralizing virus [56]. Therefore, it appears that the subclass of IgG was not so important as the absolute concentration of IgG in reducing viral shedding. We have earlier found that whole virus vaccine elicited a higher serum IgG2a/IgG1 ratio than split virus vaccine [18]. A high ratio of serum IgG2a/IgG1 may indicate a Th1 response, which is characterized by an effective cell-mediated response [57–59] including IFN-γ production which is important in blocking viral replication. Interestingly, the Th profile, shown by the IgG2a/IgG1 ratio following vaccination, appears to some degree to influence the subsequent Th response upon viral challenge. We have previously speculated that whole virus vaccine may produce a more effective cellular response than split virus vaccine. If Th1 memory cells and CTLs are able to generate effective cellular responses in the respiratory tract, whole virus vaccine may be a more appropriate vaccine in combating severe influenza infection.

In conclusion, this study shows that the presence of vaccine induced IgG was an important factor in reducing viral replication and shortening the course of infection upon viral challenge. One dose of whole virus vaccine was more immunogenic and more effective in limiting viral replication than one dose of split virus vaccine. Two doses of split virus vaccine, particularly the two highest strengths, resulted in high serum IgG concentrations which upon viral challenge appeared to be important in significantly limiting viral shedding in the upper respiratory tract. However, parenteral vaccination did not generally produce sterilizing immunity to prevent infection. In man, limited viral replication in vaccinees upon subsequent influenza infection may in fact be beneficial in inducing both mucosal and cellular responses to complement the vaccine-induced immunity.

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