Paper II
Parenteral Vaccination against Influenza Does Not Induce a Local Antigen-Specific Immune Response in the Nasal Mucosa

Karl Albert Brokstad, 1,2,3 Jens-Christian Eriksson, 3
Rebecca Jane Cox, 2 Turid Tynning, 1,3 Jan Olofsson, 3
Roland Jonsson, 1,2,3 and Åke Davidsson 1,3,4

1 Broegelmann Research Laboratory and 2 Department of Microbiology and Immunology, University of Bergen, and 3 Department of Otolaryngology/Head and Neck Surgery, Haukeland University Hospital, Bergen, and 4 Department of Otolaryngology, Örebro Medical Centre Hospital, Örebro, Sweden

The immune response in the nasal mucosa to influenza vaccination in 23 patients scheduled for tonsillectomy was studied. A statistically significant increase in influenza virus–specific serum and oral fluid antibodies was observed 7 days after vaccination. The numbers of influenza virus–specific antibody-secreting cells (ASCs) in peripheral blood also increased significantly 1 week after vaccination. The numbers of ASCs in tonsils and nasal mucosa were compared with data from a recent study of nonvaccinated volunteers. The numbers of influenza virus–specific ASCs in tonsils were significantly higher in the vaccinated group, but, surprisingly, there was no significant difference between the groups in the numbers of ASCs in nasal mucosa. This suggests that the influenza virus–specific antibodies detected in oral fluid are not produced locally in the nasal mucosa and may originate from a systemic source, indicating that the vaccination may favor a systemic immune response.

Influenza virus is a common respiratory pathogen; it causes high morbidity in the general population and high mortality in at-risk groups. The annual costs associated with influenza, including the cost of hospitalization, disease treatment, and loss of productivity, are considerable [1].

For many years, vaccination has been the major method of influenza prophylaxis. Recently, several antiviral drugs have been developed for therapeutic and prophylactic use. Despite vast investment in anti–influenza virus drugs, influenza vaccination remains the major and most cost-effective method of prophylaxis. Two factors that have been considered important to production of an effective influenza vaccine are the antigenic match between strains included in the vaccine and those circulating in the community and the vaccine’s ability to stimulate the production of serum antibodies, as tested by the hemagglutination inhibition (HAI) assay.

In previous studies, we investigated the humoral immune response induced by parenteral influenza vaccination and natural infection [2–5]. We found that parenteral influenza vaccination induced a rapid and strong systemic immune response [2, 3], whereas the local immune response was equally rapid but more transient [3, 4]. More recently, we have examined in greater detail the basal levels of influenza virus–specific antibody-secreting cells (ASCs) in blood, tonsils, and nasal mucosa [5]. The results from this study are interesting because, although none of the study subjects had been recently vaccinated or naturally infected with influenza virus, it was still possible to detect low levels of influenza virus–specific ASCs in all compartments [5]. We also found that the frequency of influenza virus–specific ASCs among the isolated lymphocytes was 10–100 times higher in the nasal mucosa than in the blood and tonsils [5].

The aim of the present study was to examine the effect of parenteral influenza vaccination on the numbers of influenza virus–specific ASCs locally in the tonsils and nasal mucosa.

Patients, Materials, and Methods

Patients and samples. Samples for immunologic testing were obtained from 23 volunteers (11 male and 12 female; mean age, 26 years) who were scheduled for tonsillectomy at the Haukeland University Hospital (Bergen, Norway) during August to December 2000. The subjects were chosen from among patients with recurrent tonsillitis who were otherwise healthy. The patients had not experienced any infection during the preceding 3 months, had no history of allergy, and had not been immunized or infected with influenza virus during the last few years. One week before the operation, peripheral blood was collected by intravenous puncture, and oral fluid was collected with an absorbent pad on a stick (Orasure; Epitope), and the subjects were vaccinated with influenza vaccine (Fluarix; SmithKline Beecham). The trivalent vaccine consisted of 15 μg of hemagglutinin (HA) from each of the following virus strains: A/
New Caledonia/20/99 (H1N1), A/Panama/2007/99 (H3N2), and B/Yamanashi/166/98. Seven days later, bilateral tonsillectomy was done. Blood and oral fluid were collected a second time from the volunteers. In addition, the adenoid tonsils and a nasal mucosal biopsy sample from the middle portion of the caudal medial part of the inferior turbinate were collected from 8 of the patients.

A blood sample was collected before the vaccination, in conjunction with the clinical examination of the patients. These samples were sent to the laboratory at Haukeland University Hospital and analyzed for antibodies to influenza A virus, influenza B virus, para-influenza viruses, and common allergens, in addition to analysis in routine preoperative tests.

Because prevaccination samples from tonsils and nasal mucosa were not available, results from postvaccination samples from tonsils and nasal mucosa were compared with data from a recent and comparable study that included 9 male and 10 female nonvaccinated volunteers (mean age, 28 years) [5]. These subjects belonged to the same patient group, had similar basal anti–influenza virus antibody levels, and had not recently been vaccinated. Age and sex distribution were similar in the 2 groups.

**HAI assay.** The vaccine strains (from the 2000/2001 influenza season) A/New Caledonia/2009 (H1N1), A/Panama/2007/99 (H3N2), and B/Yamanashi/166/98 were propagated in embryonated hens’ eggs and used as viral antigens in the HAI test. Serum was pretreated with receptor-destroying enzyme (Denka Seiken) overnight at 37°C and then incubated for 1 h at 56°C to remove nonspecific inhibitors. The HAI test was done with 8 HA units of virus and 0.7% (vol/vol) turkey erythrocytes, as described elsewhere [2, 6]. A serum HAI titer of ≥40 has been deemed to be protective [7].

**ELISA.** The concentrations of anti–influenza virus antibodies in serum and oral fluid were measured by ELISA [2–4]. Purified surface glycoproteins (gift of Medeva Pharma) from the vaccine strains were used as antigens. Ninety-six–well microtiter plates were coated with 0.1 μg of HA in 100 μL of PBS per well overnight at 4°C. The wells were blocked with 10% fetal calf serum (FCS) in PBS before serum and oral fluid samples were applied in duplicate dilution series. For detection, we used peroxidase-conjugated goat anti-human polyclonal immunoglobulins (A-8400; Sigma). The reaction was developed with 100 μL/well o-phenylenediamine (P-2045; Dako) at a concentration of 0.7 mg/mL and stopped with 100 μL of 1 M H₂SO₄ per well. The plates were thoroughly washed between each step with multiple washes of PBS. The results were read with a Mikrotek Emax microplate reader and analyzed with SoftMax Pro software (Apple Macintosh version). A standard curve was prepared with anti-human polyclonal immunoglobulins (I-8758; Sigma) as capture antibody (1:100 dilution) and purified human immunoglobulin (I-4506; Sigma) as the standard. The standard was prepared as a duplicate dilution series on each microtiter plate analyzed.

**Enzyme-linked immunospot (ELISpot) assay.** Lymphocytes were isolated from the blood, tonsils, and nasal tissue with use of Lymphoprep (Nycomed Pharma) and were analyzed by the ELISpot method [2, 8]. The ELISpot plates were coated with 1 μg of HA per well. The ELISpot assay was done aseptically. Wells were blocked with cell-culture medium (RPMI 1640 medium containing 10% FCS and 2 mM glutamine). Lymphocytes from blood and tonsils were added to the plates at concentrations of 5 × 10⁵ lymphocytes/well in multiple wells. The number of lymphocytes isolated from the nasal tissue did not allow such concentrations; instead, all isolated cells were divided into duplicate wells for each influenza virus strain (5 × 10⁴ to 1 × 10⁵ lymphocytes/well). After overnight incubation in a cell-culture incubator (37°C in air with 5% CO₂), the plates were washed, and binding of anti–influenza virus antibodies was detected by peroxidase-conjugated anti-human polyvalent immunoglobulins from goat (A-8400; Sigma). The substrate used for detection of the spots was 4-chloro-1-naphthyl (C-6788; Sigma). The plates were allowed to dry, after which the spots in each well were counted manually in a stereomicroscope (M3C, type S, Wild) at ×16–25 magnification.

**Statistical analysis.** Comparisons were made with Student’s t test (paired and unpaired), using GraphPad Prism v3.0 on an Apple Macintosh computer. Results of a statistical test were considered to be significant when P < .05 and to be borderline when .05 ≤ P ≤ .07.

**Results.** In the present study, the effect of parenterally administered influenza vaccine on systemic and local mucosal immune responses was investigated in 23 volunteers. Although the subjects had recurrent tonsillitis, they were otherwise healthy, with no infection in the 3–4 months before tonsillectomy was performed. They had not experienced influenza virus infection or been vaccinated against influenza during the previous year, and they had no history of allergy (as ascertained by oral communication with the patients). This information was also confirmed by routine diagnostic analysis (data not shown).

**Serum HAI antibody titers.** Serum samples were collected immediately before vaccination and 7 days after vaccination, when tonsillectomy was done. The serum samples were tested by the HAI assay (figure 1), a common method for assessing protective serum antibody responses. An HAI titer of ≥40 is considered to give protection against influenza virus infection [7]. None of the 23 volunteers in this study had HAI titers of ≥40 to the H1N1 virus before vaccination, whereas 30% had protective titers to the H3N2 strain and 43% to the B strain. After vaccination, these proportions increased to 83%, 70%, and 83% for the H1N1, H3N2, and B strains, respectively. The increase in HAI titers was statistically significant for all 3 virus strains (H1N1, P < .0001; H3N2, P < .0001; B, P = .0004).

**Influenza virus–specific antibody concentrations in serum and oral fluid.** The levels of influenza virus–specific antibodies in serum were measured by ELISA (figure 2). This assay tests the same immunologic response as does the HAI assay, but, because there are some fundamental differences between these 2 methods, each measures a different subpopulation of influenza virus–specific serum antibodies. Although ELISA is considerably more sensitive, a protective threshold has not yet been established, as has been done for the HAI assay.

The mean prevaccination serum concentration of antibodies to the H1N1 strain was 26 μg/mL; to the H3N2 strain, 21 μg/mL;
and to the B strain, 13 μg/mL. After vaccination, the serum antibody concentrations increased significantly, to 137 μg/mL (P = .0029), 69 μg/mL (P < .0001), and 227 μg/mL (P = .0006), respectively.

In an earlier study [4], we found that influenza virus–specific antibodies in oral fluid (saliva) consist mainly of secretory IgA1. In the present study, we measured the concentration of total influenza virus–specific antibodies in the oral fluid (figure 3). The mean levels of prevaccination influenza virus–specific antibodies were 26 ng/mL, 29 ng/mL, and 35 ng/mL, respectively, to the H1N1, H3N2, and B vaccine strains. Seven days after vaccination, the concentrations in the oral fluid of antibodies specific to the H1N1 and B strains rose significantly, to 65 ng/mL (P < .0001) and 88 ng/mL (P < .0001), respectively. The response to the H3N2 virus increased 7 days after vaccination to a mean concentration of 99 ng/mL. This change in mean concentration was borderline, not statistically significant (P = .0664), perhaps because of an outlying value.

Levels of influenza virus–specific ASCs. Lymphocytes were isolated from peripheral blood, tonsils, and nasal mucosal biopsy samples. Blood was obtained immediately before and 7 days after vaccination, whereas tonsils and nasal mucosa were collected at 7 days after vaccination only.

The numbers of influenza virus–specific ASCs against the vaccine viruses in peripheral blood were very low (mean, <1 ASC per 10⁶ lymphocytes) before vaccination (figure 4). These numbers increased significantly by 7 days after vaccination, to mean values of 1419 (P = .0011), 370 (P = .0170), and 532 (P = .0215) influenza virus–specific ASCs per 10⁶ lymphocytes for the H1N1, H3N2, and B strains, respectively.

In the tonsils, the mean numbers of influenza virus–specific ASCs were 188 (H1N1), 36 (H3N2), and 25 (B strain) ASCs per 10⁶ lymphocytes at 7 days after vaccination. When these results were compared with data from a recent study [5] of nonvaccinated subjects, we found significantly higher numbers of ASCs specific to the H1N1 (P = .0143) and H3N2 (P = .0032) strains in the vaccinated volunteers. For the B strain, this increase was borderline (P = .0582).

The frequencies of influenza virus–specific ASCs in nasal mucosal tissue were higher than the frequencies in tonsils but

Figure 1. Mean serum hemagglutination inhibition (HAI) titer against 3 vaccine viruses (H1N1, H3N2, and B strains) for each subject (n = 23) immediately before (open symbols) and 7 days after (solid symbols) vaccination. The horizontal bar in each column represents the mean value. The shaded area indicates nonprotective HAI titers (<40). *Significant difference between prevaccination and day-7 samples (P < .05, by paired t test).
lower than frequencies in peripheral blood at 7 days after vaccination. The mean numbers of influenza virus–specific ASCs per 10⁶ lymphocytes were 693 (H1N1), 673 (H3N2), and 229 (B strain). The numbers of influenza virus–specific ASCs in the nasal mucosa did not differ significantly between vaccinated and nonvaccinated subjects (H1N1, \( P = 0.1937 \); H3N2, \( P = 0.3454 \); B strain, \( P = 0.5673 \)) [5].

**Discussion**

Our long-term strategy has been to delineate the systemic and local immune responses to influenza vaccination. Such knowledge is important for future vaccine development strategies. The aim of the present study was to examine the levels of influenza virus–specific ASCs locally in tonsils and nasal mucosa after parenteral vaccination.

The subjects in this study were selected from among young adult patients scheduled to undergo tonsillectomy who were otherwise healthy. The use of young adults ensured that all subjects had been previously primed (infected) with influenza virus and thus had some level of influenza virus–specific memory cells. This study was carried out during a time of no local influenza activity (autumn 2000, Norway). None of the subjects had experienced influenza virus infection or vaccination during the preceding influenza season (1999–2000). This was experimentally supported by the HAI assay results, which showed that none of the subjects had serum HAI titers of \( > 80 \) before vaccination. The serum ELISA titers and the HAI titers showed the same trend in immunologic response. Vaccination induced a significant increase in serum anti–influenza virus antibody production (HAI and ELISA), which is in line with findings published elsewhere [2, 3].

In previous studies [2–4], we found that the influenza virus–specific antibodies in the oral fluid (saliva) consist mainly of secretory IgA1, that the antibody response is first detected as early as 5–7 days after vaccination, and that the elevated antibody response lasts for 3–5 days. We therefore decided in the present study to sample oral fluid at 7 days after vaccination and to test only for total anti–influenza virus antibodies. We observed a significant increase in levels in oral fluid of antibody to the
H1N1 and B strains after vaccination; these results support earlier findings [3, 4]. The increase in the immune response to the H3N2 strain was borderline ($P = 0.0664$). When the statistical analysis was repeated after removal of 1 far-outlying value pair, $P$ dropped to $<0.0001$.

The level of influenza virus–specific ASCs in the blood was very low before vaccination, with a frequency of $\sim 10^{-7}$ lymphocytes, compared with levels in tonsils and nasal mucosa. This level increased strongly, by a magnitude of nearly 4 log$_{10}$, after vaccination. The basal level of influenza virus–specific ASCs in the tonsils was 2–3 log$_{10}$ higher than the level in peripheral blood. Vaccination induced a significant increase in influenza virus–specific tonsillar ASCs against 2 of the viruses (H1N1 and H3N2), whereas the increase of ASCs to the B virus was borderline ($P = 0.0582$). The numbers of influenza virus–specific ASCs in blood and tonsils were similar to those observed in our previous studies [2, 3].

In all sample material tested thus far, we found a significant increase in levels of influenza virus–specific antibodies and ASCs after vaccination. It was surprising to discover that the numbers of influenza virus–specific ASCs in the nasal mucosa did not vary significantly between vaccinated and nonvaccinated subjects. The nasal mucosa is an important tissue for protection against influenza virus infection, and the observed increase in anti–influenza virus antibodies in the oral fluid may originate from a source other than lamina propria in the nasal mucosa. A likely origin is a local secondary lymphoid site (e.g., the tonsils) or discrete lymphoid tissue (e.g., salivary glands).

The most important aspect of this study is the examination of ASCs in the nasal mucosa, the main site of influenza virus infection. Data on the response of the humoral immune system in the upper respiratory tract mucosa of humans to influenza vaccination and infection are scarce. This is mainly because of the methodologic difficulties and ethical aspects associated with obtaining such samples from humans. However, several studies in animals have emerged recently that describe the immune responses in the nasal mucosa and nasal-associated lymphoid tissue. As is true for humans [2, 3, 9], parenteral influenza vaccination produces mainly a systemic response in animals, with a poor local immune response [10, 11]. When mice were immu-

**Figure 3.** Total concentration of influenza virus–specific antibodies to 3 vaccine viruses (H1N1, H3N2, and B strains) for each subject ($n = 23$) in oral fluid immediately before (open symbols) and 7 days after (solid symbols) vaccination. The horizontal bar in each column indicates the mean value. *Significant difference between prevaccination and day-7 samples ($P < 0.05$, by paired $t$ test).
nized by the oral or nasal route, either with live attenuated vaccine or with killed virus combined with an adjuvant, the local immune response induced specific T and B cell responses with an enrichment of these cell types in the local mucosa [12–15]. The kinetics of these immune responses [11, 13] was similar to that seen in our previously published studies in humans [2–4]. In addition, parenteral influenza vaccination appears to favor a Th2 response, whereas infection promotes a Th1 response [14, 15].

An important observation in our study is that the frequency of influenza virus–specific ASCs in the nasal mucosa was 10–100 times higher than the frequency found in peripheral blood and tonsils without any recent influenza virus stimulation and at a time during which no influenza activity was seen in the local community [5]. We have suggested that the basal level of influenza virus–specific ASCs in the upper respiratory tract mucosa may be important (crucial) in maintaining protection against influenza. A decrease in this level below a certain threshold may take several years after an infection to occur and renders the subject susceptible to infection. Nasal immunization can induce a long-lasting response lasting for >8 months in mice [16]. Whether this long-lasting response is due to the presence of long-lived (>1 year) plasma cells or to continuous unspecific restimulation and activation of the plasma/memory cells is still to be determined [11, 16, 17].

The absence of immune response in the nasal mucosa may indicate a lack of appropriate local influenza virus stimulation. An apparent drawback to traditional parenteral (intramuscular or subcutaneous) vaccines is that they induce a weak and short-lived local mucosal immune response. Another concern associated with the current vaccine strategy is that repeated annual vaccination may drive the type of immune response from a local to a systemic response. This may also explain why, in some studies, the effectiveness of the influenza vaccine is poor. It is therefore our opinion that future influenza vaccine development should include vaccines that more closely imitate natural infection and/or stimulate the local mucosa in the upper respiratory tract.

Figure 4. No. of influenza virus–specific antibody-secreting cells (ASCs) to 3 vaccine viruses (H1N1, H3N2, and B) for each subject in peripheral blood, tonsils, and nasal mucosa immediately before vaccination (or in nonvaccinated subjects) (open symbols; day 0) and 7 days after (solid symbols) influenza virus vaccination. (Because we lacked prevaccination samples from tonsils and nasal mucosa, comparable data from nonvaccinated subjects were obtained from a recent study [5].) The horizontal line in each column indicates the mean value. *Significant difference between this sample and the paired basal level sample (P < .05, by paired t test for blood and by unpaired t test for tonsils and nasal mucosa).
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References
