ATRAUMATIC ORAL SPRAY IMMUNIZATION WITH
REPLICATION-DEFICIENT VIRAL VECTOR VACCINES

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ABSTRACT

Development of needle-free vaccines is one of the recently defined “Grande Challenges in Global Health”. To explore whether a natural pathway to the inductive site of the mucosa-associated lymphatic tissue could be exploited for atraumatic immunization purposes, replication-deficient viral vector vaccines were sprayed directly onto the tonsils of rhesus macaques. Tonsillar immunization with viral vector vaccines encoding simian immunodeficiency virus (SIV) antigens induced cellular and humoral immune responses. Viral RNA levels after a stringent SIV challenge were reduced providing a similar level of protection as observed after systemic immunization with the same vaccines. Thus, atraumatic oral spray immunization with replication-deficient vectors can overcome the epithelial barrier, deliver the vaccine antigen to the mucosa-associated lymphatic tissue, and avoid induction of tolerance providing a novel approach to circumvent acceptability problems of syringe and needle vaccines in children and developing countries.
INTRODUCTION

First immunization studies in humans with viral vectors encoding vaccine antigens have demonstrated induction of cellular and humoral immune responses (7, 20, 29, 30, 33). Several vector vaccines based on adenoviruses or poxviruses have shown promising preclinical results and are currently at different stages of clinical development for prophylactic or therapeutic use against infectious diseases or cancer.

In children, syringe and needle administration of vaccines faces acceptability problems, while in developing countries inappropriate re-use of needles and syringes is associated with an increased risk of infection. A non-invasive oral vaccination strategy could greatly facilitate world-wide access to vaccines by for example enabling trained teachers to administer the vaccine. So far, only live attenuated vaccines have been used for oral vaccination in humans. The efficacy of these vaccines depends on subsequent replication and spread of the vaccines in the gastro-intestinal tract. Although highly efficient, spread of live attenuated vaccines to contact persons of the vaccinees or reversion of the vaccines to virulent forms limit the applicability of the live attenuated vaccine approach for many infectious diseases. Oral vaccination with replication-deficient viral vector vaccines might be able to substitute for the live-attenuated vaccines, but it is questionable whether they can elicit substantial immune responses given that the excess amount of antigens taken up as food on a regular basis mostly leads to tolerance rather than immunity. In addition, if only the superficial layers of the mucosa are transduced by the viral vector vaccines, shedding of the transduced cells prior to expression of the vaccine antigen could be expected.

The epithelial barrier of the oral cavity to be passed by viral vector vaccines consists of a multilayered, non-keratinized squamous epithelium. Below the epithelium, the oral cavity contains the Waldeyer’s ring, an important member of the mucosa-associated lymphatic tissue (MALT), including the lingual and palatine tonsils. The crypts of these tonsils are lined by a lymphoepithelium with interspersed M cells that facilitate controlled entry of antigens.
through the epithelial barrier. Since the oral mucosa and, especially the crypt epithelium of the tonsils are also rich of dendritic cells (DCs), delivery of the vaccine via the tonsillar crypts could be a promising vaccination approach.

Administering SIV onto the tonsils of rhesus macaques led to efficient infection of exposed animals providing a valuable atraumatic mucosal challenge model (36). Tonsillar delivery of live-attenuated SIV vaccines also provided protection against subsequent challenge with homologous SIV and an SIV-HIV hybrid virus (35, 38). Since protection induced by these live-attenuated vaccines most likely has been due to systemic spread of the vaccine virus, we now analysed the immunogenicity and efficacy of replication-deficient viral vector vaccines after immunization by the tonsillar route and compared them to systemic administration. Adenoviral vectors were selected for tonsillar immunizations, assuming that the natural infection pathway of adenoviruses would also allow efficient delivery of the vector-encoded vaccine antigens. For priming we also used a single-cycle immunodeficiency virus vaccine (SCIV). The SCIVs were produced by transient transfection of an SIV genome, which was made replication-deficient by mutations in the primer binding site and a deletion of vif. (23). To allow a single round of replication, the primer-binding site mutations were complemented in trans by a matched tRNA expression plasmid in vif-independent 293 producer cells. After administration to the vaccinees, the SCIVs can undergo only a single round of replication leading to the production of non-infectious virus-like particles in vivo (12, 23). To increase in vivo expression levels, we pseudotyped the single cycle immunodeficiency virus vaccines (SCIV) with the G-protein of vesicular stomatitis virus, which mediates efficient entry into a broad spectrum of cells (6) including dendritic cells (16). After repeated systemic immunizations with SCIVs, SIV-specific humoral and cellular immune responses were observed, peak viremia during primary infection with the SIVmac239 challenge virus was significantly reduced (13, 23), and in a long-term vaccination experiment persistent suppression of SIVmac239 viral load was achieved (24).
We now observed that adenoviral vectors delivered by a simple atraumatic spray procedure onto the tonsils led to expression of the encoded antigen in close proximity to the inductive site of the MALT. Oral immunization with the adenoviral vectors was sufficient to induce cellular and humoral immune responses to encoded SIV vaccine antigens, but the adenoviral vector vaccine alone did not reduce challenge virus load. By contrast, a tonsillar prime-boost regimen of SCIV and adenoviral vector vaccines induced higher levels of cellular immune responses and reduced viral RNA levels after challenge with neutralization-resistant SIVmac239.
MATERIAL AND METHODS

Preparation of the vaccines

The 293T (293ts/A1609) (10), 293A (strongly adherent subclone of 293, Quantum Biotechnologies, Montreal, Canada), 293T-Rex (Invitrogen, Karlsruhe, Germany), and S-MAGI cells (8) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (FCS), penicillin, streptomycin and glutamine. The SCIV vaccines were produced by transient transfection of 293T cells as previously described (23). Viral particles from the conditioned media were thereafter concentrated by low speed centrifugation (27). The preparation contained 235 µg/ml RT as measured by a commercial RT assay (Roche, Penzberg, Germany). Further characterization of the SCIV preparation revealed a similar ratio of Gag and Env bands in Western blot analyses as previously reported (23) and readily detectable levels of VSV-G. For determination of the infectious titer, S-Magi indicator cell lines, containing the beta-galactosidase gene downstream of the HIV-1 LTR, were seeded at a density of 2.5x10⁴ cells per well of a 24-well plate. One day later, 200 µl of serial dilutions of the concentrated SCIV particles were incubated with the S-Magi cells for three hours prior to addition of fresh medium. Two days later, S-MAGI cells were stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) essentially as described (8). The vector titer was calculated from the number of stained cells per well and was expressed as lacZ-forming units (LFU) / ml. The two SCIV preparations had infectious titers of 3.5x10⁹ and 2.3x10⁸ infectious units/ml. The construction of the recombinant adenoviral vector expressing the codon-optimized SIV gag-pol (Ad-Sgpsyn) has been described (25). For the construction of the recombinant adenovirus expressing the SIV envelope (Ad-Senv-co), a codon-optimized version of the SIVmac239 envelope gene encoding a membran-anchored Env with a truncated (amino acid 733 to 879) C-terminus of the cytoplasmic domain (TM207, (41)) was cloned into the HindIII-XbaI digested pShuttle-TetO2 (25) generating pS-Senv-co. The leader peptide of SIV was replaced by the leader peptide of tissue plasminogen activator. The full-
length adenoviral vector plasmid was generated by homologous recombination of the
plasmids pS Senv-co and pAdEasy1 (21) in BJ5183 cells (Stratagene, Amsterdam, The
Netherland) using standard procedures. Correctly-recombined plasmids were transfected into
293T-Rex cells and the recombinant viral vectors growing out from the transfected cells were
analysed for expression of the vaccine antigen by Western blot analyses. A second adenoviral
vector (Ad-Srtenvco) encoding Rev and a secreted version of SIV Env was constructed in
pShuttle-CMV by deleting the transmembrane domain of Env. The construct contains the
SIVmac239 nucleotides 6696 to 6859 (first exon of rev, numbering according to GeneBank
entry M33262), the first 25 codons of the tissue plasminogen activator leader sequence,
SIVmac239 env codons 23 to 683 in a codon-optimized version, and wildtype SIV
nucleotides 9002 to 9499 (codons 715 to 879 of SIV env and second exon of rev). Full-length
adenoviral vector constructs were generated by homologous recombination with pAdEasy1
and vector stocks were produced in 293 cells as described above. After purification of
adenoviral vectors by CsCl-gradient centrifugation, the particle concentration was measured
by the optical density. The adenoviral vaccine preparations of Ad-Sgpsyn, Ad-Senvco, and
Ad-Srtenvco had particle concentrations of 4.9x10^{12} /ml, 4.8x10^{12}/ml, and 2.4x10^{12}/ml,
respectively. The Ad-GFP vector was kindly provided by Dr. Kirsten Bender (Bochum) and
had been constructed by homologous recombination using the pAdTrack-CMV plasmid and
pAD-Easy1 (21). For the adenoviral neutralization assay, a second adenoviral vector,
designated Ad-EGFP, was generated by cloning the EGFP gene of pEGFP-1 (Clontech,
Moutain View, CA, USA) into pShuttle-CMV (Qbiogene, Irvine, CA, USA) and subsequent
homologous recombination with pAD-Easy1.

Animal experiments
Thirty-four purpose-bred young adult rhesus macaques were used in the present studies.
Twenty-two of them were of Indian origin, the 12 monkeys for the second immunization
experiment had been imported from China. Animals were housed at the German Primate Centre and animal care and use were performed in compliance with the German Animal Protection Law and relevant institutional guidelines.

Just prior to oral immunization, animals were treated intravenously (i.v.) with glycopyrroniumbromide at a dose of 40 µg. In the “Ad-oral” experiment, Ad-Sgpsyn and Ad-Senvco were each adjusted to a final concentration of $2 \times 10^{11}$ particles/ml and administered in a total volume of 0.5 ml. In the prime-boost experiment, the adenoviral vectors Ad-Sgpsyn and Ad-Srtenvco were administered by oral spray immunization (0.5 ml total volume) or intramuscular immunization (final volume of 3 ml injected at five different sites) at doses of $0.5 \times 10^{11}$ and $3 \times 10^{11}$ particles per construct, respectively. For intravenous immunization with SCIV, $2 \times 10^9$ LFU were injected in a final volume of 10 ml. Challenge virus exposure was performed with pathogenic SIVmac239 by the tonsillar route by touching the tonsils lightly with a cotton-wool swab soaked with culture medium containing approximately 6000 median tissue culture infectious doses of cell-free SIVmac239 virus as described previously (24).

Immunohistochemistry and in situ hybridisation

Routine histology: The tissues obtained by biopsy or autopsy were divided into 2 parts. One part was fixed overnight in 4% neutral buffered formalin and embedded in paraffin. The sections were stained with hematoxylin and eosin and Giemsa for routine histology. Portions of the fresh tissue were embedded in tissue freezing medium (Leica, Nussloch, Germany), snap frozen in liquid nitrogen and stored at -70°C until use.

Immunohistochemistry: The staining for the detection of GFP was performed on frozen sections. The cryostat sections were fixed with acetone for 30 min and then incubated with a rabbit polyclonal antibody GFP-Ch1P (1:300; Abcam, Cambridge, UK) at room temperature for 30 min. The antibody binding was visualized after incubation of the sections with a biotinylated goat anti-rabbit immunoglobulin (DakoCytomation, Copenhagen, Denmark).
followed by Streptavidin-APAAP (Dako). The color was developed with the alkaline
phosphatase antialkaline phosphatase technique with New Fuchsin as red chromogen.

In situ hybridization: SIV RNA in the axillary lymph nodes was detected on paraffin sections
with a $^{35}$S-labeled single stranded (antisense) RNA probe (Lofstrand Laboratories,
Gaithersburg, MD, USA) as described in detail before (39). From each biopsy, 6-8 sections
were hybridized. Spleen sections from naive infected monkeys were used as a positive control.
As a negative control, sections from each lymph node specimen were hybridized with a sense-
strand $^{35}$S-labeled probe. The slides were dipped in photo emulsion (NBT2; Kodak) and
exposed in the dark at 4°C for 7 days. The slides were developed (D19; Kodak), fixed and
counterstained with hematoxylin and mounted. Examination of the sections was performed
with a Zeiss Axiophot microscope (Carl Zeiss Jena, Germany) equipped with transmission
and epiluminescent illumination. Cells expressing SIV RNA were counted in all sections and
the mean value per section was calculated.

Immune monitoring and viral load measurement

To determine SIV-specific T-cell responses an IFNγ-ELISPOT was employed essentially as
described (37). The following antigenic stimuli were used: a 20-mer peptide pool of the
SIVgag p27 region (EVA ARP714.1-22; 22 peptides); a pool of 15-mer peptides of
SIVmac251/32H-Gag (EVA7066.1-16; 16 peptides); a pool of 15-mer peptides of
SIVmac251/32H-Rev (EVA7068.1-8; 8 peptides); four pools of 15-mer peptides of
SIVmac239 Env (NIH AIDS Research & Reference Reagent Program, Cat# 62049)
comprising 55-56 peptides each (overall 218 env peptides). As an SIV-unrelated control
stimulus a pool of six 20-mer peptides derived from the gHCV NS3 gene (aa1138-1157, aa
1198-1217, aa 1208-1227, aa 1458-1477, aa 1528-1547, aa 1538-1557) was applied.
To measure humoral SIV-specific immune responses a standard ELISA for the detection of
antibodies against the SIV polypeptides gp130 SU and p27 CA (Stolte et al. 2006) in a
limiting dilution format and a yield reduction assay for the determination of neutralising antibodies (24) were employed. Recombinant SIVgp130 (EVA670, NIBSC) and SIVp27 (EVA643) for ELISA were kindly provided by Programme EVA. Adenovirus-neutralizing antibodies known to be induced in vaccination regimes employing adenoviral vectors were determined in a green fluorescent foci reduction assay as previously described (14) with slight modifications. Briefly, inactivated monkey sera were analysed in serial four-fold dilutions starting at a 1:8 dilution. 120 µl of diluted serum and 100 µl of a replication-deficient Ad5 vector expressing EGFP containing 2x10⁵ PFU were incubated in triplicates at 4°C for 2h. Then 150µl of this mixture was transferred onto 96-well flat-bottom plates which had been pre-seeded with 293 cells and the plates were incubated at 37°C in 5% CO₂ for two days. Thereafter the plates were examined under a fluorescent microscope. Sample dilutions exhibiting >50% reduction of green-fluorescent foci compared to infected controls incubated with autologous monkey sera obtained before immunisation were considered positive for Ad5 neutralising antibodies. Viral RNA copy numbers in plasma were determined by real-time PCR as described (38).

Statistical analyses

The mean number of spots per 10⁶ PBMCs was determined from triplicate cultures for each peptide pool and the mean number of spots in control cultures with an irrelevant HCV peptide pool or lacking a peptide was subtracted. If data from several time points during the same treatment period (e.g. prior to immunization, after 1st, 2nd, or 3rd immunization) were available the mean ELISPOTs were calculated for this period for each peptide pool and each animal. The mean and standard deviation from each treatment period was determined for all animals and each peptide pool within the same group. A One way ANOVA analysis followed by pairwise multiple comparison using the Tukey Test was performed for each peptide pool to test whether there is a significant difference in the ELISPOTs prior to immunization and after
each immunization. Significantly enhanced ELISPOT responses during immunization within a treatment group were compared to the control group using the two-sided t-test. The t-test (two-sided) was also used to analyse differences in the peak viral load, early early set point RNA levels (mean of week 16, 20, and 24 of each animal), and late set point RNA levels (mean of week 28 to 48). The Mann-Whitney Rank Sum Test was used to evaluate differences in the median values of the log 2 antibody titers.
RESULTS

Adenoviral vector immunization by the tonsillar route

Given the tropism of adenoviruses of the subgenus C for the upper respiratory tract and their persistence in submucosal lymphatic tissues, we first analysed whether a replication-deficient adenoviral vector would allow expression of vaccine antigens in close proximity of the oral MALT. A replication-deficient adenoviral vector expressing GFP was therefore sprayed directly onto the tonsils of four rhesus monkeys. The adenoviral vector was administered at a concentration of $2 \times 10^{11}$ particles/ml in total volume of 0.5 ml using a spray pump system (Pfeiffer, Radolfzell, Germany). Since ketamin anaesthesia induced hypersalivation, monkeys were treated with glycopyrroniumbromide 10 to 15 minutes prior to the oral spray application, in order to enhance access of the vector preparations to the lymphoepithelium of the tonsils.

Two days later, immunohistological staining revealed GFP-positive cells with epithelial cell-like morphology at the basal cell layer of the squamous epithelium of the tonsils in three of four animals. The close proximity to cells with lymphoid morphology (Fig. 1 A to B) suggests that antigens could be delivered atraumatically to the induction site of the oral MALT by adenoviral vector immunization through the tonsillar route.

To explore whether oral immunization with replication deficient vectors would indeed elicit immune responses, 10 rhesus monkeys received two doses of an adenoviral vector vaccine at week 0 and 4 by oral spray immunization (Fig. 2A). The SIV adenoviral vector vaccine (Ad-SIV) consisted of two adenoviral vectors expressing $gag$-$pol$ and $env$, respectively. Four weeks after the first immunization, low antibody titers against Env and Gag were detected in a minority of immunized monkeys (Fig. 2B). After the second immunization, significantly higher antibody titers to p27CA (Mann-Whitney Rank Sum Test: $p=0.009$) and gp130 (p<0.001) were observed than prior to immunization. Four of the 10 animals received a third oral immunization with the same adenoviral vector vaccine at week 16. After the third
immunization, Env antibody titers increased significantly (p=0.023), while the increase in the Gag antibody titers did not reach statistical significance.

Different overlapping peptide pools spanning Gag and Env of SIV were used in an IFN-γ ELISPOT assay to monitor cellular immune responses after immunization (Fig. 2C). Since Rev is not encoded by the vaccines used, a Rev-peptide pool was also included as a negative control. To avoid arbitrary definitions of thresholds for ELISPOT positivity, which could bias statistical analyses, the ELISPOT values of non-stimulated control cultures of each monkey at each time point were subtracted from the values obtained after stimulation with different overlapping peptide pools. In non-immunized animals and in animals prior to immunization, this led to low positive or negative values depending on the peptide pools added suggesting either weak unspecific stimulatory and inhibitory effects or random variation. For further statistical analyses, the mean of the ELISPOT response measured prior to immunization (week -4 and 0) was compared to the mean of the ELISPOT response at week 2 and 4 and at week 6 and 8 in order to determine the effect of the first and second adenoviral vector immunization, respectively. Since the dynamics of the immune responses in individual animals were rather heterogeneous, with different animals showing the strongest ELISPOT responses at different time points after immunization, the average of the results from both time points analysed after each immunization is given. Cellular immune responses could already be detected by the IFN-γ ELISPOT assay after a single oral adenoviral vector immunization, but in contrast to the antibody response the second immunization did not booster the IFN-γ ELISPOT responses (Fig. 2C). In the four animals that received a third oral immunization a booster effect on the IFN-γ ELISPOT response (mean of week 14 and 16) was observed for the Gag peptide pool.

In the IFN-γ ELISPOT assay 7 different peptide pools deduced from SIV genes and consisting of 264 single peptides were used. To exclude the possibility, that the increase in IFN-γ ELISPOTs is due to stimulation of T cells originally raised against non-SIV antigens by
some of the peptides used, non-immunized control animals were also analysed in the IFN-γ ELISPOT assay. With the exception of the Env3 peptide pool, no significant differences between the pre and mock values were detected in the control group (Fig. 2D). Importantly, the IFN-γ ELISPOT response to the Gag, p27, and Env2 peptide pool after the first, second and/or third immunization was significantly higher in the orally immunized than in the non-immunized group (see arrows, Fig. 2C). Thus, oral spray immunization with adenoviral vectors induces cellular and humoral immune responses to the encoded vaccine antigen.

**Challenge of monkeys immunized with adenoviral vectors by the oral route**

The four monkeys receiving three oral immunizations with the adenoviral vector and eight control monkeys were subsequently challenged orally with the SIVmac239 virus, a neutralization-resistant molecular clone homologous to the vaccine antigens. An oral challenge was performed, since this provides a reliable, truly atraumatic, mucosal infection route (36). All animals became infected and peak viremia and set point RNA levels did not differ significantly between these two groups (Fig. 2E). Thus, the immune responses induced by oral immunization with the adenoviral vector did not protect from subsequent SIV challenge.

**Oral Prime-boost regimen**

To improve efficacy of the tonsillar immunization against SIV, we investigated whether a heterologous prime-boost regimen consisting of a SCIV prime and adenoviral vector boost would be beneficial. In addition, we compared oral spray with systemic vaccine application side by side. One group of four rhesus monkeys (systemic p/b) was immunized by priming with SCIV at week 0 by the intravenous route and intramuscular boosting with the adenoviral vectors encoding *gag-pol, env*, and *rev* at week 8 (Fig. 3). A second group of four rhesus monkeys (oral p/b) was immunized exclusively by spraying the vaccine preparations of SCIV
and the adenoviral vectors directly onto the tonsils of monkeys (Fig. 3). For the oral vaccination, each vaccine was given twice to compensate for the lower dose administered due to volume restrictions. To control for unspecific immunostimulatory effects of the adenoviral vector vaccines prior to challenge, two control monkeys each received an adenoviral vector encoding GFP by either the oral (week 8 and 12) or the intramuscular (week 8) route. All groups were challenged orally at week 20 with SIVmac239.

**Systemic spread of SCIV transduced cells after oral immunization**

We previously observed systemic infection after oral exposure to replication competent SIV (36) indicating that the tonsils provide a port of entry for SIV particles. To explore, whether systemic spread would also occur after oral immunization with VSV-G pseudotyped replication-deficient SCIVs, axillary lymph nodes were removed four days after immunization and a highly sensitive in situ hybridisation technique was used to analyze expression of SCIV. A low number of SIV RNA positive cells (1.87 RNA+ cells per section) could be detected in the lymph nodes of 3 of the 4 immunized animals (Fig. 1E–H). The low number of positive cells did not allow further characterization of the vaccine expressing cells by immunohistochemistry. However, cell morphology indicated that these cells were lymphocytes. The positive cells were present in the T cell-dependent zone, the germinal centres and the sinuses. Importantly, we were able to detect such cells in the efferent lymphatics. These findings indicate that the vaccine reached all immunologically important regions of the node.

**Humoral immune responses**

Priming with SCIV by the oral or intravenous route resulted in detectable antibodies to Gag in only one animal from each group, while Env antibodies were detectable at low levels in two animals after systemic immunization (Fig. 4). After the two oral boosts with the adenoviral
vector an increase in Gag antibody titers was observed in all four animals and three of the
four animals also developed Env antibodies. After boosting the systemic vaccination group
with the adenoviral vector a sharp increase in antibody titers was observed, which exceeded
those seen after tonsillar immunization (Fig. 4). The anti-Env antibodies induced in the
systemic group were able to neutralize the SIVmac251 strain, which had been used in the
neutralization assay due to the neutralization-resistant phenotype of the SIVmac239 virus,
while neutralizing antibodies in the oral p/b group remained undetectable (data not shown).

Cellular immune responses

Cellular immune responses after immunization were monitored using the IFN-γ ELISPOT
assay (Fig. 5). For statistical analyses, the mean of the ELISPOT response measured prior to
immunization (week -4 and 0) for each group was compared to the mean of the ELISPOT
response measured after priming (week 4 and 8) and boosting (week 10 and 12). After oral
priming with SCIV, the ELISPOT response to the various peptide pools did not exceed the
background response seen prior to immunization. However, after the first boost with the
adenoviral vectors by the oral route a significant increase in the IFN-γ ELISPOT responses
after stimulation with the p27 and Env3 peptide pools was observed (Fig 5A). Although
considerably higher mean ELISPOT numbers after boosting were also seen by stimulation
with the other peptide pools, this increase did not reach statistical significance. The systemic
prime-boost regimen induced a broad IFN-γ ELISPOT response to Gag, Rev, and various Env
peptide pools (Fig. 5B). Side by side with the vaccinated animals, ELISPOT responses were
also measured for the control monkeys (Fig 5C). With one exception, there were no significant
differences between the boost values obtained for the control group and its pre values
excluding that a systematic error due to variations in culture conditions is responsible for the
positive ELISPOT responses seen after boosting in the vaccinated animals. A significant
change in the ELISPOT response of the control group was only observed for the Env3 peptide
pool after priming, suggesting fluctuation of T cells cross-reacting with a peptide of the Env3 peptide pool. Nevertheless, the increase in the Env3 IFN-γ ELISPOTs in the immunized groups seems to be vaccine induced, since the number of ELISPOTS after boosting was significantly lower in the control group than in the immunized groups. The ELISPOT response to the p27CA peptide pool after the oral booster immunization was also significantly higher than the ELISPOT response measured in parallel for the control group. Thus, the ELISPOT response to these peptide pools after the oral prime boost immunization is significantly higher than the preimmunization values of the same group and the ELISPOT responses measured in parallel in the non-immunized control group. Using the same criteria for the systemic SCIV prime adenoviral vector boost immunization a broader reactivity was observed (see arrows, Fig. 5B), with only two peptide pools not showing a statistically significant ELISPOT response.

**Viral load after challenge**

To investigate the efficacy of the oral prime-boost vaccination approach, monkeys were challenged with SIVmac239 by the tonsillar route, thus providing a mucosal challenge with a stringent form of SIV. All animals became infected (Fig. 6), but peak viral RNA load (week 2), early set point RNA levels (mean viral RNA load week 12 to 24), and late set-point RNA levels (mean viral RNA load week 28 to 48) were 83, 35, and 43-fold lower, respectively in orally immunized monkeys than in the control animals. The differences in peak viral load between the two groups were statistically significant (p< 0.05). Of note, no differences in viral load levels were observed between orally and systemically immunized monkeys.

**Neutralizing antibodies against adenovirus**

Antibody titers to SIV after oral immunization with the adenoviral vector vaccine increased after the second administration (Fig. 2). This implies that oral immunization with adenoviral
vectors does not prevent expression of the vaccine antigen after a second dose of the same adenoviral vector. Since it has been previously observed, that intramuscular immunization with adenoviral vector vaccines limited the efficacy of booster immunization with homologous adenoviral vectors, we compared induction of neutralizing antibodies to the adenoviral vector after oral and intramuscular (systemic) immunization (Fig. 7). A single intramuscular injection of $6 \times 10^{11}$ adenoviral vector particles induced antibody titers exceeding $1/100$ in all monkeys (Fig. 7B). In contrast, a single oral immunization with $1 \times 10^{11}$ or $2 \times 10^{11}$ particles did not induce neutralizing antibodies, although high titered neutralizing antibodies were detected after the oral booster immunization with the adenoviral vector (Fig. 7A, B). However, the neutralizing antibody titers declined rapidly. A third oral immunization with the adenoviral vectors 12 weeks after the second clearly boosted humoral immune responses to SIV antigens, indicating that neutralizing antibodies to the adenoviral vector did not prevent expression of the vaccine antigen after repeated oral administration.
DISCUSSION

Simple spraying of replication-deficient adenoviral vectors onto the tonsils either alone or after priming with SCIV induced cellular and humoral immune responses to the encoded vaccine antigens indicating that the viral vectors can overcome the epithelial barrier, deliver the vaccine antigen to the inductive sites of the MALT, and avoid induction of tolerance frequently observed after oral delivery of protein antigens. Viruses have evolved mechanisms to transit tight cell layers by a cellular transcytosis pathway without replicating in the cells they pass (1, 5, 22, 31, 34). Since even enveloped viruses are shuttled in this way through the cell and secreted at the basolateral site in an infectious form, transduction of basal cell layers by viral vector vaccines seems possible. In addition, the lymphoepithelium in the depth of the tonsillar crypts with its interspersed M-cells should facilitate uptake of antigens. Following oral SCIV vaccine application, we could detect SIV-RNA positive cells in non-draining lymph nodes. The localisation of the SIV-RNA positive cells in the sinuses of the lymph nodes and the efferent lymphatics suggests that SCIV infected cells carry the viral vector vaccines through out the lymphatic tissues. Although we can not formally exclude replication of SCIV in vivo, we consider this to be highly unlikely since i) the primer complementation approach used to generate the SCIVs reduces infectivity in single round replication assays at least $10^4$-fold (17, 18); ii.) the SCIVs have an additional deletion of $vif$, which severely impairs replication in primary cells and rhesus macaques (9, 32), and iii) we were never able to recover replication-competent virus from SCIV injected animals despite repeated attempts (23).

For the adenoviral vector vaccine, we provide evidence of transduction of cells with epithelial cell-like morphology at the basal layer of the squamous epithelium. The close proximity of the transduced cells to lymphoid cells suggests that the tonsillar spray immunization indeed leads to atraumatic delivery of vaccine antigen to the oral MALT. The detection of adenoviral vector transduced cells underneath intact superficial layers of the squamous epithelium...
provides first in vivo evidence for transcytosis of adenoviral particles through these superficial layers.

Another atraumatic route used for immunization has been the nasal application. Although the nasal MALT might be well suited for induction of immune responses, the anatomic link between nose and brain via the olfactory nerve suggests a note of caution particularly for the use of viral vector vaccines, since retrograde axonal transport of viral capsids has been well documented (15, 26).

Oral spray immunization with the adenoviral vectors alone also revealed induction of humoral and cellular immune responses. While the oral booster immunization with the adenoviral vectors four weeks after priming enhanced humoral immune responses, ELISPOT responses were not increased by this early boost. Since a third oral booster immunization with the adenoviral vector seemed to stimulate humoral and cellular immune responses a longer time period between immunizations might be beneficial. However, the number of animals responding to the oral adenoviral vector immunization alone was lower than after the prime-boost regimen. This suggests that the SCIV given orally primed for immune responses, although anti-SIV responses were mostly not detectable until after the adenoviral vector booster immunization. Since SIV-specific immune responses induced by adenoviral vector vaccines might inhibit the infectivity of the SCIV vaccine, the SCIVs, and not the adenoviral vectors, were used for priming.

Preexisting immunity to the vector could limit the use of adenoviral vectors in humans (40). In addition, neutralizing antibodies induced by priming with adenoviral vectors reduce the efficacy of booster immunizations with the same adenoviral vector serotype (3). We therefore compared induction of neutralizing antibodies to the adenoviral vector after oral and intramuscular immunization. While a single intramuscular injection of the adenoviral vector vaccine induced neutralizing antibodies, substantial neutralizing antibodies to the adenoviral vector were only observed after two oral immunizations. Consistently, a strong booster effect
of the second oral adenoviral vector immunization was evident as humoral immune responses
to the vector encoded vaccine antigens were only observed after the second oral adenoviral
vector immunization. Even a third oral adenoviral vector immunization given 12 weeks after
the second boosted immune responses to the vaccine antigens in four out of four monkeys
suggesting that repeated administration of the same adenoviral vector is feasible for the oral
immunization route. A rapid decline in neutralizing antibody titers to the adenoviral vector
might explain the booster effects observed after the third oral adenoviral vector immunization.
In addition to extended waiting periods between booster immunizations, recent advances with
chimeric adenoviruses and/or the use of vectors based on adenovirus serotypes with low
prevalence should also be able to overcome potential problems of oral adenoviral vector
immunization with preexisting immunity in humans (reviewed in (2)).
In addition to the immunogenicity of the oral SCIV prime adenoviral boost immunization, we
also observed substantial suppression of viral load after challenge with SIVmac239 in the
absence of a sterilizing immunity. This indicates that oral immunization induced systemic
immune responses inhibiting SIV replication. The mechanisms mediating this reduction in
viral load remain to be defined. Neutralizing antibodies to the SIVmac239 challenge virus
could not be detected and there was no obvious inverse correlation between the magnitude of
the IFN-γ ELISPOT responses and the viral load after challenge. A more detailed
characterization of polyfunctional CD4 and CD8 T cells responses (4, 19) might provide
better correlates of protection. Whether the oral prime-boost immunization is indeed more
efficient than oral immunization with adenoviral vectors alone can not be concluded with
certainty. While the adenoviral vector vaccine of the first study expressed a membrane-bound
form of SIV Env, the adenoviral vector vaccine of the second study encoded Rev and a
secreted form of SIV Env. Furthermore, rhesus monkeys of Indian origin were used in the
first study, which are more susceptible to fatal consequences of SIV infection than the rhesus
monkeys of Chinese origin used in the second study (28). Nevertheless, given the difficulties
in inducing protective immune responses particularly against the neutralisation resistant SIVmac239 virus, a 35 to 83-fold reduction in peak and set-point RNA levels after oral immunization is a striking observation, even in rhesus monkeys of Chinese origin.

Interestingly, subtopical delivery of Modified Vaccinia Virus Ankara vaccines into the palatine tonsils of rhesus macaques via a needle-free injection device also induced substantial immune responses to the vector encoded antigens and suppression of viral load after SHIV89.6P challenge (11). Thus, further studies on the efficacy of oral vaccination with viral vector vaccines against systemic and particularly respiratory tract infections seem warranted.
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**FIGURE LEGENDS**

**Figure 1. Detection of replication-deficient viral vectors in the lymphoid tissue.** GFP-expression after tonsillar administration of an adenoviral vector (Ad-GFP) was detected by immunohistochemistry (red) in the crypt (A) and squamous epithelium (B) of the tonsil. The arrow (B) points to a large, non-lymphoid cell showing positivity for GFP in the lymphoid tissue. In a control macaque, tonsils were not stained with the antibody to GFP (C). As positive control, 293T cells transfected with a GFP expression plasmid were also stained (D). SCIV producing cells in axillary lymph nodes four days after i.v. inoculation (E) or tonsillar application (F-H) of the vaccine. Cells expressing SIV RNA (greenish blue colour with the combined reflected light and transillumination) are in the T-dependent zone (E), the germinal center (GC) and the efferent lymphatic vessel (G and H). This vessel harbouring the SCIV positive cell (G; arrow) is shown with higher magnification (H). The morphology of the positive cell (H; arrow) demonstrates that it is a lymphocyte (transillumination only, the silver grains are black). The number of hybridization signals appears low since the silver grains of the signal are located in a higher focal plane than the cells of the tissue section. Original magnifications: A, C, E-G = x100; B and H = x160; D = x50.

**Figure 2. Adenoviral vector immunization by the tonsillar route.** (A) Experimental outline. The scale indicates the number of weeks after the first immunization. Type of vaccine or challenge virus is given above the time line. Four of the 10 monkeys of the Ad-oral group received a third oral immunization at week 16. (B) Mean titer and standard deviation of antibodies binding p27 CA or gp130 SU in 10 (week 0 to 12) and 4 (week 16 to 48) macaques of the Ad-oral group. The vertical dotted line in (B) indicates the time of challenge. (C) ELISPOT responses to the indicated peptide pools in the Ad-oral group were determined 4 and 0 weeks prior to immunization (Pre), and two and four weeks after the first (1st), second (2nd), and third (3rd) oral immunization with the adenoviral vector. ELISPOTs of
unstimulated control cultures from each time point were subtracted and the means of all available time points were used as a single Pre, 1st, 2nd and 3rd value for each animal. The mean and standard deviation of the Pre, 1st, 2nd, and 3rd values of all animals after stimulation with the indicated peptide pools is shown. To determine whether there is a statistically significant difference between the mean of the Pre, 1st, and 2nd ELISPOT values for each peptide the one way ANOVA test was used followed by pairwise multiple comparison using the Tukey test. Numbers above the horizontal bars give the respective p values, if a significant difference between two time points was obtained in the ANOVA test. Columns marked with an arrow indicate ELISPOT responses that are significantly higher (p<0.05, t-test) than the mock ELISPOT response to the respective peptide pool of the Ad-control group (Fig. 2D).

For the 4 of the 10 animals that received a third (3rd) oral immunization, a paired t-test (“#”) was used to determine significant increases in ELISPOT responses between the second and third immunization. (D) IFN-γ ELISPOT responses in six control animals were determined on week -4, and 0 (Pre), and on week 8 (mock) as described in Fig. 2c. The t-test was used to determine statistically significant differences between the Pre and mock ELISPOT responses for each peptide. * Due to failure of the equal variance test, ANOVA on ranks followed by pairwise comparison using Dunn’s method was performed. (E) RNA load after challenge with SIVmac239. The mean and standard deviation of the viral RNA load in the four animals receiving three oral adenoviral vector immunizations (Ad-oral) and eight control monkeys infected in parallel is shown.

**Figure 3. Prime boost immunization with viral vector vaccines.** Names of the different treatment groups and number of animals per group are given. The scale indicates the number of weeks after the first immunization. Type of vaccine or challenge virus, route of immunization, and dosages are given above the time line.
Figure 4. Antibody titers to SIV p27 CA (left panel) and gp130 SU (right panel) after oral or systemic immunization and in control monkeys. The five digit numbers in the legend are monkey designations, arrows mark the time points of immunization. The vertical dotted lines indicate the time of challenge.

Figure 5. IFN-γ ELISPOT response after prime boost immunization by the oral (A), or systemic (B) route. ELISPOT responses to the indicated peptide pools were determined 1 and 3 weeks prior to immunization (Pre), at week 4 and 8 (Prime), and at week 10 and 12 (Boost). ELISPOT responses are presented as described in figure legend 2. Numbers above the horizontal bars give the respective p values, if a significant difference between two time points was obtained in the ANOVA test. Columns marked with an arrow indicate that the ELISPOT response is significantly higher (p<0.05, t-test) than the ELISPOT response to the respective peptide pool of the control group at the same time point (C). * Due to failure of the normality test, ANOVA on ranks followed by pairwise comparison using Dunn’s method was performed.

Figure 6. RNA load after challenge. A) The mean and standard deviation of the three groups is shown. B to D) The viral RNA load in monkeys immunized orally (B), systemically (C) or in the non-immunized control group (D) is also shown for each of the macaques. The five-digit numbers in the legend are monkey designations.

Figure 7. Neutralizing antibodies to the adenoviral vector. (A) Neutralizing antibody titers to the adenoviral vector before the first (Pre) and third (Pre 3rd) and two weeks after the first (1st), second (2nd), and third (3rd) adenoviral vector application (dose: 2x10^{11} particles) by the tonsillar route (for immunization regimen see Fig. 2A). (B) Neutralizing antibody titers to the adenoviral vector were determined before (Pre) and four weeks after the first (Prime) and
second (Boost) adenoviral vector application by the tonsillar (oral, dose: $1 \times 10^{11}$) or intramuscular (systemic, dose: $6 \times 10^{11}$) route. The two control macaques receiving Ad-GFP either orally of systemically (for immunization regimen see Fig. 3A) were included in the analyses.