Available online at www.sciencedirect.com
SCIENCE DIRECT®
Vaccine 23 (2005) 2994–3004
Vaccine
www.elsevier.com/locate/vaccine

Type I IFN is a powerful mucosal adjuvant for a selective intranasal vaccination against influenza virus in mice and affects antigen capture at mucosal level

Laura Bracci[a,*], Irene Canini[a], Simona Puzelli[b,1], Paola Sestili[a], Massimo Venditti[a], Massimo Spada[a], Isabella Donatelli[b], Filippo Belardelli[a], Enrico Proietti[a]

[a] *Department of Cell Biology and Neurosciences, Parasitic and Immune-mediated Diseases, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy*
[b] *Department of Infectious, Parasitic and Immune-mediated Diseases, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy*

Received 6 September 2004; received in revised form 10 November 2004; accepted 9 December 2004
Available online 31 December 2004

Abstract

In view of the increasing interest in mucosal vaccination, we investigated whether type I IFN could act as adjuvant of an intranasally administered influenza vaccine. A single intranasal administration of IFNαβ-adjuvanted vaccine in anesthetized C3H/HeN mice was capable of protecting the totality of animals against virus challenge, while vaccine alone was only partially effective. To mimic intranasal vaccine administration in man and to limit vaccine delivery strictly to nasal mucosa, we used a second method of vaccination based on vaccine fractionation in six doses and intranasal instillation in non-anesthetized mice. By using this vaccination schedule, IFNαβ-adjuvanted vaccine also prevented mice from disease development and induced an efficient long lasting immune response. Further experiments showed that IFNαβ increased the percentage of antigen-associated phagocytes in the nasal mucus layer, thus suggesting a new possible mechanism of action for type I IFN as an adjuvant.

Keywords: Influenza; Intranasal; Type I IFN

1. Introduction

Effective needle-free delivery systems for vaccine administration avoid the multiple risks and drawbacks of the parenteral vaccination. The potential benefits of mucosal vaccine delivery, consisting of simplicity of administration, improved access and compliance, avoidance of infection from inappropriate re-use of needles, would represent an important advance for vaccination campaigns. Moreover, the respiratory mucosa interfaces broadly with the environment and is the initial site of many infections. Nasal immunization is commonly used to induce immunity along the respiratory tract and is optimal for protection from respiratory infections. This route of immunization induces secretory IgA and systemic IgG Ab responses and is crucial for achieving local protection at the pathogen entry site and for preventing dissemination of the infection to extra pulmonary sites [1,2]. Nevertheless, under normal circumstances, soluble proteins non associated to replicating microorganisms, delivered through intact mucosal surfaces, do not provoke strong immune reactions, but tend to induce a state of hyporesponsiveness [3–5]. The inflammatory events occurring during natural viral or bacterial infections, which include the production of certain cytokines, can break tolerance and prime a specific immune response.

Type I IFNs are a cytokine family endowed with pleiotropic effects: under physiological conditions, they are

* Corresponding author. Tel.: +39 06 4990 2140; fax: +39 06 4990 3641.
E-mail address: lbracci@iss.it (L. Bracci).
[1] Fax: +39 06 4990 2082.

0264-410X/$ – see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.vaccine.2004.12.006

spontaneously produced at low levels but their expression is highly increased in response to viral and bacterial infections [6]. Originally described as antiviral cytokine, type I IFN possess a variety of immunomodulatory activities. These include activation of B cells and promotion of fast and polyclonal Ab responses [7,8], enhancement of NK and T cell cytolytic activity [9], up-regulation of histocompatibility antigen class I expression [10], induction of proliferation and long-term survival of memory CD8+ T cells [11,12], promotion of Th1-responses in terms of IgG2a Ab production and CD4+ T cells activation [13], rapid induction of in vitro and in vivo differentiation of monocytes into functionally active dendritic cells (DC) [14–16]. In particular, some authors reported that type I IFN secreted by human [17,18] and murine DC [19] in response to pathogen-associated signals can act in an autocrine manner promoting survival of DC precursors [20] and stimulating expression of type I IFN-induced genes for DC activation [21,22]. All these elements contribute to the concept that type I IFN constitutes a link between innate and adaptive immunity and this concept can be exploited for further studies on the modulation of the immune response to pathogens.

In a previous study, we demonstrated that endogenous type I IFN is necessary for adjuvant-induced Th1 responses and that type I IFN itself is an unexpectedly powerful adjuvant when administered with the human influenza vaccine, inducing a Th1 type immune response and protection from virus challenge [23]. In the present study, we investigated type I IFN adjuvant activity during intranasal vaccination with a currently used human influenza vaccine, comparing the classical method of intranasal delivery in anesthetized mice to an alternative method more closely resembling the one used for mucosal delivery in man. We have also evaluated the effect of the intranasal administration of type I IFN on antigen entrapment at mucosal level.

2. Materials and methods

2.1. Mice

IFN-IR knock-out (KO) C3H/HeN mice and their wild-type counterparts were generated at the Institut Curie (Orsay, France), as previously reported [23] and kindly provided by late E. De Maeyer (Institut Curie, Orsay, France). C3H/HeN were purchased from Charles River (Calco, Italy). Mice were housed in the facilities of the Department of Virology at Istituto Superiore di Sanità and were used at the age of 7–8 weeks. Wild-type and IFN-IR KO mice were kept under specific pathogen-free conditions. All work with animals conformed to European Community guidelines.

2.2. Antigen

The monovalent subunit influenza vaccine “Agrippal” IVR-116, in use since 2000/2001 vaccination campaign (Chiron Vaccines, Siena, Italy), was prepared from influenza virus A/NewCaledonia/20/99 (H1N1). The hemagglutinin (HA, mol. wt.: 72,000) concentration in the subunit vaccine, calculated by single radial diffusion, was 260 μg/ml.

2.3. Interferon

High titer IFN-α/β (2×10^7 U/mg of protein) was prepared in the C243-3 cell line following a method adapted from Tovey et al. [24]. IFN was concentrated and partially purified by ammonium sulfate precipitation and dialysis against PBS as previously described [23].

2.4. Intranasal immunizations

In the first set of experiments (*anesthetized mouse model*), mice were anesthetized and instilled into alternate nostrils in dropwise fashion with 50 μl of a solution containing 25 μl of vaccine (200 μg HA/ml) and 25 μl of saline or type I IFN. In another set of experiments (*non-anesthetized mouse model*), nostrils of awake mice were moistened along the day of treatment (at 10 min intervals) with six doses (8 μl each) of vaccine with or without type I IFN for a total amount comparable to that of the previous immunization protocol. Vaccination was performed on days 0 and 14 unless specifically indicated.

2.5. Tracking of intranasally-delivered solutions

C3H/HeN mice were inoculated intranasally (i.n.) following the above mentioned immunization protocols, with a solution containing CFSE (Molecular Probes, Eugene, OR)-labeled murine RBL-5 cells (2×10^7 cell/ml) in association or not with type I IFN. Thirty minutes after vaccine administration was completed, mice were sacrificed, broncho-alveolar lavage (BAL) and nasal lavage (NL) were performed and analyzed by a FACScan (Becton Dickinson).

2.6. Uptake of fluorescently labelled dextran by mucosal phagocytes

C3H/HeN mice were inoculated i.n. with a solution containing 250 μg/ml of dextran-FITC (mol. wt.: 70,000, Molecular Probes, Eugene, OR) and type I IFN (4 kU for anesthetized mice and 40 kU for non-anesthetized) following the above described immunization models. One and six hours after vaccine administration was completed, mice were sacrificed and mucosal (broncho-alveolar and nasal) washings were performed for the analysis of cell-associated dextran-FITC (DXT-FITC) by a FACScan (Becton Dickinson). Percentage of phagocytes was calculated on a gate restricted to FSC^{hi} SSC^{hi} DXT-$FITC^{+}$ cells as described in [25].

2.7. Determination of influenza-specific antibodies

To measure specific Ab levels in serum, broncho-alveolar lavage (BAL) and nasal lavage (NL), standard direct ELISA were performed as previously described [23]. Briefly, 96

well flat-bottom microtiter plates (Immulon 4HBX, Dynatech, Chantilly, VA) were coated with 100 μl of a solution containing 2.5 μgHA/ml influenza vaccine. Samples were serially diluted in PBS 3% BSA. The following dilutions of peroxidase-conjugated secondary Abs were used for anti-HA Ab detection: anti-mouse IgG (H + L chain; Pierce, Rockford, IL), 1/75,000; anti-mouse IgG2a (Cappel, ICN Biomedicals, Ohio), 1/200; anti-mouse IgG1 (Cappal, ICN Biomedicals, Ohio), 1/400; anti-mouse IgM (Pierce, Rockford, IL), 1/500 and anti-mouse IgA (Kirkegaarde and Perry, Guilford, UK), 1/1500. *Ortho*-phenylenediamine (Sigma) was used as enzymatic substrate and the reaction was stopped by addition of 50 μl 4N H_2SO_4 after 10 min incubation at dark. Optical densities (OD) were read in a microplate autoreader at 490 nm wavelength. Results are expressed as reciprocal endpoint titers where a threshold of positivity for OD values was calculated for each Ab isotype as the average + 3 S.D. of all dilutions from three control mouse sera (from saline-treated mice). For a given sample, the endpoint titer was determined as the first dilution below the threshold of positivity.

2.8. Broncho-alveolar lavage (BAL) and nasal lavage (NL)

For broncho-alveolar lavage (BAL), mice were sacrificed and the trachea was exposed and incannulated by using a 19-gauge blunt-ended needle. Lavage was performed by introducing 1 ml of sterile 5 mM EDTA in phosphate-buffered saline into the lungs, followed by aspiration, re-injection and re-aspiration two additional times. BAL is suitable for analyzing cells and Abs contained in the secretions of the lower respiratory tract. For nasal lavage (NL), mice were sacrificed, the skin was removed from the head and the lower jaw was cut away. Lavage was performed by introducing a 19-gauge blunt-ended needle into the nasal cavity and washing the mucosa with 1 ml of sterile 5 mM EDTA in phosphate-buffered saline. NL is suitable for analyzing cells and Abs contained in the secretions of the upper respiratory tract.

2.9. Virus and virus challenge

The original H1N1 influenza virus A/NewCaledonia/20/99 (supplied by NIBSC, Hertfordshire, UK) was adapted to mouse after six blind intranasal passages as previously described [23]. The final virus titer was 1024 HAU/ml or 1.17×10^8 PFU/ml. One LD_{50} corresponded to a dilution of 1:300 of the stock virus suspension. For virus challenge, anesthetized mice were instilled i.n. with 50 μl of a virus suspension containing $10LD_{50}$.

2.10. Statistical analysis

Data were analyzed by the Student's *t*-test and by Wilcoxon rank-sum test.

3. Results

3.1. Type I IFN dose-dependent adjuvant effect in intranasal influenza vaccine delivery

Two i.n. immunizations of anesthetized C3H/HeN mice with influenza vaccine in association with high doses (4 and 40 kU) of type I IFN resulted in a strong induction of HA-specific Abs, while only a modest humoral response was observed with vaccine alone (Fig. 1). Four kilounits of type I IFN proved to be the most effective dose to achieve the optimal IgGtot, IgG1, IgM and IgA titers, while IgG2a titer was further increased at higher type I IFN concentrations. Of note, mice vaccinated twice with 5 μg HA and 4 kU of type I IFN showed complete protection from virus challenge 45 days after the last vaccine administration, while the use of vaccine alone or mixed with low dose type I IFN did not prevent mice from infection and death (data not shown). Thus, we chose 4 kU of type I IFN as the selected dose for the subsequent vaccination experiments.

3.2. A single intranasal type I IFN-adjuvanted influenza vaccine administration results in a sustained Ab production and protection from influenza virus infection

A single i.n. immunization of C3H/HeN anesthetized mice with 5 μg HA mixed with type I IFN was capable of inducing a significant increase (with respect to vaccine alone) in total IgG, IgG2a and serum IgA levels already 13 days after vaccine administration (Fig. 2A). In particular, IgG2a reached their maximum of induction 13 days after vaccination and remained at plateau levels until day 30. IgA levels decreased, after a peak level at day 13, to levels comparable to those obtained with vaccine alone. The administration of type I IFN without vaccine did not alter the specific Ab profile with respect to saline-treated animals. A subsequent challenge of all groups of mice 60 days after vaccination with $10LD_{50}$ of influenza virus showed that only mice instilled with type I IFN-adjuvanted vaccine were totally protected from disease development (as indicated by the lack of any virus-induced decrease in mouse weight) and death, while 100% of mice treated with vaccine alone developed a disease and 60% of them died (Fig. 2B).

3.3. Specificity of type I IFN adjuvant effect

The specificity of the type I IFN-induced adjuvant effect was evaluated by using an inbred strain of type I IFN receptor knock-out C3H/HeN (IFN-IR KO) mice and their wild type (WT) counterparts as control. For this purpose, anesthetized IFN-IR KO and WT mice were immunized on days 0 and 14 with 5 μg HA or 5 μg HA + 4 kU type I IFN chosen as the optimal dose for the induction of HA-specific Abs (Fig. 1). Specific HA responses were analyzed at different times. As expected, no increase in Ab production was observed in IFN-IR KO animals immunized with type I IFN as an adjuvant

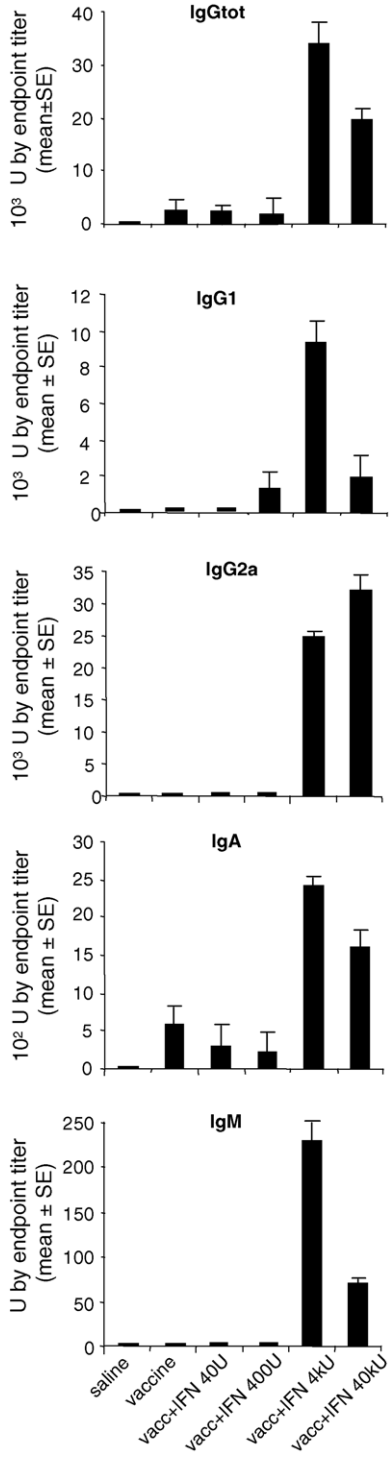

Fig. 1. Dose-dependent adjuvant effect of type I IFN in i.n. immunization of anesthetized mice. Anesthetized 7–8-week-old C3H/HeN mice were instilled i.n. on days 0 and 14 with 50 μl of a saline solution containing 5 μg HA, 5 μg HA + different doses of type I IFN (40 U, 400 U 4 kU, 40 kU) or nothing as control; 14 days after the second immunization, sera were collected by retro-orbital bleeding and subsequently analyzed for the HA-specific Ig content. Results are expressed as the mean ± standard error (S.E.) of five samples per group tested in duplicate.

either after one (Fig. 3A) or two immunizations (Fig. 3B). Interestingly, comparative analysis of Ab levels after a single administration of vaccine alone in WT and IFN-IR KO mice (Fig. 3A) showed that IFN-IR KO mice did not develop any measurable Ab response while WT mice did, indicating that the absence of type I IFN signals rendered mice much less responsive to a weak antigenic challenge. Moreover, the use of type I IFN as adjuvant did not result in any protection from disease development in IFN-IR KO mice, while WT animals receiving two vaccine administrations with antigen plus type I IFN did not show any significant loss of weight and survived to virus challenge. No differences were found in susceptibility to virus infection between KO and WT mice treated with saline alone (Fig. 3C).

3.4. Adjuvant effect of type I IFN in mice subjected to selective intranasal delivery by fractionated vaccine doses

In order to limit antigen delivery only to the nasal mucosa, thus preventing any possible pulmonary involvement in vaccine absorption, we set up a different immunization strategy, mimicking aerosol administration, in which non-anesthetized mice received an overall amount of vaccine, alone or mixed with type I IFN, comparable to that used in previous vaccination experiments, but fractionated in six subsequent doses (8 μl each) delivered at 10 min intervals. Fig. 4A shows the immune response obtained after two vaccinations with influenza vaccine alone or mixed with different amounts of type I IFN, following this new immunization strategy. Similarly to the previous experiments, type I IFN induced a significant dose-dependent increase in Ab response to vaccine antigens, with particular effect on IgG2a and IgA Ab subclasses. No plateau effect was observed in this experimental setting indicating that the highest available type I IFN dose (40 kU) was the most effective in increasing all the Ig isotypes. A subsequent challenge of the same mouse groups with 10LD_{50} of influenza virus, 60 days after the last vaccine administration, showed that 100% of mice receiving vaccine mixed with both 4 and 40 kU of type I IFN survived, even though the group receiving the intermediate dose (4 kU) had a little weight loss, indicating a lower degree of protection against disease development. Among the other groups, mice receiving vaccine alone or mixed with low dose type I IFN (400 U) had significantly decreased survival rates. All mice treated with saline died after influenza virus challenge (Fig. 4B).

3.5. Long term persistence of influenza-specific antibodies: comparison of intranasal immunization in anesthetized versus non-anesthetized mice

To assess whether type I IFN had an effect on the persistence of immunological memory after intra-nasal administration with influenza vaccine, HA-specific IgG were measured up to 180 days after the second vaccine intranasal administration. The data presented in Fig. 5 show that nasal im-

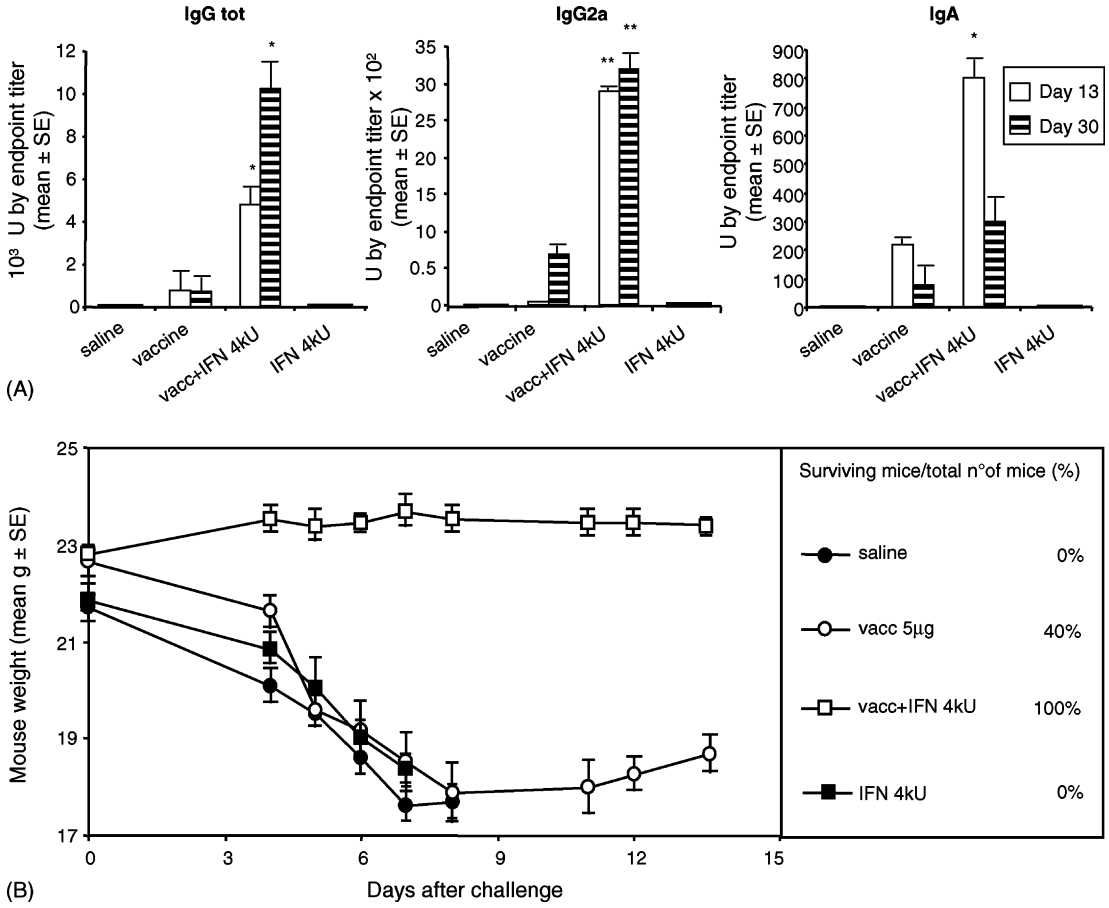

Fig. 2. Adjuvant effect of type I IFN after one immunization in anesthetized mice. Anesthetized 7–8-week-old C3H/HeN mice were instilled i.n. on day 0 with 5 μg HA, 5 μg HA + IFN 4 kU, IFN alone or saline as controls; (A) 13 and 30 days after vaccination, sera were collected by retro-orbital bleeding and for the HA-specific Ig content. Results are expressed as the mean ± standard error (S.E.) of five samples per group tested in duplicate. (B) Thirty days after vaccination C3H/HeN mice were infected with $10LD_{50}$ of influenza virus and weight loss and survival rate were recorded. Data represent the mean weight course ± standard error (S.E.) of five mice per group; $^{*}p < 0.05$; $^{**}p < 0.001$ (vs. vaccine alone).

munization of both anesthetized and non-anesthetized mice with vaccine alone was effective in inducing anti influenza IgG Abs, but IgG titer declined constantly until day 180. On the contrary, type I IFN-adjuvanted vaccine administered in anesthetized mice was extremely effective in keeping Ig titers high up to 6 months, while the selective delivery of vaccine mixed with type I IFN to nasal mucosa in non-anesthetized mice proved less effective than in anesthetized mice, but still superior to vaccine alone. The analysis of IgG2a Ab subclass did not show relevant differences from the previous data referred to total IgG.

3.6. Secretory IgA induction by type I IFN

To evaluate secretory IgA (s-IgA) production in the respiratory tract after i.n. vaccination comparing vaccine delivery in anesthetized and non-anesthetized mice, nasal (NL) and broncho-alveolar (BAL) lavages were performed 14 days after the second immunization. Mice treated with vaccine mixed with type I IFN showed a strong s-IgA induction in the upper respiratory tract (as measured in NL) in both the immunization models, while mice treated with vaccine alone had barely or no detectable s-IgA levels (Fig. 6A). After instillation in anesthetized mice, type I IFN-adjuvanted vaccine did not induce a considerable increase in s-IgA concentration in the lower respiratory tract, as measured in BAL (Fig. 6B, left panel) even at higher doses (40 kU, data not shown). On the contrary, when administered selectively to nasal mucosa in non-anesthetized mice, the vaccine effect on s-IgA induction was greatly enhanced by type I IFN (Fig. 6B, right panel). In particular, despite inducing comparable systemic responses (Figs. 1 and 4), higher s-IgA titers were obtained in non-anesthetized mice, thus indicating the more selective character of this immunization schedule and its local effectiveness compared to the administration in anesthetized animals.

3.7. Distribution of intranasally delivered solutions in anesthetized and non-anesthetized mice and type I IFN effect on antigen capture

To investigate the possible mechanisms associated to the different vaccine administration models, we tracked the dis-

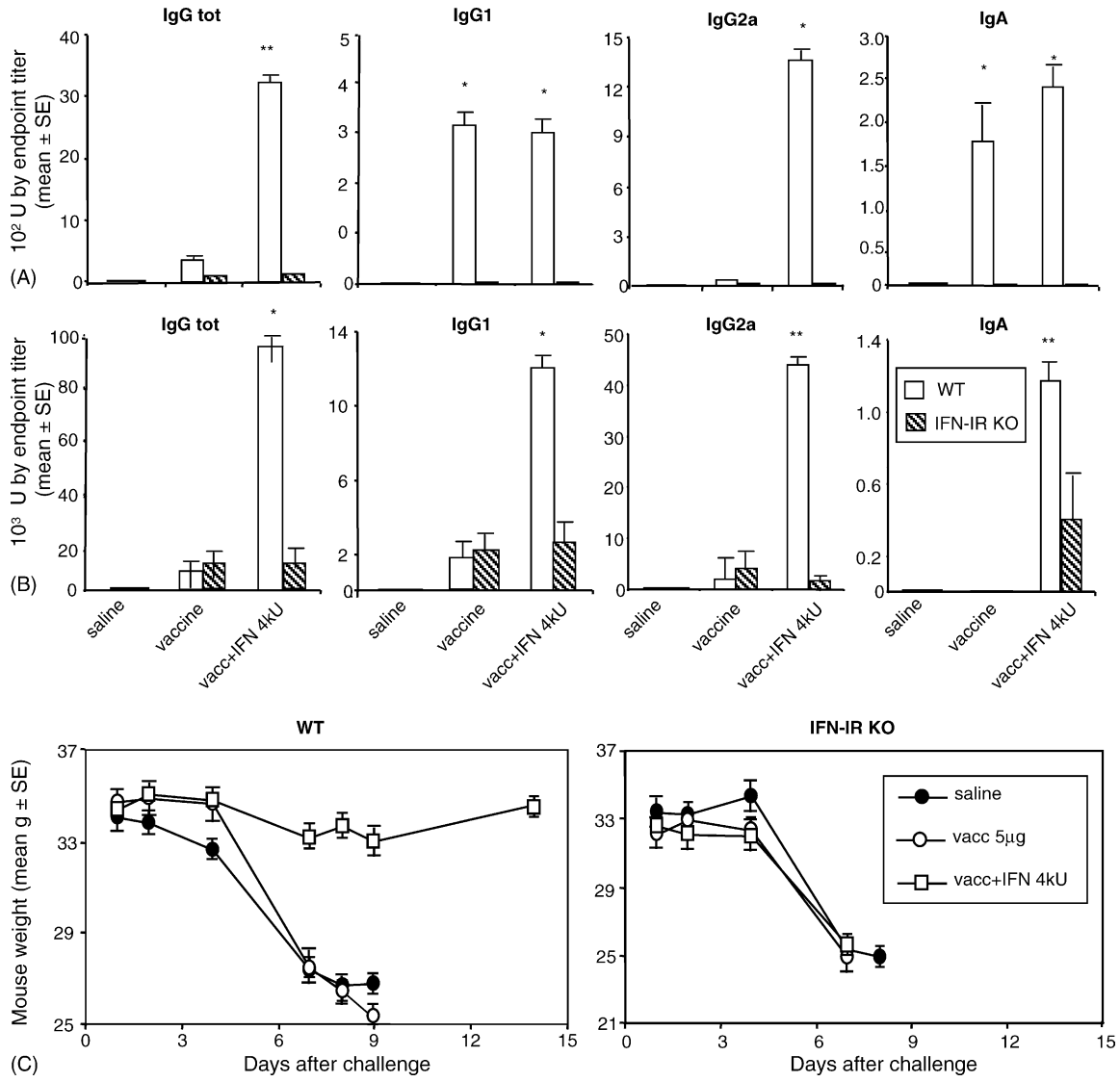

Fig. 3. Specificity of IFN-induced adjuvant effect. Anesthetized 7–8-week-old IFN-IR KO C3H/HeN and their wild type controls were vaccinated i.n. on days 0 and 14 with 5 μg HA, 5 μg HA + IFN 4 kU or saline as control; (A) 13 days after the first immunization and (B) 40 days after the second immunization, sera were collected by retro-orbital bleeding and analyzed for their HA-specific Ig content. Results are expressed as the mean ± standard error (S.E.) of five samples per group tested in duplicate. (C) Forty-five days after the second immunization, wild type and IFN-IR KO C3H/HeN mice were infected with $10LD_{50}$ of influenza virus and weight loss and mean survival rate were recorded. Data represent the mean weight course ± standard error (S.E.) of five mice per group; $^{*}p < 0.05$; $^{**}p < 0.001$ (vs. vaccine alone).

tribution of particulate suspensions following i.n. inoculation in anesthetized versus non-anesthetized mice. In a first experiment, 50 μl of a solution containing 2×10^7 CFSE-labeled cells/ml was inoculated i.n. into anesthetized mice; 30 min after the administration, nasal and broncho-alveolar mucosa were repeatedly washed with fixed volumes of PBS and analyzed for fluorescence emission. Following this protocol, the cell particulate distributed evenly between the upper and lower respiratory tracts as revealed by 45 ± 3% CFSE-labeled cells rescued from BAL and 37 ± 5% CFSE-labeled cells rescued from NL (Fig. 7A). In contrast, using the second protocol of immunization in non-anesthetized mice, the particulate suspension localized only to the nasal mucosa as assessed by the detection of 36 ± 2% CFSE-labeled cells in this compartment versus no fluorescent cell particulate rescued from the BAL (Fig. 7B). This method proved useful to clarify the differences in the distribution and retention of particulate suspensions instilled i.n. in anesthetized and non-anesthetized animals.

To further investigate whether type I IFN had some role in antigen uptake by alveolar phagocytes and immobilization in the mucus layer during i.n. delivery, we instilled anesthetized and non-anesthetized mice, respectively, with one-dose or a fractionated dose of a solution containing dextran-FITC (DXT-FITC) instead of HA, mixed with type I IFN or saline as control. One and six hours after vaccine administration, BAL and NL were performed and DXT-FITC uptake by alveolar phagocytes was evaluated by a FACScan. As

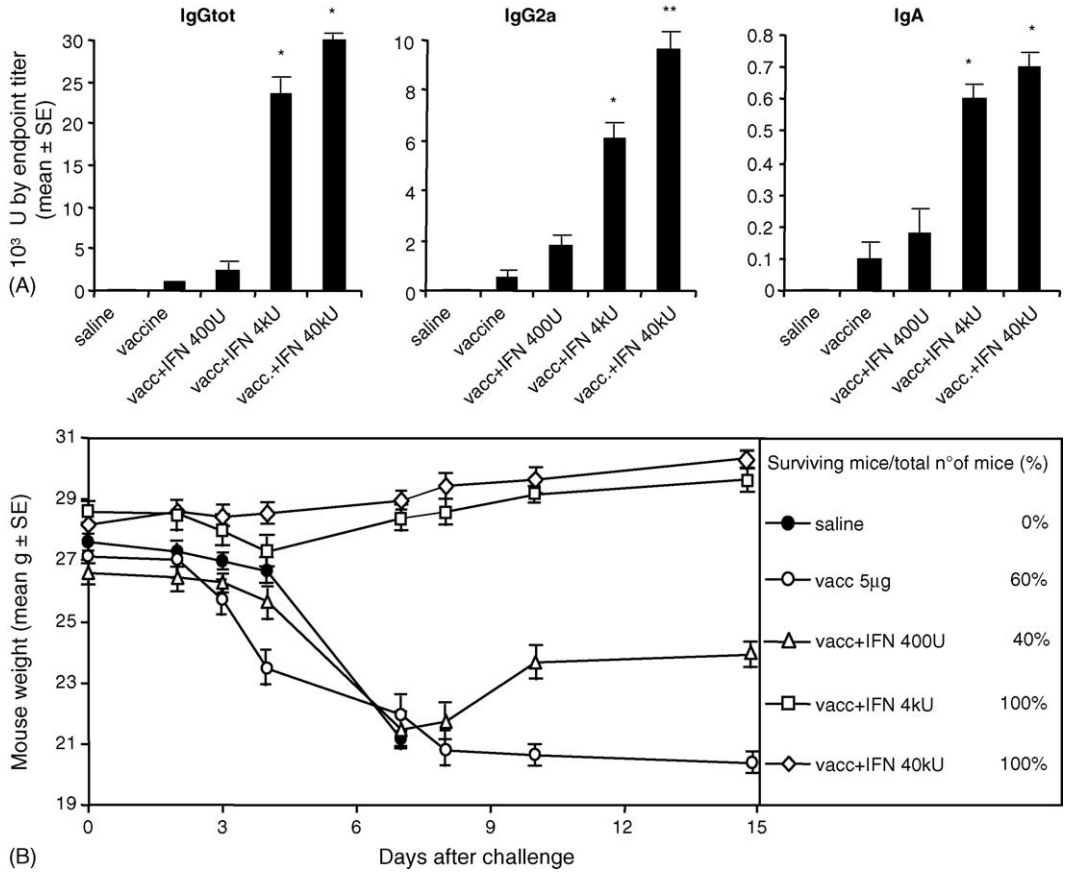

Fig. 4. Adjuvant effect of type I IFN in i.n. immunization of non-anesthetized mice. Non-anesthetized 7–8-week-old C3H/HeN mice were instilled i.n. on days 0 and 14 with 5 μg HA, 5 μg HA + different doses of type I IFN (400 U, 4 kU, 40 kU) or saline as control. (A) Forty-five days after the second immunization sera were collected by retro-orbital bleeding and analyzed for their HA-specific Ig content. Results are expressed as the mean ± standard error (S.E.) of five samples per group tested in duplicate. (B) Forty-five days after the last vaccination C3H/HeN mice were infected with $10LD_{50}$ of influenza virus and weight loss and survival rate were recorded. Data represent the mean weight course ± standard error (S.E.) of five mice per group; $^{*}p < 0.05$; $^{**}p < 0.001$ (vs. vaccine alone).

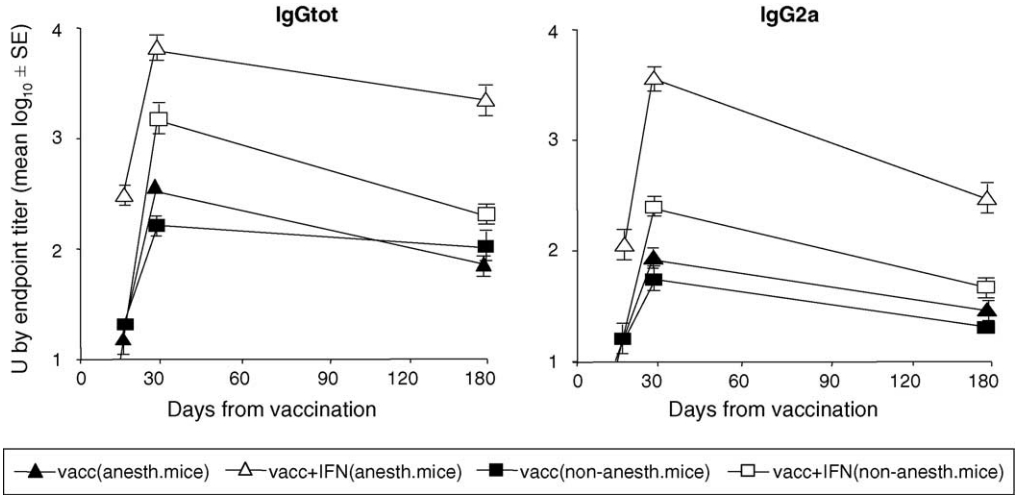

Fig. 5. Long term persistence of HA-specific Ig in anesthetized and non-anesthetized mice. Anesthetized and non-anesthetized 7–8-week-old C3H/HeN mice were instilled i.n. on days 0 and 14 with 5 μg HA or 5 μg HA + IFN (4 kU for anesthetized mice and 40 kU for non-anesthetized mice). 13, 90 and 180 days after vaccination sera were collected by retro-orbital bleeding and analyzed for their HA-specific Ig content. Results are expressed as the mean ± standard error (S.E.) of five samples per group tested in duplicate.

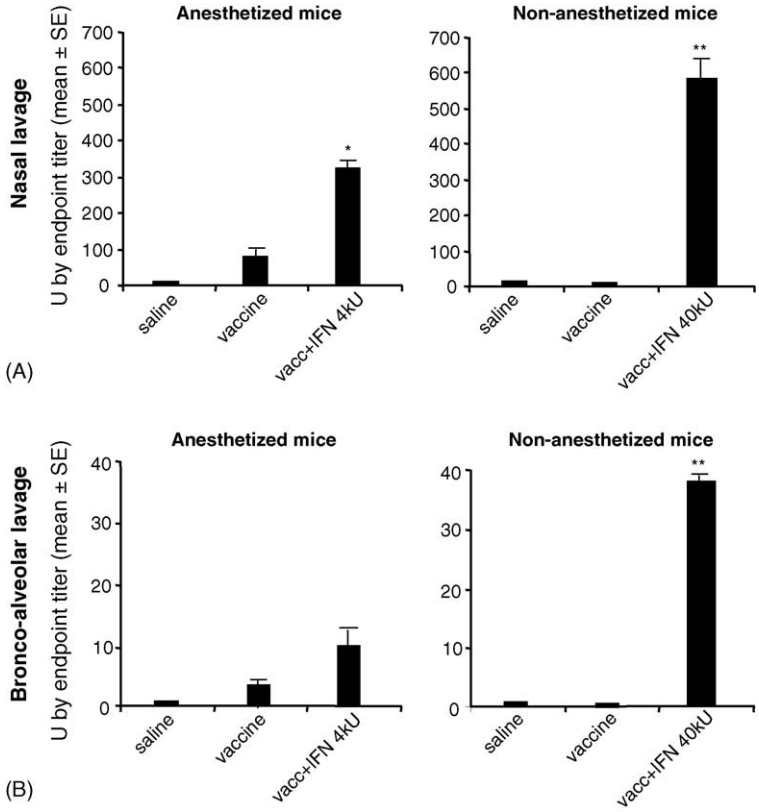

Fig. 6. Secretory IgA levels in upper and lower respiratory tract. Anesthetized and non-anesthetized 7–8-week-old C3H/HeN mice were instilled i.n. on days 0 and 14 with 5 μg HA or 5 μg HA + IFN (as indicated) or saline as control; (A) 14 days after the second immunization broncho-alveolar and (B) nasal lavages were performed and analyzed for their HA-specific IgA content. Results are expressed as the mean ± standard error (S.E.) of five samples per group tested in duplicate; $^{*}p < 0.05$; $^{**}p < 0.001$ (vs. vaccine alone).

shown in Fig. 7, DXT-FITC^{+} cells were found in both BAL and NL of anesthetized mice (Fig. 7C and E), in contrast, only NL, contained cell-associated DXT-FITC cells when non-anesthetized mice were administered with fractionated doses of the same solutions (Fig. 7D and F). Of particular interest, the percentage of DXT-FITC uptaking phagocytes in both BAL and NL was significantly increased when animals were instilled with an IFN-containing solution with respect to DXT-FITC alone. Type I IFN resulted particularly effective in increasing DXT-FITC capture by alveolar phagocytes in BAL and NL 1 h after mouse treatment (Fig. 7C and D). Six hours after nasal instillation, no differences were found in BAL from anesthetized mice treated with IFN-containing solution with respect to dextran alone (Fig. 7E), while in NL from non-anesthetized mice the differences between the two treatment groups remained extremely high (Fig. 7F).

4. Discussion

In this study, we investigated the adjuvant activity of type I IFN when administered i.n. together with an influenza vaccine in mice and evaluated the optimal modalities of mucosal delivery and the possible mechanisms. To this end, we used the commercially available sub-unit vaccine against A/New Caledonia influenza strain adopted in the recent 2001/2002 vaccination campaign. Given i.n. in anesthetized mice, type I IFN-mixed influenza vaccine resulted in a very effective Ab response of all Ig subtypes. Of interest, while excessive IFN doses could revert the adjuvant effect for some Ig subclasses, as demonstrated by a dose–response curve, IgG2a Abs, which are indicative for Th1 immune response in mice, were further increased by enhancing IFN doses (Fig. 1). Although two IFN-mixed vaccine administrations were optimal for inducing the maximal Ab response and a complete protection from influenza virus infection (data not shown), a single immunization with IFN-mixed vaccine was already capable of inducing considerable amounts of immunoglobulins and, particularly, IgG2a and IgA Abs. These Ig levels fully correlated with a complete in vivo protective effect from both disease development and death after a challenge with 10LD_{50} of mouse-adapted A/New Caledonia influenza virus (Fig. 2). Vaccine alone was also capable of inducing some Ab response, which resulted in a partial recovery of 40% infected mice, but it could not prevent mice from developing infection, as demonstrated by mouse weight loss (Fig. 2). This finding is different from our previous observations on parenteral influenza vaccine administration, where a single i.n. vaccine injection was completely ineffective [23]; however, it is in agreement with the general knowledge on the effi-

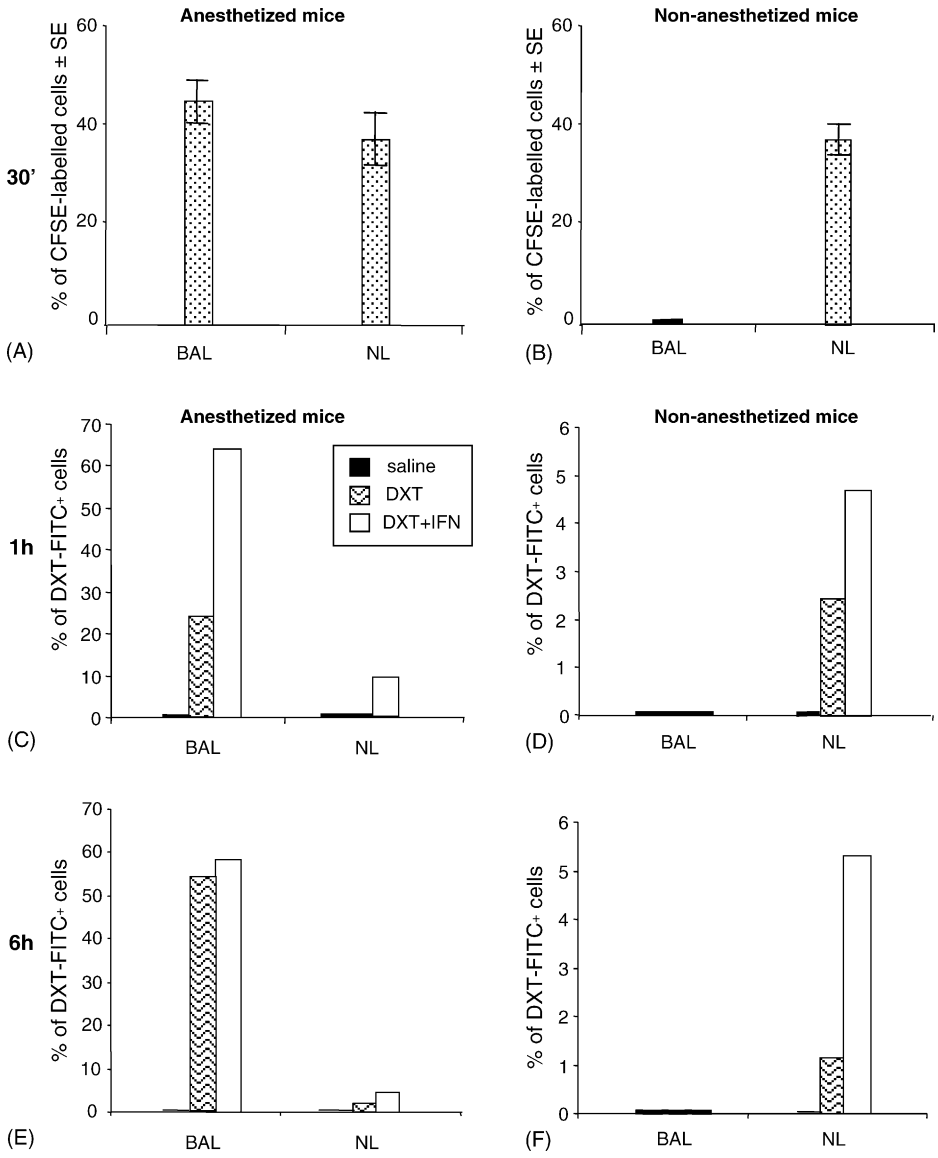

Fig. 7. Differences in antigen delivery and in IFN-induced antigen uptake between the two vaccination models. Upper panel, 7–8-week-old anesthetized and non-anesthetized C3H/HeN mice were instilled i.n. with 50 μl (overall amount) of a solution containing CFSE-labeled cells; 30 min after vaccine administration was completed, mice were sacrificed and the nasal and pulmonary mucosa was repeatedly washed and subsequently processed for flow cytometric analysis of fluorescent cells. Data represent the percentage of CFSE-labeled cells in BAL and NL of (A) anesthetized and (B) non-anesthetized mice. Data are expressed as the mean ± standard error (S.E.) of three similar experiments. Lower panel, 7–8-week-old anesthetized and non-anesthetized C3H/HeN mice were instilled i.n. with 50 μl of a solution containing dextran-FITC (DXT-FITC) ± IFN-I; 1 and 6 h after vaccine administration was completed, mice were sacrificed and the nasal and pulmonary mucosa was repeatedly washed and subsequently processed for the analysis of DXT-FITC$^+$ cells on a FACScan. Data represent the percentage of phagocytes calculated on a gate restricted to FSC^{hi} SSC^{hi} DXT-FITC$^+$ cells, in BAL and NL of anesthetized (C and E) and non-anesthetized (D and F) mice. One representative experiment out of three is shown.

cacy of the intranasal route of administration for influenza vaccine [26–28] and might be explained by the particular architecture of the nasal lymphoid system [29]. As our IFN preparation still contained impurities, we assessed the specificity of the IFN adjuvant effect by using type I IFN receptor knock out (IFN-IR KO) mice. In this model, the absence of the type I IFN-dependent machinery resulted in a minor extent of Ab production after a weak antigenic challenge (after one vaccine administration, Fig. 3A). Of interest, the finding that HA-specific IgG2a isotype was increased in wild type (WT), but not in IFN-IR KO mice, only by IFN-mixed vaccine, confirmed our previous observation on the importance of type I IFN system in achieving a preferential Th1 type of immune response [23]. Finally, type I IFN had no adjuvant activity in IFN-IR KO mice, while it maintained its adjuvant properties in inducing HA-specific antibodies in WT animals after both one or two i.n. immunizations (Fig. 3 A and B). In in vivo experiments, IFN-mixed influenza vaccine failed to protect IFN-IR KO mice from virus challenge, thus confirming the specificity of the adjuvant effect of our

IFN preparation. We have further confirmed these results by using a purified recombinant mouse IFNα preparation (data not shown). Of note, the lack of IFN-α/β signalling in IFN-IR KO mice did not affect influenza virus replication with respect to WT animals, in agreement with previous results [30,31].

A previous work demonstrated that cell particles presented as drops of a liquid suspension onto the nares of anaesthetized mice fill the lower airways 10^3 times more, compared to non-anesthetized animals, and that intrapulmonary antigen presentation, as a part of an i.n. immunization strategy, is of importance for systemic but not for mucosal Ab responses [32]. Therefore, we studied the adjuvant effect of type I IFN in an alternative immunization model in which fractionated vaccine doses were administered to non-anesthetized mice thus mimicking a vaccine delivery system suitable for i.n. vaccine administration in man. Using this immunization protocol, two administrations of type I IFN-mixed vaccine determined a significant raise in HA-specific Ab titers with respect to vaccine alone, and no "plateau" effect was observed in a dose–response experiment, thus indicating that higher type I IFN doses could induce even more significant effects (Fig. 4A). Antibody titers correlated with in vivo protective activity in influenza virus-infected animals; as shown in Fig. 4B, all the IFN doses used determined an advantage in terms of survival rate with respect to vaccine alone, and the highest IFN dose induced a complete resistance to virus challenge with no signs of symptomatic lung infection, as demonstrated by weight gain. Unlike the vaccine delivery in anesthetized mice, a single i.n. immunization of fully awake animals was not sufficient to induce a complete in vivo protection against virus challenge (data not shown). Moreover, the analysis of HA-specific Abs 6 months after vaccination showed that intranasal vaccine administration in anesthetized mice induced a far longer Ab persistence with respect to non-anesthetized animals (Fig. 5). These data indicate that lung involvement during mucosal vaccine administration results in a more sustained and prompt systemic immune response. This observation is confirmed by others who demonstrated that, during mucosal vaccination, lung involvement increases the systemic IgG response [32]. On the other hand, mucosal immunity against influenza is considered more efficient than systemic immunity due to specific secretory IgA (s-IgA) antibodies in the respiratory tract [33]. In our experimental system, administration of vaccine alone in anesthetized mice induced measurable levels of s-IgA, while no detectable s-IgA levels were found in fully awake animals, thus confirming the strictly local s-IgA induction in selective mucosal vaccination of the upper airways of non-anesthetized animals. Type I IFN-mixed influenza vaccine proved extremely active in increasing s-IgA levels in both upper (NL) and lower airways (BAL) of non-anesthetized mice, while s-IgA levels in anesthetized animals were only slightly increased with respect to vaccine alone (Fig. 6). These findings are in agreement with the observation that mucosal IgA in the upper airways are actively secreted across the nasal mucosa, which is rich of lymphoid stations where locally delivered type I IFN can act as a powerful mucosal adjuvant.

The local nature of vaccine activity in non-anesthetized mice may depend on the fact that in fully awake mice, when reflexes are active, fluid applied to the nares is not easily inhaled, whereas volumes of 20 μl or more are inhaled rapidly during anesthesia as demonstrated by Yetter et al. [34]. This observation was confirmed also in our model where tracking of cell particulate after nasal delivery showed no cell particulate at lung level in mice instilled without anesthesia (Fig. 7A and B). Interestingly, using dextran FITC (DXT-FITC), a compound with molecular weight and uptake pathway similar to the HA molecules present in influenza vaccine [35,36], we found that the number of DXT-FITC uptaking cells was significantly increased when type I IFN was added to the solution (Fig. 7C–F). These experiments suggest the existence of potentially relevant new mechanisms of action for type I IFN which may be responsible for its adjuvant effect at the mucosal level. In addition, no DXT-FITC-associated cells were detectable at lung level in non-anesthetized mice, thus confirming our previous observation on the selective delivery of vaccine to the nasal mucosa with this immunization model. This aspect is not of secondary relevance if one considers that total respiratory tract immunization might actually do more harm, when involves lung mucosa instead of being confined to the upper airways. In fact, since some vaccines lead to pulmonary inflammation [37–39], nasal vaccines should be presented in a way to avoid their being deeply inhaled. In humans, this may be achieved by giving them as nasal drops [40,41]. As demonstrated in the present study, type I IFN administered by this inhalation procedure proved to be a powerful vaccine adjuvant suitable to induce high titers of specific antibodies at both systemic and mucosal level. Taking into account that type I IFNs are cytokines with a long record of clinical use [42], the finding that they can act as powerful adjuvants in intranasal vaccination against infectious diseases can disclose practical implications for new strategies in vaccine development.

Acknowledgements

This work was supported by grant Bio QLK2-CT-2002-0846 from the European Community. We thank Dr. Maurizio Tomasi and Dr. Giovanna Schiavoni for helpful discussion and suggestions. We are grateful to Cinzia Gasparrini and Angela Fresolone for excellent secretarial assistance.

References

[1] Cartner SC, Lindsey JR, Gibbs-Erwin J, Cassell GH, Simecka JW. Roles of innate and adaptive immunity in respiratory mycoplasmosis. Infect Immun 1998;66(8):3485–91.

[2] Reuman PD, Keely SP, Schiff GM. Rapid recovery in mice after combined nasal/oral immunization with killed respiratory syncytial virus. J Med Virol 1990;32(1):67–72.

[3] Akbari O, DeKruyff RH, Umetsu DT. Pulmonary dendritic cells producing IL-10 mediate tolerance induced by respiratory exposure to antigen. Nat Immunol 2001;2(8):725–31.
[4] Jones HP, Hodge LM, Fujihashi K, Kiyono H, McGhee JR, Simecka JW. The pulmonary environment promotes Th2 cell responses after nasal-pulmonary immunization with antigen alone, but Th1 responses are induced during instances of intense immune stimulation. J Immunol 2001;167(8):4518–26.
[5] Bilsborough J, George TC, Norment A, Viney JL. Mucosal CD8alpha+ DC, with a plasmacytoid phenotype, induce differentiation and support function of T cells with regulatory properties. Immunology 2003;108(4):481–92.
[6] Belardelli F. Role of interferons and other cytokines in the regulation of the immune response. Apmis 1995;103(3):161–79.
[7] Le Bon A, Schiavoni G, D'Agostino G, Gresser I, Belardelli F, Tough DF. Type I interferons potently enhance humoral immunity and can promote isotype switching by stimulating dendritic cells in vivo. Immunity 2001;14(4):461–70.
[8] Braun D, Caramalho I, Demengeot J. IFN-alpha/beta enhances BCR-dependent B cell responses. Int Immunol 2002;14(4):411–9.
[9] Hunter CA, Gabriel KE, Radzanowski T, Neyer LE, Remington JS. Type I interferons enhance production of IFN-gamma by NK cells. Immunol Lett 1997;59(1):1–5.
[10] Nistico P, Tecce R, Giacomini P, et al. Effect of recombinant human leukocyte, fibroblast, and immune interferons on expression of class I and II major histocompatibility complex and invariant chain in early passage human melanoma cells. Cancer Res 1990;50(23):7422–9.
[11] Tough DF, Borrow P, Sprent J. Induction of bystander T cell proliferation by viruses and type I interferon in vivo. Science 1996;272(5270):1947–50.
[12] Marrack P, Kappler J, Mitchell T. Type I interferons keep activated T cells alive. J Exp Med 1999;189(3):521–30.
[13] Finkelman FD, Svetic A, Gresser I, et al. Regulation by interferon alpha of immunoglobulin isotype selection and lymphokine production in mice. J Exp Med 1991;174(5):1179–88.
[14] Luft T, Pang KC, Thomas E, et al. Type I IFNs enhance the terminal differentiation of dendritic cells. J Immunol 1998;161(4):1947–53.
[15] Santini SM, Lapenta C, Logozzi M, et al. Type I interferon as a powerful adjuvant for monocyte-derived dendritic cell development and activity in vitro and in Hu-PBL-SCID mice. J Exp Med 2000;191(10):1777–88.
[16] Montoya M, Schiavoni G, Mattei F, et al. Type I interferons produced by dendritic cells promote their phenotypic and functional activation. Blood 2002;99(9):3263–71.
[17] Siegal FP, Kadowaki N, Shodell M, et al. The nature of the principal type 1 interferon-producing cells in human blood. Science 1999;284(5421):1835–7.
[18] Cella M, Jarrossay D, Facchetti F, et al. Plasmacytoid monocytes migrate to inflamed lymph nodes and produce large amounts of type I interferon. Nat Med 1999;5(8):919–23.
[19] Hochrein H, Shortman K, Vremec D, Scott B, Hertzog P, O'Keeffe M. Differential production of IL-12, IFN-alpha, and IFN-gamma by mouse dendritic cell subsets. J Immunol 2001;166(9):5448–55.
[20] Kadowaki N, Antonenko S, Lau JY, Liu YJ. Natural interferon alpha/beta-producing cells link innate and adaptive immunity. J Exp Med 2000;192(2):219–26.
[21] Cella M, Salio M, Sakakibara Y, Langen H, Julkunen I, Lanzavecchia A. Maturation, activation, and protection of dendritic cells induced by double-stranded RNA. J Exp Med 1999;189(5):821–9.
[22] Hashimoto SI, Suzuki T, Nagai S, Yamashita T, Toyoda N, Matsushima K. Identification of genes specifically expressed in human activated and mature dendritic cells through serial analysis of gene expression. Blood 2000;96(6):2206–14.
[23] Proietti E, Bracci L, Puzelli S, et al. Type I IFN as a natural adjuvant for a protective immune response: lessons from the influenza vaccine model. J Immunol 2002;169(1):375–83.
[24] Tovey MG, Begon-Lours J, Gresser I. A method for the large scale production of potent interferon preparations. Proc Soc Exp Biol Med 1974;146(3):809–15.
[25] Gonzalez-Juarrero M, Shim TS, Kipnis A, Junqueira-Kipnis AP, Orme IM. Dynamics of macrophage cell populations during murine pulmonary tuberculosis. J Immunol 2003;171(6):3128–35.
[26] Brokstad KA, Eriksson JC, Cox RJ, et al. Parenteral vaccination against influenza does not induce a local antigen-specific immune response in the nasal mucosa. J Infect Dis 2002;185(7):878–84.
[27] Kuper CF, Koornstra PJ, Hameleers DM, et al. The role of nasopharyngeal lymphoid tissue. Immunol Today 1992;13(6):219–24.
[28] Sminia T, van der Brugge-Gamelkoorn GJ, Jeurissen SH. Structure and function of bronchus-associated lymphoid tissue (BALT). Crit Rev Immunol 1989;9(2):119–50.
[29] Asanuma H, Thompson AH, Iwasaki T, et al. Isolation and characterization of mouse nasal-associated lymphoid tissue. J Immunol Methods 1997;202(2):123–31.
[30] Price GE, Gaszewska-Mastarlarz A, Moskophidis D. The role of alpha/beta and gamma interferons in development of immunity to influenza A virus in mice. J Virol 2000;74(9):3996–4003.
[31] Gresser I, Tovey MG, Maury C, Bandu MT. Role of interferon in the pathogenesis of virus diseases in mice as demonstrated by the use of anti-interferon serum. II. Studies with herpes simplex, Moloney sarcoma, vesicular stomatitis, Newcastle disease, and influenza viruses. J Exp Med 1976;144(5):1316–23.
[32] Janakova L, Bakke H, Haugen IL, et al. Influence of intravenous anesthesia on mucosal and systemic antibody responses to nasal vaccines. Infect Immun 2002;70(10):5479–84.
[33] Ito R, Ozaki YA, Yoshikawa T, et al. Roles of anti-hemagglutinin IgA and IgG antibodies in different sites of the respiratory tract of vaccinated mice in preventing lethal influenza pneumonia. Vaccine 2003;21(19–20):2362–71.
[34] Yetter RA, Lehrer S, Ramphal R, Small Jr PA. Outcome of influenza infection: effect of site of initial infection and heterotypic immunity. Infect Immun 1980;29(2):654–62.
[35] Jansson B, Bjork E. Visualization of in vivo olfactory uptake and transfer using fluorescein dextran. J Drug Target 2002;10(5):379–86.
[36] Peppelenbosch MP, DeSmedt M, Pynaert G, van Deventer SJ, Grooten J. Macrophages present pinocytosed exogenous antigen via MHC class I whereas antigen ingested by receptor-mediated endocytosis is presented via MHC class II. J Immunol 2000;165(4):1984–91.
[37] Saunders NB, Shoemaker DR, Brandt BL, Moran EE, Larsen T, Zollinger WD. Immunogenicity of intranasally administered meningococcal native outer membrane vesicles in mice. Infect Immun 1999;67(1):113–9.
[38] Simecka JW, Jackson RJ, Kiyono H, McGhee JR. Mucosally induced immunoglobulin E-associated inflammation in the respiratory tract. Infect Immun 2000;68(2):672–9.
[39] Szarka RJ, Wang N, Gordon L, Nation PN, Smith RH. A murine model of pulmonary damage induced by lipopolysaccharide via intranasal instillation. J Immunol Methods 1997;202(1):49–57.
[40] Berstad AK, Holst J, Froholm LO, et al. A nasal whole-cell pertussis vaccine induces specific systemic and cross-reactive mucosal antibody responses in human volunteers. J Med Microbiol 2000;49(2):157–63.
[41] Haneberg B, Dalseg R, Wedege E, et al. Intranasal administration of a meningococcal outer membrane vesicle vaccine induces persistent local mucosal antibodies and serum antibodies with strong bactericidal activity in humans. Infect Immun 1998;66(4):1334–41.
[42] Pfeffer LM, Dinarello CA, Herberman RB, et al. Biological properties of recombinant alpha-interferons: 40th anniversary of the discovery of interferons. Cancer Res 1998;58(12):2489–99.