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Safety and immunogenicity, after nasal application of HIV-1 DNA gagp37 plasmid vaccine in young mice

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ABSTRACT

Background: There is a need for safe and potent adjuvants capable of delivering vaccine candidates over the mucosal barrier, with good capacity to stimulate both mucosal and systemic cell-mediated and humoral immunity. An adjuvant aimed for intranasal delivery should preferably deliver the antigen and minimize the transfer into the close proximity of the central nervous system, thus avoiding damage on the olfactory tissues. Advantages with a mucosal delivery route would be to provide mucosal and systemic immunity, requiring lower vaccine doses then when given parentally. The aim of this study was to study if the N3 adjuvant intranasally administered with HIV DNA plasmids would be transferred into the olfactory tissues and cause local inflammation and tissue damage.
Results: The N3 adjuvant alone and when combined with HIV-1 DNA gag plasmid and delivered intranasally did not cause detectable damage to the nasal epithelium or the olfactory epithelium or bulb over a period of 3 days after delivery. The intranasal administration of HIV-1 gagp37 DNA induced both a humoral and a cell-mediated immunity against the gag antigen. Significantly higher HIV-1-specific humoral, but not cell-mediated immune responses were seen in DNA/N3-immunized mice in comparison with HIV-1 DNA/saline-immunized animals.
Conclusions: A safe and convenient intranasal mode of HIV-1 DNA plasmid and adjuvant delivery was shown not to interfere with the tissues in close proximity to the central nervous system. The N3 adjuvant combined with HIV-1 plasmids enhances the HIV-1-specific immunogenicity and merits to be clinically tested.

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1. Background

In spite of all fascinating and promising mucosal delivery modes for pharmacological drugs and vaccines their potential risk factors need to be considered. In lately performed intranasal vaccine trials side effects such as Bell's palsy or damaged olfactory nerves and nasal epithelium (NE) have been described [1–3]. These unwanted side effects still should not discourage from developing vaccines and adjuvants for nasal administration, since there are several advantages in using this route. First, the possibility of avoiding needle and syringe and the risks associated, secondly the nasopharyngeal-associated lymphoid tissues (NALT) contains a rich immunological environment including the nasopharyngeal tonsils (Waldeyers ring). Second, immunizing via the nasal route have further shown capable of providing immunity both locally and in more distant mucosal and systemic regions which has been shown to result in both cytotoxic CD8+ T-cells, and secretory IgA [4,5].

HIV-1 infection is commonly spread via mucosal transmission [6]. Thus the mucosal surfaces in the genital and rectal regions are critical entry sites for the HIV, and in these compartments an efficient blockade of transmission would have a strong impact on the transmission rate and risk. If a HIV vaccine could be developed which could elicit an adaptive mucosal and systemic humoral and cell-mediated immunity against conserved, neutralizing HIV-1 envelope epitopes this would be a truly prophylactic vaccine. Previous studies have shown that passive transfer of HIV- or SIV-specific immunoglobulins can provide protective immunity to mucosal SHIV challenge [7] and that highly HIV-1- and -2-exposed individuals can develop HIV-specific neutralizing IgA in serum and mucosa [8,9]. These studies suggests that it could be possible to induce a potentially protective immunity with HIV-1 DNA plasmids

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as shown in our own [10–12] and studies by others [13–16]. These and other results suggest that needle-free intranasal HIV-1 DNA immunization followed by a heterologous recombinant or peptide antigen boost could be a powerful vaccine strategy which merit to be evaluated in clinical studies.

2. Methods

2.1. Immunizations, antigens and adjuvants

For the safety study C57Bl/6 mice were immunized intranasally once with HIV-1 plasmid encoding gag of subtype B combined with 2% N3 adjuvant or with empty adjuvant as control and one-third group with saline.

In the immunogenicity study female mice were immunized three times with 4-week intervals with the same HIV-1 DNA plasmids intranasally. Group 1 with DNA and the 2% N3 adjuvant, group 2 with DNA in saline and the third group with saline only. At 6 weeks after the final immunization serum and mucosal upper airway washings and spleens were collected for analysis. The plasmids used have been described elsewhere [17–19]. In brief, the plasmids are driven by an immediate early CMV promoter, the gene coding sequence which is followed by the HPV-16 late poly(A) signal [17,19]. Adjuvant preparation [20]; 0.4 g of the 1:1 (molar ratio) mixture of mono-olein and oleylamine, 9.6 ml 0.1 M Tris buffer, pH 8.0 added in a beaker. The N3 emulsion was formed by sonication for 2 min and the pH adjusted to 8.0. The final vaccine formulation was 1:1 (v/v) mixture of a obtained 4% N3 emulsion and a DNA solution having a concentration of 20 μg DNA/20 μl.

2.2. Preparation of nasal, olfactory and cerebral samples for histological analyses

Mice were sacrificed by CO_2. The head were cut off and the skin was removed and the head was immediately placed in 4% paraformaldehyde (pH 7.4).

2.2.1. Fixation

The head was fixed in 4% paraformaldehyde for 24 h at room temperature (RT).

2.2.2. Decalcification

After fixation the head was washed in Tris buffer (pH 7.6) and then placed in 8% EDTA (pH 7.6) for decalcification. The decalcification was performed in RT and the EDTA buffer was changed every 24th hour 6 times.

2.2.3. Paraffin embedding

The heads were washed in tap water to remove the EDTA and then cut in three pieces. Each pieces was placed in a cassette, plastic holders and dehydrated in a series of ethanol and xylene baths. The dehydrate process is important to get out all of water from the tissue so that the paraffin can penetrate through it. Three baths of paraffin at 60 °C were performed before the paraffin embedded tissues were placed in steel containers, filled up with paraffin and let to cold down at ice.

2.2.4. Sectioning of paraffin closes

A microtome (Leica, Mannheim, Germany) was used for sectioning of the paraffin closes. Five micrometers thick sections were cut, placed on a water bath and catch up on glass slices. Incubation for 1 h in 37 °C to attaches the tissue slice to glasses.

2.3. Immunohistochemical detection

This technique was used to detect if the adjuvant and DNA vaccine may result in inflammatory reactions in the nose epithelium in olfactory bulb (OB) or CNS. Antibody against a common mouse leukocyte antigen (CD45) was used (BD Sciences, US). To trace the anti-mouse leukocyte antigen we used enzyme coupled secondary antibodies, one was biotinylated avidin–biotin complex (ABComplex, DAKO) and the other was alkaline phosphates coupled (DAKO Cytomation, Dakopatts, Copenhagen, Denmark). Endogenous alkalic phosphatase was blocked by Levamisol and was performed simultaneously with the colour development. For the biotinylated antibody we used AP substrate, and for the alkaline phosphates antibody we use fast red substrate (3,3-diaminobenzidin-tethrahydrochloride). All glasses were stained in hematoxylin (Histolab, Gothenburg, Sweden) colour bath and they were then mothered and microscopically examined.

2.4. Serum and mucosal samples

The mice were immunized i.n.a. with plasmid DNA or saline with or without adjuvant. The immunological assays used were for the cellular responses were IFN-gamma ELISpot. The HIV-specific humoral response was analysed by ELISA, which was performed on serum IgG and IgA, fecal pellet IgG and IgA and vaginal wash IgA.

2.5. Enzyme-linked immunosorbent assay for detection of IgG and IgA antibodies

Ninety-six well plates (NUNC-Maxisorp, Odense, Denmark) were coated with HIV-1 recombinant p24 antigens (Protein Sciences, Meriden, CT). Mouse sera were diluted in phosphate buffer saline (pH 7.4) with 0.5% bovine serum albumin (BSA, Boehring Mannheim, Mannheim, Germany) and 0.05% Tween 20 (Sigma–Aldrich, Sweden, AB) and 100 μl of dilutions 1/100, 1/1000, 1/10 000 and 1/100 000 were added to each well and incubated at +37 °C for 1.5 h.

Mucosal samples were tested as previously described [21,18,22]. Horseradish peroxidase conjugated anti-mouse IgG (Biorad, Richmond), dilution 1/3000 or anti-mouse IgA (Southern Biotechnologies, Birmingham, AL) dilution 1/1000, was added and incubated at +37 °C for 2 h and OPD (2 mg/ml orthophenylenediamine in 0.05 M sodium citric acid pH 5.5 with 0.003% H_2O_2) was added as substrate at 100 μl/well. After a 30 min incubation period, the reaction was stopped by adding 100 μl/well 2.5 M H_2SO_4 and absorbances were measured at 490 nm. Sera, vaginal secretions and feces from pre-immunized animals were used as negative controls.

2.6. Cell-mediated immunity

2.6.1. Secretion of cytokines in mucosal washings

No significant differences in amounts of secreted local IFN-gamma in the nasal tissues could be found between the study groups 1 and 2.

2.7. Cell-mediated immunity assayed by ELISpot

Mice were anaesthetised with isoflourane and blood collected by cardiac-punction. Blood was immediately placed in EDTA tubes. EDTA is a chelator who binds calcium and are there of an inhibitor for blood coagulation. Spleen was collected and cut into small pieces and then squeezed through a steel mesh down to a petri-dish containing RPMI 1640 supplemented with L-glutamine, antibiotics

and 10% fetal calf serum cell medium (Gibco, Life Sciences, Paisley, Scotland). The spleen cells were frozen in liquid nitrogen until further analysed by ELISpot.

The ELISpot assay was used to perform interferon-gamma T-cells response analyses after DNA immunization with and without the N3 adjuvant. All the three groups of mice were tested 4 weeks after the last immunization. Each test was performed in triplicate and negative and positive controls were used. The assay was performed according with the recommendations of the manufacturer (MabTech, Nacka, Sweden). In brief, 96-well plate was prepared with antibody against the IFN-gamma. After incubation at 4–8 °C over night, blocking was performed with cell medium (containing 10% serum). After blocking, 100 μl of cell suspension containing 200 000 cells was added and incubated in 37 °C and 5% CO_2 for 24 h. A biotinylated detection antibody was used against the antigen. Thereafter a complex of streptavidine–alkaline phosphatase is added, and when substrate is added to alkaline phosphates, a coloured spot will develop. The plates were studied under a light microscope and reading is performed automatic in an ELISpot reader (AID, San Diego, CA).

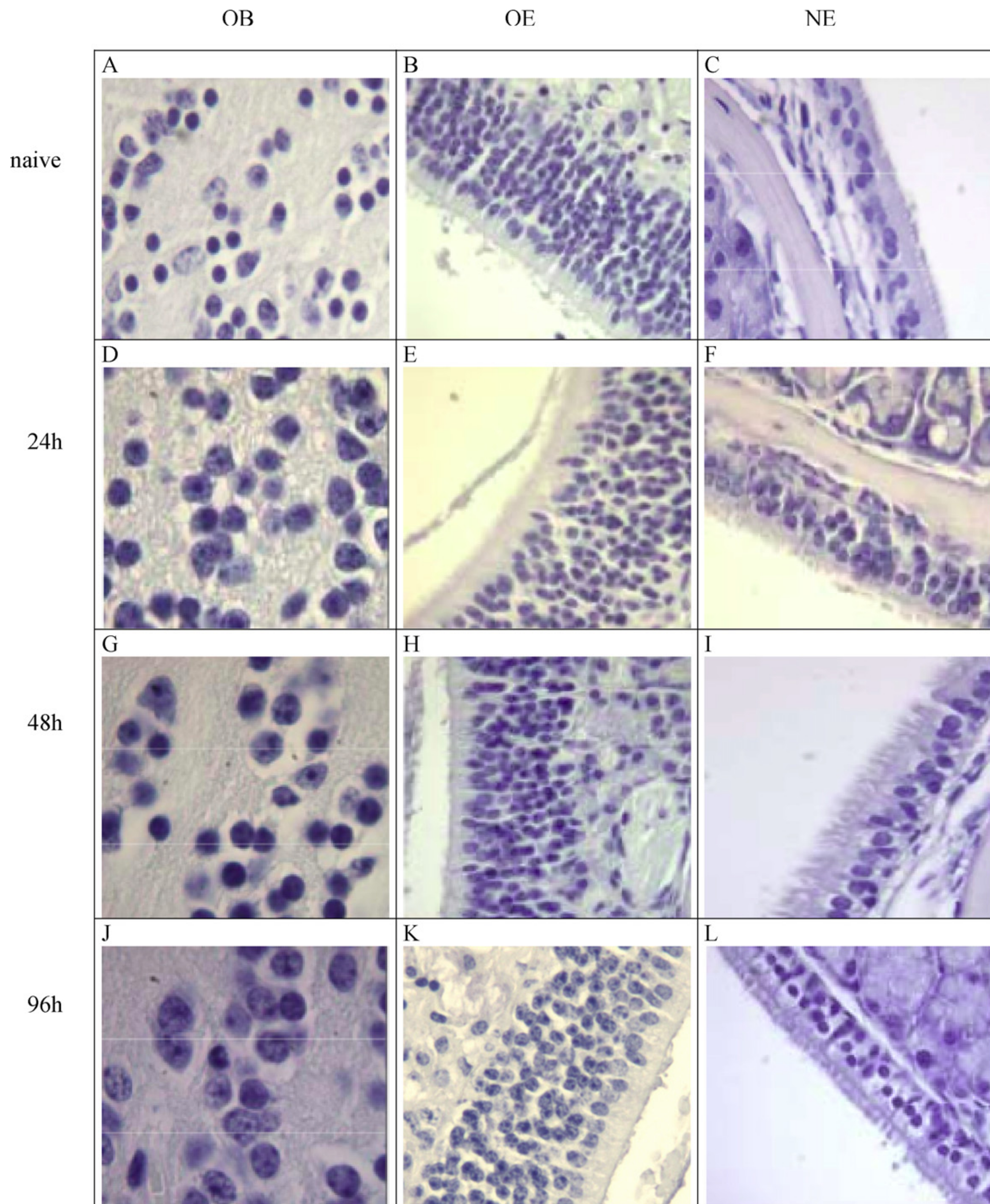

Fig. 1. The typical tissue pattern in histochemical analyses of the upper airways of mice immunized with 10 μg plasmid DNA dissolved in Tris buffer containing 2% N3 adjuvant after 24, 48 and 96 h. Figures (A)–(C) represents tissues from untreated normal mice with (A), (D), (G) and (J) represent olfactory bulb (OB) tissues; (B), (E), (H) and (K) represent olfactory epithelium (OE) tissues; (C), (F), (I) and (L) represent nasal epithelium (NE) tissues. The horizontal figures (A)–(F) show the tissue 24 h after exposure to plasmid DNA and 2% N3 adjuvant, (G)–(I) show the tissues 48 h after exposure and (J)–(L) show tissues 96 h after exposure to plasmid DNA and 2% N3.

2.8. Statistical analyses

Statistical comparisons between the groups were performed using the nonparametric Mann–Whitney *U*-test. A significant difference was considered when a *p*-value of <0.05 was obtained. One-way ANOVA with Dunnetts post-test was performed using GraphPad Prism version 4.0a for MacIntosh, OS 9, Apple Graphic-Pad Software, San Diego, CA, was used for comparisons of medians between groups at $p < 0.001$ and <0.05 levels.

3. Results

3.1. Olfactory nerve and tissues

No inflammation or abnormal tissue structures after intranasal exposure to the 2% N3 adjuvant or 10 μg pDNA gagp37 and 2% N3 adjuvant was seen at 24, 48 or 96 h. In Fig. 1A–K a comparison of representative tissue slides from naive mice and N3 adjuvant-exposed mice after 24–96 h in tissues from olfactory bulb, nasal epithelium or olfactory epithelium (OE) is shown. No differences between the tissues were detected in tissues from the three different study groups suggesting that the adjuvant nor the pDNA gagp37 induces changes or inflammation in the nasal or olfactory tissues of treated animals.

When performing immunohistochemistry with antibodies against the murine CD45-expressing cells to elucidate if N3 adjuvant-immunized animals would have an increased invasion of potentially inflammation causing cell populations in the nasal and olfactory tissues 24–96 h after administration of the adjuvant (or adjuvant and plasmid DNA) no differences in tissues from naive untreated and immunized mice could be shown (data not shown).

3.2. Immunoglobulins against HIV-1 in serum

HIV-1 DNA-induced systemic humoral immunity after three immunizations was strong in the mice when adjuvant N3 was used (Table 1). The difference between using adjuvant was seen, in mice immunized with DNA and N3 the serum IgG and IgA titers against p24 antigen became significantly higher then when no adjuvant was used ($p < 0.05$).

3.3. Fecal stool immunity in adult mice

Mucosal (fecal) immune responses were found in animals immunized via the intranasal routes, the highest HIV-1-specific IgA titers found with adjuvanted vaccine where fecal IgG titers against p24 were significantly higher ($p < 0.01$)

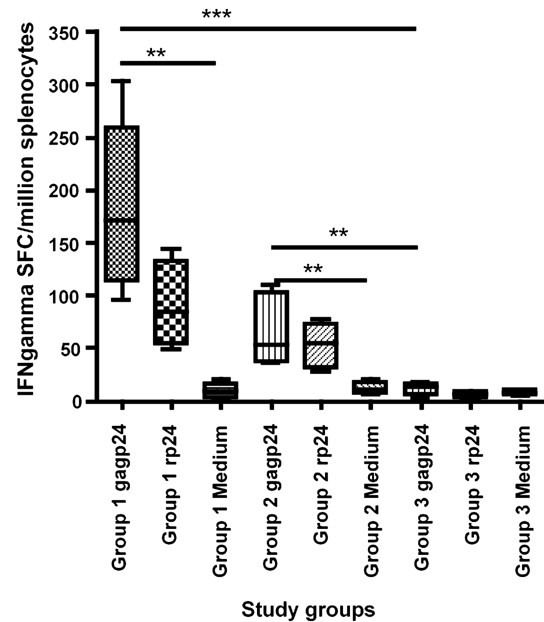

Fig. 2. Interferon-gamma secreting cells/million splenocytes shown by ELISpot and compared between three groups of intranasally-immunized mice. Box plots show the group median and 25% and 75% percentiles for each analysed group. Significant differences between the groups are indicated by the (*) symbols where $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

3.4. Nasal and vaginal wash immunity

Nasal group 1 anti-gag IgA titers were significantly higher then group 2 anti-gag IgA titers ($p < 0.05$). Nasal IgG titers were not significantly different (Table 1).

Vaginal IgG titers in group 1 were significantly higher than in group 2 mice ($p < 0.05$). The vaginal IgA titers were not significantly different between groups 1 and 2. Both groups 1 and 2 mucosal anti-gag titers were significantly stronger then the control group Ig anti-gag reactivities ($p < 0.001$).

3.5. Cell-mediated immunity

Interferon-gamma secreting spleen cells were significantly higher in plasmid-DNA and N3 adjuvant-immunized mice ($p < 0.01$) compared with N3 only or naive mice (Fig. 2).

3.6. Behavioural observations

Early after intranasal administration of the HIV-1 DNA and N3 adjuvant or N3 adjuvant only was it possible to see that the mice

Table 1
Humoral immunity against gagp24 in mice immunized with gagp37 DNA with and without N3 adjuvant

Group	Immunization	Adjuvant	Serum IgG rp24	Serum IgA rp24	Fecal IgG rp24	Fecal IgA rp24	Nasal wash IgG rp24	Nasal wash IgG rp24	Vaginal wash IgG rp24	Vaginal wash IgA rp24
1	pDNA-p37 3×	2% N3	15,300	2780	144	40	7	6	7.7	5
	95% CI		10,531–20,069	1502–4064	60–226	23–60	3.5–10	4–8	3–12	2.5–7.5
2	pDNA-p37 3×	No	2680	900	17	24	3.7	2	1.6	0.7
	95% CI		2008–3525	600-1190	7–26	1.6–46	2–5	0–4	0–3.7	0–2.4
3	Saline	2% N3	<100	<100	<4	<4	<2	<2	<2	<2
	95% CI		N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
4	–	–	<100	<100	<4	<4	<2	<2	<2	<2
	95% CI		N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

Group mean titers and 95% CI.

felt discomfort with the vaccine administration. The signs were sneezing and increased polishing of the nostrils and whiskers. This discomfort was seen for approximately 1 h after the vaccine administration, thereafter there was no difference in behaviour between the vaccines and the control mice left untouched.

4. Discussion

Even though side effects have been seen with mucosally delivered experimental vaccines they seem often to be associated with the chosen adjuvant combined with the vaccine itself [1–3]. Thus there is a great need to find safe but sufficiently potent adjuvants/vaccines which provide both immediate immunity and a long-lasting immune memory. In this category of substances a fascinating area of research is among the natural or endogenous candidates such as the fatty acid based adjuvants used in this and previous work the N3 and L3 adjuvants [20,22,23].

In this study we show that the tested adjuvant the N3 no detectable tissue damage on the nasal epithelium or on the olfactory pathways the nasal and central nervous connecting tissues. Further we show that the use of an adjuvant significantly enhances immune responses against the p24 antigen especially the humoral immune responses. The data indicate that the delivery of DNA with N3 adjuvant may mainly be an increased efficacy to deliver a larger amount of DNA plasmid over the mucosa and to the antigen-presenting cells rather then causing local inflammation and thereby recruiting and enhancing the immunogenicity. The inflammation and immune recruitment may instead take place in draining lymph nodes. This may be an advantage especially in individuals with small upper airways where an inflamed and even slightly swollen nasal tissue may de inconvenient, unwanted and disturbing, such as in small children. However, it needs to be pointed out that the architecture of the nasal cavity of a mouse is very different from a human so direct comparisons are not possible. This is in concordance with our previous studies with this and similar adjuvants, but with other HIV-1 DNA plasmids such as the envelope gene-expressing plasmids [22,23]. In this report we therefore focus more on the gag-antigen directed immunity a more conserved and therefore attractive CD8+ CTL and CD4+ T-helper cell target antigen. In earlier studies with this pDNA gagp37 antigen we have shown that the expressed antigen is a very immunogenic vaccine candidate, with or without adjuvant [24]. It was therefore not surprising that the IFN-gamma secreting cells were non-significantly different between the adjuvant and non-adjuvant-immunized animals (Fig. 2). Further we have seen that when combining the DNA plasmid-expressing gagp37 with the gp160-gene-expressing plasmids at the same injections site suppression of the HIV-1-specific immunity was noticed [25,26]. These previous studies were performed by syringe and needle or Biojector administration, and have not yet been studied when mucosal administration was performed. It would therefore be desirable to perform a clinical trial with intranasally or otherwise mucosal delivery of a HIV-1 vaccine candidate consisting of a combination of DNA plasmids representing a broad HIV-1 clade repertoire. The gag-gene have an obvious place in such a combination vaccine due to its relatively high conservation between clades, its strong correlation with reduced disease progression rates in patients responding persistently and strong against it [27,28]. With this study, using intranasal delivery with a novel endogenous adjuvant an alternative to parenteral immunization seem safe and possible.

5. Conclusion

Intranasal administration of HIV-1 DNA gagp37 with the endogenous N3 adjuvant is safe and does not cause detectable damage in the nasopharyngeal and olfactory tissues. The N3 adjuvant delivered HIV-1 DNA vaccine induces significantly higher gagp24-specific humoral immune responses, both systemically and on mucosal surfaces. Thus the combination of HIV-1 DNA vaccines with 2% N3 adjuvant seems safe for intranasal administration.

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References

[1] Mutsch M, Zhou W, Rhodes P, Bopp M, Chen RT, Linder T, et al. Use of inactivated intranasal influenza vaccine and the risk of Bell's plasy in Switzerland. N Engl J Med 2004;350:896–903.

[2] Couch RB. Nasal vaccination. *Escherichia coli* enterotoxin, and Bells palsy. N Engl J Med 2004;350:860–1.

[3] Van Ginkel FW, Jackson RJ, Yuki Y, McGhee JR. Cutting edge: the mucosal adjuvant cholera toxin redirects vaccine proteins into olfactory tissues. J Immunol 2000;165:4778–82.

[4] Belyakov IM, Derby MA, Ahlers JD, Kelsall BL, Earl BL, Moss B, et al. Mucosal immunization with HIV-1 peptide vaccine induces mucosal and systemic cytotoxic T lymphocytes and protective immunity in mice against intrarectal recombinant HIV-1 vaccinia challenge. Proc Natl Acad Sci USA 1998;95:1709–14.

[5] Neutra MR, Kozlowski PA. Mucosal vaccines: the promise and the challenge. Nat Rev Immunol 2006;6:148–58.

[6] Pope MA, Haase AT. Transmission acute HIV-1 infection and the quest for strategies to prevent infection. Nat Med 2003;9:847–52.

[7] Baba TW, Liska V, Hofmann-Lehman R, Vlasak J, Xu W, Ayehunie S, et al. Human neutralizing monoclonal antibodies of the IgG1 subtype protect against mucosal simian-human immunodeficiency virus infection. Nat Med 2000;6:200–6.

[8] Devito C, Hinkula J, Kaul R, Kimani J, Kiama P, Lopalco L, et al. Cross-clade HIV-1 specific neutralizing IgA in mucosal and systemic compartments of HIV-1 exposed, persistently seronegative subjects. J AIDS 2002;30:413–20.

[9] Lizeng O, Nilsson C, Sourial S, Andersson S, Larsen O, Aaby P, et al. Potent neutralizing serum immunoglobulin A (IgA) in human immunodeficiency virus type 2 exposed IgG seronegative individuals. J Virol 2004;78:7016–22.

[10] Ljungberg K, Rollman E, Eriksson L, Hinkula J, Wahren B. Enhanced immune responses after DNA vaccination with combined envelope genes from different HIV-1 subtypes. Virology 2002;302:44–57.

[11] Rollman E, Hinkula J, Arteaga J, Zuber B, Kjerrstrom A, Liu M, et al. Multi-subtype gp160 DNA immunization induces broadly neutralizing anti-HIV antibodies. Gene Ther 2004;11:1146–54.

[12] Hinkula J, Rollman E, Lundholm P, Benthin R, Okuda K, Wahren B. Genetic immunization with multiple HIV genes provides protection against HIV-1/MuLV pseudovirus challenge in vivo. Cells Tissues Organs 2004;177:169–84.

[13] Hamajima K, Sasaki S, Fukushima J, Kaneko T, Xin KQ, Kudho I, et al. Intranasal administration of HIV-DNA vaccine formulated with a polymer carboxymethylcellulose, augments mucosal antibody production and cell-mediated immune response. Clin Immunol Immunopathol 1998;88:205–10.

[14] Jounai N, Okuda K, Kojima V, Hamajima K, Ohba K, Klinman D, et al. Contribution of the rev gene to the immunogenicity of DNA vaccines targeting the envelope glycoprotein of HIV. J Gene Med 2003;5:609–17.

[15] Sasaki S, Hamajima K, Fukushima J, Ihata A, Ishii N, Gorai I, et al. Comparison of intranasal and intramuscular immunization against HIV-1 with a DNA-monophosphoribosyl lipid A adjuvant vaccine. Infect Immun 1998;66:823–6.

[16] Singh M, Vajdy M, Gardner J, Briones M, O'Hagan D. Mucosal immunization with HIV-1 gag DNA on cationic microparticles prolongs gene expression and enhances local and systemic immunity. Vaccine 2001;20:594–602.

[17] Hinkula J, Svanholm C, Schwartz S, Lundholm P, Brytting M, Engstrom G, et al. Recognition of prominent viral epitopes induced by immunization with human immunodeficiency virus type 1 regulatory genes. J Virol 1997;71: 5528–39.

[18] Asakura Y, Lundholm P, Kjerrstrom, Benthin R, Lucht E, Fukushima J, et al. DNA-plasmids of HIV-1 induce systemic and mucosal immune responses. Biol Chem 1999;380:375–9.

[19] Rollman E, Bråve A, Boberg A, Gudmundsdotter L, Engstrom G, Isaguliants M, et al. The rationale behind a vaccine based on multiple HIV antigens. Microbes Inf 2005;7:1414–23.

[20] Schröder U, Svenson SB. Nasal and parenteral immunizations with diphtheria toxic using monoglyceride/fatty Ac-id lipid suspensions as adjutants. Vaccine 1999;17:2096–100.

[21] VanCott TC, Kaminski RW, Mascola JR, Kalyanaraman VS, Wassef NM, Alving CR, et al. HIV-1 neutralizing antibodies in the genital and respiratory tracts of mice intranasally immunized with oligomeric gp160. J Immunol 1998;160:2000–10.

[22] Devito C, Zuber B, Schroder U, Benthin R, Okuda K, Broliden K, et al. Intranasal HIV-1-gp160-DNA/gp41 peptide prime-boost immunization regimen results in long-term HIV-1 neutralizing humoral mucosal and systemic immunity. J Immunol 2004;173:7078–89.

[23] Hinkula J, Devito C, Zuber B, Benthin R, Ferreira D, Wahren B, et al. A novel DNA adjuvant, N3, enhances mucosal and systemic immune responses induced by HIV-1 DNA and peptide immunizations. Vaccine 2006;24: 4494–7.

[24] Rollman E, Ramqvist T, Zuber B, Tegerstedt K, Kjerrstrom-Zuber A, Klingstrom J, et al. Genetic immunization is augmented by murine polyomavirua VP1 pseudocapsid. Vaccine 2003;21:2263–7.

[25] Bråve A, Ljungberg K, Rollman E, Hinkula J, Boberg A, Wahren B. Multigene/multi-subtype HIV-1 vaccine induces potent cellular and humoral immune responses by needle-free intradermal delivery. Mol Ther 2005;12:1197–205.

[26] Bråve A, Ljungberg A, Boberg A, Rollman E, Engstrom G, Hinkula J, et al. Reduced cellular immune responses following immunization with a multi-gene HIV-1 vaccine. Vaccine 2006;24:4524–6.

[27] Rinaldo C, Huang XL, Faz ZF, Ding M, Beltz L, Logar A, et al. High levels of anti-human immunodeficiency virus type 1 (HIV-1) memory cytotoxic T-lymphocyte activity and low viral load are associates with lack of disease in HIV-1-infected long-term non-progressors. J Virol 1995;69:5838–42.

[28] Harrer T, Harrer E, Kalams SA, Elbeik T, Staprans SI, Feinberg MB, et al. Strong cytotoxic T cell and weak neutralizing antibody responses in a subset of persons with stable nonprogressing HIV type 1 infection. AIDS Res Hum Retrovirus 1996;12:585–92.