HIV-1 specific humoral immune responses in healthy volunteers after HIV DNA prime - HIV MVA boost immunization

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Since the discovery of HIV-1, more than 60 million individuals have been infected and 3 millions are infected every year. Several antiviral drugs are available that greatly suppress the viral replication and prolongs the life of the infected individual, but the drugs cannot cure the host from the virus. In order to stop the HIV-epidemic there is an urgent need of a vaccine. However, so far all traditional vaccine strategies have shown to be inefficient (and potentially dangerous) and new vaccine approaches are being explored. Our group has designed a genetic vaccine encoding different parts of the HIV-1 virus. The HIV-1 genes are carried by DNA plasmids and by the viral vector, modified vaccinia virus Ankara (MVA). In the first phase I trial with the vaccine, 40 Swedish, healthy volunteers were immunized by three priming plasmid DNA immunizations and 6 months later boosted once with the MVA. Several years later, 24 of the volunteers received a second boost with MVA. The result after the 1st MVA immunization was highly successful and all but one volunteer responded with HIV-1 specific immune responses. In this project, the humoral responses were investigated against different HIV-1 structures: capsid/matrix (Gag), envelope (Env) and the enzyme reverse transcriptase (RT), after the 2nd MVA boost. The result showed that a 2nd boost of MVA was required in order to elicit detectable Env-specific antibodies. Gag-specific antibodies were relatively high already after the 1st MVA boost, but increased after the 2nd. A few vaccinees developed a response against RT. The volunteers were vaccinated differently in order to investigate which delivery method that would be preferred for the vaccine. The volunteers were either immunized with different amounts of vaccine intradermally or intramuscularly and with or without the adjuvant GM-CSF. Due to low numbers of individuals in each group, no clear conclusion could be made regarding the best delivery strategy.

The results presented here show that the vaccine can induce Env- and Gag-specific antibodies, in addition to the already reported cellular responses, and in addition to an ongoing phase I trial in Tanzania, the vaccine will shortly enter a new phase I trial in Sweden as well as two phase 2 trials in Tanzania and Mozambique.
INTRODUCTION

Human immunodeficiency virus (HIV)

In the early 80’s several cases of diseases, which normally only occur in immunodeficient persons, were reported among homosexual men in the US. The patients’ immune systems and especially the CD4+ T lymphocytes were depleted, and this new disease was given the name acquired immunodeficiency syndrome (AIDS). In 1983, the French scientists Francoise Barré-Sinoussi and Luc Montagnier isolated a novel retrovirus from the lymph node of an AIDS patient and the virus was identified as the causative agent of AIDS [4, 33, 44]. The retrovirus received its name, HIV, in 1985 [37] and the French scientists Montagnier and Barré-Sinoussi were honoured with the Nobel Prize in 2008 for their discovery.

Since the start of the epidemic, approximately 60 million people have been infected with HIV-1 and today, 33 million individuals live with the disease, 3 millions are newly infected and 2 millions die every year due to AIDS-related diseases [67].

The virus can be divided into two types; HIV-1 and HIV-2. Both originate from African simian immunodeficiency viruses (SIVs) that infect non-human primates and according to phylogenetic studies, the jump from monkeys to humans is believed to have occurred during the early 20th century [28]. HIV-1, which is the cause of the world wide epidemic, is believed to originate from the chimpanzee SIV (SIVCPZ). The HIV-2, which is mainly found in Western Africa is believed to originate from sooty mangabey SIV (SIVSM) [21, 56]. HIV-1 can further be divided into three different groups: M (main), O (outlier) and N (non-M non-O), where M is most common. Viruses within group M can be further divided into subtypes (or clades) A-K, which are geographically distributed. The subtype B virus mainly circulates in the western part of the world [47], whereas subtype C is the most common subtype in the world and is mainly found in southern Africa and India [67].

Structure and replication

HIV-1 is a Lentivirus, a genus within the Retroviridae family. It is a spherical, enveloped virus with a diameter of 110 nm. The genome is approximately 10 kB and consists of two positive single stranded RNA molecules, which contain nine genes. Like other retroviruses, HIV-1 has the genes env, gag and pol. In addition, HIV-1 carries regulatory genes: transactivator of viral transcription (tat), regulator of RNA transport (rev); and accessory genes: viral infectivity factor (vif), viral protein R (vpr), viral protein U (vpu) and the negative factor ( nef) [18].

The envelope consists of a lipid bilayer that is obtained from the host cell during budding and within the membrane the viral glycoproteins gp120 and gp41 protrudes (Figure 1). Trimeric gp120 is non-covalently associated with trimers of the transmembrane protein gp41. The two proteins derive from the polyprotein gp160, which is encoded by the viral env-gene [31]. The capsid (p24) and the nucleocapsid (p7) build up the viral core and they protect and encapsulate the viral genome. The nucleocapsid is in turn surrounded by the matrix protein p17, which stabilizes the virus. These three proteins derive from the polyprotein p55Gag that is encoded by the gag-gene. From the pol-gene, the three viral enzymes are transcribed and translated; reverse transcriptase (RT), protease (PR) and integrase (IN) (Figure 1) [31].
Infection begins when the gp120 envelope protein binds to the CD4 molecule on the target cells like T cells, monocytes and dendritic cells (DCs) (Figure 2). This causes conformational change of the protein, allowing for the gp120 to bind to the chemokine co-receptors CXCR4 and CCR5. This interaction brings the virus closer to the cell and allows for the gp41 protein to penetrate the cell membrane resulting in fusion of the viral with the cellular membrane. When it enters the cytoplasm, the virus’ own RT turns single stranded (ss) RNA to double stranded (ds) DNA which later is integrated into the host cell genome by the enzyme IN. The RT enzyme does not have the capacity of proof-reading (as cellular polymerases) and that leads to a high mutation frequency [18, 62]. Some of the HIV-1 genes are first transcribed and translated into precursor proteins, which enhance the transcription and translation of the late structural genes. The gp160 protein is glycosylated in the endoplasmic reticulum (ER) and cleaved into gp120 and gp41 in the golgi apparatus. The proteins are later assembled by the cell membrane. The pol and gag genes are transcribed and translated into the polyproteins p55Gag and p160GagPol. The newly formed proteins are cleaved and assembled at the cell membrane. Thereafter, new viruses bud from the surface (Figure 2) [19].
Figure 2. HIV-1 replication. (1) Replication starts when the virus binds to the CD4 molecule and the co-receptor (CCR5, CXCR4) and fuses with the cell membrane. (2) In the cytoplasm, the capsid is released and the viral RNA is transcribed into DNA by its own RT. (3) The viral DNA is later transported into the nucleus and integrated into the host genome. (4) Once integrated, the viral genome is transcribed by the cellular transcription machinery, and the transcripts are translated in the cells cytoplasm into precursor proteins (5), which are cleaved during assembling and budding (6) of the virus.
**Infection and immunology**

HIV-1 can be transmitted through several routes, but the most common is through unsafe vaginal or anal sex. Other routes are by transfer of blood (using unclean needles, during transplantation or transfusion and from mother to child (during pregnancy, birth or breastfeeding). The risk of acquiring HIV-1 infection during vaginal intercourse is relatively low, approximately one out of 100 exposures, but the risk depends on viral load of the infected partner and presence of other genital infections [36, 65].

The infection occurs when the virus enters the bloodstream or is transmitted over a mucosal surface. Here, the virus either directly infects or attaches to antigen-presenting cells (APCs) such as DCs or macrophages (MØ), but the virus can infect any cell that expresses both the primary receptor CD4 and any of the co-receptors CCR5 or CXCR4. DCs transport the virus to the lymph nodes, where it can infect T lymphocytes. The virus has a preference for infecting CD4⁺ T cells (especially memory T cells) and the initial infection is massive with high viral replication. This stage is called the acute phase, which can last for 2-3 months and often manifests as flulike symptoms [27]. The cell-mediated immune response against HIV-1 consists of activated CD4⁺ T cells that help CD8⁺ T cells to mature to cytotoxic T cells (CTLs). The CTLs kill HIV-1 infected cells and suppress the virus. They also secrete different chemokines (RANTES, MIP-1α/β) which have a protecting function against the HIV-1 virus. The viral load decreases to a stable level termed the viral set-point and is a good indicator of disease progression. The patient now enters the chronic phase that can last for several years (16).

The humoral immunity includes B cells and their production of antibodies (abs), which are located on or secreted by different types of B cells. Anti-HIV ab can be found a few weeks after infection and most of the abs are directed to the viral envelope proteins. The majority of produced abs often are non-neutralizing (they cannot neutralize the virus) and the development of neutralizing abs takes several months [68].

As the infection progresses, the CD4⁺ T cells are eliminated, and when the CD4 T cell count reaches below 200 cells/mm³ blood the patient develop AIDS and opportunistic infections appear (Figure 3) [16, 22]. The early destruction of memory T cells is believed to play a substantial role in the progress to AIDS.

![Figure 3. The clinical course of HIV-1 infection.](image)

When infection occurs, the CD4⁺ T cell number drops and the viral load increases. This stage lasts for weeks and is called the acute phase. The next phase is a latency stage and at this stage, the viral number decreases to a stable level (set-point). CD4⁺ T cell number declines during this stage and when reaching 200 cells/mm³, the disease is called AIDS.
HIV-1 treatment

Despite efforts, HIV-1 is still spreading and for every two people starting treatment, another five are newly infected [67].

The first promising drug, *azidothymidine* (AZT), was licensed 1989 but as we know today monotherapy against HIV-1 is inefficient due to the high mutation rate of the virus. To drugs targeting different parts of the virus is essential this type of therapy is called Highly Active Antiretroviral Therapy (HAART). The most common drugs target RT and protease but novel drugs have recently been licences that target other steps in the viral life cycle, such as integrase inhibitors and chemokine inhibitors [43]. HAART does not cure the infected individual but it can very efficiently reduce the viral load and increase the life expectancy and the quality of life for the infected person. Since the introduction of HAART, the overall mortality and viral spread have decreased significantly, especially in the developed world. Yet, the virus can survive, replicate and mutate despite all the drugs. The drugs are expensive and thus non-available in poor countries in an adequate extent. [26].

Vaccines against HIV-1

Vaccination is a process where the immune system is instructed to combat a certain pathogen (or tumor). A vaccine can be achieved in different ways and forms with the aim to produce a long-lived pathogen-specific immunity. The adaptive cellular immune response consists of CD4\(^+\) and CD8\(^+\) T cells. The humoral response is composed of B cells that generate neutralizing antibodies (Nab), which can directly inhibit the pathogen. When a vaccinated individual is exposed to the pathogen, the immune system is reactivated and able to clear the infected agent faster than if the individual is unvaccinated. Compared to therapeutic treatment vaccination is highly cost-effective [20, 38]. In the case of HIV-1, so far all the traditional vaccine strategies have been unsuccessful and all HIV-1 vaccine candidates have failed in clinical trials [3, 20]. Resistance against HIV-1 infections exist in some healthy individuals. A number of people lack the co-receptor CCR5, due to a deletion in the *CCR5* gene, which makes them immune to the virus [53]. Also in some HIV-1 infected persons, the infection can be controlled without treatment. They often have a powerful cell-mediated and humoral immunity [51, 6]. Non-Nabs are abundant in all HIV-1 infected individuals, but these non-Nabs do not kill the virus. However, some infected persons develop Nabs several months after infection during the latency stage. It has been shown that Nabs have a protective function and can reduce the progression towards AIDS [42, 68].

Live attenuated virus vaccines

Live attenuated virus vaccines contain replicating virus that have undergone a gradient loss of virulens, meaning that they no longer cause disease. The great advantage of this type of vaccines is that it elicits both strong cell-mediated and humoral immunity. This strategy is considered too dangerous for HIV-1. This was evident in a cohort of patients in Australia, where patients by mistake were given blood containing *nef*-deficient HIV-1. Initially, the patients were able to control the viremia and they did not progress to AIDS. However, the virus was able to revert to a pathogenic form and ultimately caused clinical disease and AIDS [32]. Moreover, in a non human primate study, a *nef* and *vpr* deficient SIV vaccine was evaluated but also here the virus reverted and caused the monkeys to develop AIDS [1].
Inactivated virus vaccines

In inactivated vaccines, the virus is inactivated, either chemically or by heat, before administration. It induces strong humoral responses, but only weak cell-mediated protection. The use of inactivated viruses has not shown to be protective in any study, but can be utilized ex-vivo as antigens to monitor HIV-1 responses [20, 34].

Subunit vaccines

Subunit vaccines are composed of proteins or peptides, which have been synthesized from HIV-1. Peptides are easier to design and produce, but are less immunogenic than proteins. Subunit vaccines induce mainly strong antibody (ab) response [20].

Protein vaccines have been extensively evaluated against HIV-1, especially vaccines containing the monomeric gp120 protein. In several studies, increasing CD4$^+$ T cells and neutralising antibodies (Nabs) against laboratory adapted HIV-1 strains were induced (35, 55). A larger clinical trial with a recombinant gp120 subunit vaccine including subtype B and E (AIDSVAX) was performed by the company Vaxgen, but the vaccine showed no protection from infection or reduction in viral load after infection [14, 17].

Genetic vaccines

In the beginning of the 1990s, it was shown that direct delivery of influenza genes into mice could confer protection from subsequent viral challenge [63]. So called naked genetic vaccines contain DNA or RNA from infectious agents. In the case of naked DNA vaccines, the genes are often delivered by a stable bacterial plasmid. The delivery unit is taken up by various cells, which transcribes and translates the gene into protein. Genetic immunization has some advantages over conventional vaccines. It can both induce humoral and cell-mediated immunity, it is very safe and the plasmids and insert can easily be constructed and adjusted [20, 29, 64].

Viral vectors

To enhance the immune response of DNA vaccines, viral vectors are often used for boosting responses [9]. There are viral vectors based on DNA and RNA viruses, but the most frequently used are various adenoviruses and attenuated pox-viruses such as ALVAC [61] NYVAC [24] and modified vaccinia virus Ankara (MVA) [40]. Because the Ad5 virus is prevalent in the human population, problems with anti-vector immunity arose and in the fall of 2007, a large trial failed [10, 39]. The focus now lies on modified Ad5 viruses or other, less immunogenic Adenoviruses [13].

In this study, we use the MVA as a vector vehicle. This virus belongs to the orthopoxviruses and contains a large and stable double stranded DNA genome. An advantage is that the MVA has fewer problems with anti-vector immunity than many other viral vectors [7, 23, 54, 59, 60].

Trials of genetic vaccines

In the first human clinical trial, DNA plasmids encoding Nef, Rev, Tat and Env were administrated into HIV-1 infected individuals. The vaccine was able to induce novel T cell responses [12]. DNA-priming can prime for an ab response of better quality than other vaccine modalities. This effect can be explained by that the expressed protein is correctly
folded and presented as the process is mimicking what is happening during a native infection. Together with viral vector boosting, which induces strong T cell responses that also resembles what happens during natural infections, this HIV-1 vaccine strategy has become very popular. The potential and capacity of prime-boost immunizations were first discovered in one study with the non-human macaques, which were primed with vaccinia virus expressing SIV gp160 and then boosted with recombinant SIV gp160 produced in baculo-virus infected cells [25].

A number of larger clinical trials have been performed and evaluated. After extensive research, a canarypox-based vector (ALVAC) encoding HIV-1 gp120 Gag, fragments of Gag and Pol were developed and evaluated in clinical trials, 2007. It induced both cellular and humoral responses, but the responses were overall low both for CD8+ T cells responses and abs [52]. Recently a larger clinical trial was completed. It was carried out on a large number of volunteers in Thailand, who were administered with both the viral vector ALVAC and the subunit vaccine AIDSVAX (recombinant gp120). The results were promising with a vaccine efficacy of 30% [46]. Another promising vaccine trial that got a lot of attention was the STEP study. The trial included adenovirus 5 vectors encoding HIV-1 Gag, Pol and Nef. It was administrated several times in 3000 non-infected participants, but the vaccine did not prevent the infection or lower the viral loads [10].

Enhancing vaccine potency

Adjuvants

A problem with genetic HIV-1 vaccines is that they are less immunogenic, especially in larger animals, than the more classical vaccines. Thus, there is a need for compounds that can augment vaccine potency and help to induce a strong and long-lived immune response. These compounds are called adjuvants and have been used in vaccination since early 1920s. With these enhancers, one can also direct the immune system towards a specific type of response. Moreover, the amount of antigen used and number of immunizations can be decreased if a proper adjuvant is used. The first adjuvant used was ALUM, an alum hydroxide/phosphate salt that is still employed today [41].

Our group has used the recombinant granulocyte macrophage colony-stimulating factor (rGM-CSF) as an adjuvant. This cytokine is produced by a number of cells including macrophages, activated T cells and mast cells, and the protein stimulates stem cells to produce white blood cells like granulocytes and macrophages and also stimulates professional APCs to mature. As an adjuvant, it has shown to increase T cell and ab responses in mice and non-human primates [8, 48, 49], but in humans the results have not looked good. Delivered with either HIV-1 peptides or HIV-1 DNA vaccine, the GM-CSF does not affect nor has even a negative effect on the immune response [54]. Today, only a few adjuvants are licensed for human use and include alum and certain oil-in-water emulsion adjuvants [41].

DNA vaccine delivery methods

A DNA-vaccine can be administrated in different ways. The first delivery methods used were needles and syringes and the gene gun. The latter induces cellular but predominantly humoral immune responses and the DNA, which is coated onto gold beads, is delivered by helium-propelled acceleration. Lately, novel delivery techniques have been invented and one used in our clinical vaccine trials is the Biojector. This CO2-propelled device is needle-free and the DNA is injected to target cells with the aid of a sturdy liquid flow. However, the delivery method which has shown most potential so far is electroporation [57]. Here, the DNA is
transferred to cells by electric pulses that subvert cellular membranes. The technique has shown an increased breath in immune responses and will likely be used in future clinical trials [50, 15].

HIVIS

The HIVIS trials are a collaboration between the Swedish Institute for Infectious Disease Control, Karolinska Institute, the Muhimbili University College of Health Sciences in Tanzania and the Walter Reed Army Institute for Research in the US. The phase 1 clinical trial comprises healthy volunteers from both Sweden and Tanzania, who have been administered a HIV-1 vaccine consisting of DNA prime and MVA boost. The vaccine contain seven different plasmids encoding HIV-1 Env subtype A, B and C, Gag subtype A and B, Rev and RT subtype B. The viral vector MVA has been developed by the US National Institutes of Health and the Walter Reed Army Institute of Research and contains HIV-1 Env subtype E and Gag/Pol subtype A [8] (Figure 4).

In the Swedish trial, 40 healthy volunteers between the ages 18-40, male and female, were divided into four different treatment groups. They were first primed with different amounts of DNA-vaccine by Biojector injection on days 0, 30 and 90 and two groups also received the GM-CSF adjuvant either by intradermal (ID) needle injection or intramuscular (IM) Biojector injection. Six months after the last DNA injection, the volunteers were given either a single ID HIV-1 MVA injection of 10⁷ plack forming units (pfu) or IM vaccination of 10⁸ pfu HIV-1 MVA (Figure 5). The aim of the HIVIS trials in Sweden was to study safety and immunogenicity before designing a similar trial in Tanzania. The results were successful with more than 97% HIV-1 specific cellular immune responders. Yet, only a few developed abs against HIV-1 [54].

A 2nd second MVA boost was administrated as 10⁸ pfu IM into 24 of the volunteers during summer 2009 (Figure 5).
Figure 5. Immunization schedule. DNA was injected in 40 volunteers at days 0, 30 and 90 either intradermally (ID) or intramuscularly (IM). Six months after the last DNA injection, the volunteers were given either a single ID HIV-1 MVA injection of $10^7$ plaque forming units (pfu) or IM vaccination of $10^8$ pfu HIV-1 MVA. A 2nd MVA boost was administered as $10^8$ pfu IM into 24 volunteers three years after the 1st MVA boost. Immune response tests were performed two and forty weeks after the 1st MVA and two and four weeks after the 2nd MVA boost.

AIMS

This study had the following aims:

- To detect the amount of (titer) IgG abs against Gag, Env and RT after a second boost of MVA in 23 healthy volunteers.
- To detect epitope-specific abs against gp41 after a second boost of MVA in 23 healthy volunteers
MATERIALS AND METHODS

ELISA

Indirect ELISA was carried out on plasma from 24 Swedish, participating in the HIVIS-vaccine trial. The group of volunteers consisted of male and female between the ages 18-40. Ninety-six well ELISA plates (Nunc Maxisorp, Odense, Denmark) were coated with gp160 (1.0 ug/mL, MicroGenesys Inc, Meriden CT, USA), recombinant p17/p24 (1.5 ug/mL, FIT Biotech, Tartu, Estonia), RT (1.0 ug/mL, Centre for AIDS reagents (CFAR), NIBSCH, Herts, UK) or gp41 peptides (10 ug/mL, Thermo Hybaid D-89077 Ulm Germany) from HIV-1 subtype B in 0.05 M NaCO₃ pH 9.6. Plates were incubated over night in room temperature and then stored at 4°C until further use. The ELISA-plates were washed three times with 0.9 % NaCl (Merck, Darmstadt, Germany) supplemented with 0.05 % Tween 20 (Sigma-Aldrich, Stockholm, Sweden). Plates were then blocked with 5 % fat-free milk (Semper, Sweden) in phosphate buffered saline (PBS) for one hour at 37°C. Plasma samples were diluted in 2.5 % fat-free milk in PBS, added to the plates and incubated over night at 4°C. Plates were then washed four times and rabbit anti-human IgG abs conjugated to horse radish peroxidase (HRP) (DAKO P0214, Glostrup, Denmark), diluted 1:5000 in 1.25 % fat-free milk in PBS was added. Subsequently, the plates were washed and O-phenylenediamine dihydrochloride tablets in 0.1 M NaCitrate buffer were activated by 0.3 % H₂O₂ (Sigma-Aldrich, Stockholm, Sweden) and added for development, 15-30 minutes for colour reaction. The reaction was stopped with 2.5 M H₂SO₄ and the optical density (OD) was read at 490 nm using a spectrophotometer.

Table 1. ELISA set up. ELISA plates were coated with the HIV-1 Gag, Env and RT antigens. Plasma from 23 immunized volunteers was analyzed from different time-points and in different dilutions.

<table>
<thead>
<tr>
<th>Antigen and plasma dilution</th>
<th>Baseline</th>
<th>2w post 1 MVA</th>
<th>40 w post 1 MVA</th>
<th>2 w post 2 MVA</th>
<th>4 w post 2 MVA</th>
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<tr>
<td>Env 1:100 1:200 1:400 1:800 1:1600</td>
<td>x</td>
<td>x</td>
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<td>x</td>
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<tr>
<td>Gag 1:50 1:250 1:1250 1:6250 1:31250</td>
<td>x</td>
<td>x</td>
<td>x</td>
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<tr>
<td>RT 1:40 1:80 1:160 1:320</td>
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Statistical analysis

Laboratory data were entered and analyzed in Microsoft Excel. The serological cut-off was calculated as the mean OD plus three standard deviations (SD) of the negative human samples. The endpoint titer was defined as the point where the plasma dilution drops below the cut-off. Statistical analyzes were performed using the GraphPad Prism 5.0 software. Comparison between means of two groups was performed by the non-parametric Mann-Whitney U test. A P-value of < 0.05 was considered significant.
RESULTS

HIV-1 specific abs were found in all immunized volunteers

HIV-1 specific IgG abs were detected by using indirect ELISA. Blood plasma collected at different time-points during the immunization protocol (baseline, 2 weeks post 2 MVA, 4 weeks post 2nd MVA) from 23 healthy, immunized volunteers was screened against three antigens from HIV-1: gp160Env, p24/p17Gag and RT. Originally, 24 volunteers underwent the whole immunization and received their 2nd boost with MVA, but one sample was excluded from the analysis due to lack of baseline sample. The end-point titer, which is the highest sample dilution that shows positive reactivity, was determined for each sample from each individual. The cut-off for positive reactivity was calculated as the mean optical density (OD) plus three standard deviations (SD) of the negative human samples from all the individuals.

After the 2nd MVA immunization, all volunteers developed abs against Env and Gag, but only a few responded to RT. The volunteers responded the strongest against Gag, both after the 1st and 2nd MVA (Appendix 1).

Figure 6. Endpoint titers of HIV-1 specific antibodies found in 23 healthy, Swedish volunteers immunized with a HIV-1 DNA prime-MVA boost vaccine. (a) Abs against env were detected with ELISA after a 2nd boost of MVA (n = 23). (b) Abs directed towards gag were identified already after a 1st MVA boost, (n = 11) but the number of abs increased after a 2nd MVA boost (n = 23). (c) Several volunteers developed abs against RT after a 2nd MVA boost (n = 10), but a few already had RT ab responses before MVA-boost (n = 3). The criterion for statistical significance was p < 0.05.
Env abs were not found after the 1st MVA immunization, but were readily detected after the 2nd MVA boost (Figure 6a). The end-point titer values after the 2nd MVA varied from 22 to 1723 in the volunteers. No difference was observed between titers at 2 weeks and 4 weeks after the 2nd MVA, but the reactivity was significantly higher (p < 0.0001) than both baseline reactivity and reactivity after the 1st MVA values. Half (12/23) of the individuals developed abs against Gag already after the 1st MVA boost and this was considered significant as compared to the baseline value (p < 0.0001) (Figure 6b). The 2nd MVA immunization significantly induced and increased end-point titers of Gag abs in all volunteers (p < 0.0001), resulting in end-point values varying from 165 to 8566. As was observed for the Env responses, no difference in Gag responses was seen between samples from 2 weeks and 4 weeks post 2nd MVA (Figure 6). The increase in ab-response against Env and Gag over the course of immunization protocol can be observed in figure 7a and b. Interestingly, one individual shows an opposite trend, with a decreasing titer against Gag after the second MVA boost (volunteer 12, depicted as a yellow line in Figure 7b). The 2nd MVA boost induced RT-specific ab titers in 10 out of 23 individuals and the titers after the 2nd MVA were significantly higher (p = 0.0149) than baseline values and values after the 1st MVA boost (Figure 6c). The RT-specific end-point titers after the 2nd MVA varied from 41 to 839. Three volunteers had anti-RT abs already in their baseline samples. Volunteers who developed RT abs showed an analogous trend as Env and Gag responses when RT ab responses were plotted over time (Figure 7c). Figure 8 shows the correlation between Gag and Env ab titers.

Figure 7. HIV-1 specific ab responses over time in 23 healthy, Swedish volunteers. (a) Env ab titers over time, showing ab response after a 2nd MVA boost (n = 23). (b) Gag ab levels increased after a 2nd boost of MVA (n = 23). (c) A number of volunteers (n = 10) had increased RT response after a 2nd MVA boost.
Responses according to treatment

The twenty-three volunteers were randomized into 4 groups, which were immunized according to different protocols. They received the DNA plasmids either ID or in IM, with or without the adjuvant GM-CSF. The individuals were subsequently divided further to receive the 1st MVA boost either ID or IM. To determine which delivery route that would be preferred in a future vaccine, the end-point ab titers were compared for the different groups. Individuals immunized with DNA IM developed slightly better Gag ab titers than volunteers receiving DNA ID, but the difference was not significant ($p = 0.2045$) (Figure 9a). Moreover, volunteers receiving the 1st MVA boost IM had higher Gag-specific ab titers than individuals immunized ID ($p = 0.0256$). There was no difference in env ab titers when comparing IM or ID injection (Figure 9b). Volunteers with the highest ab responses against both Env and Gag were immunized with DNA and MVA IM without any adjuvant (Figure 9).

Figure 9. Group specific Gag ab response in 23 healthy, Swedish volunteers. (a) Volunteers receiving the DNA prime IM developed a better Gag ab response than DNA ID, but was not considered significant (0.2045). Volunteers immunized with MVA IM had better Gag ab titers than individuals receiving the MVA ID ($p = 0.0256$). (b) No difference in immunization delivery strategy was seen when analyzing Env ab titers. Black spots represent volunteers who did not receive the adjuvant G-CSF. Purple dots are volunteers immunized with the adjuvant. IM = Intramuscular. ID = Intradermal. The criterion for statistical significance was $p < 0.05$. 
All 23 volunteers developed Env abs when samples were analyzed using whole gp160 protein. In order to determine the specificity of the abs, the samples were analyzed using individual peptides from the gp41 protein. ELISA plates were coated with different peptides with the length of 15 amino acids. Although the positive control samples (plasma from HIV-1 infected patients) worked in the assay, none of the volunteers had responses against any of the peptides (data not shown).
DISCUSSION

In this project I have investigated the humoral immune response in 23 healthy, Swedish volunteers who were immunized with a genetic vaccine. The individuals were first primed with DNA plasmids encoding HIV-1 antigens and thereafter boosted twice with a recombinant viral vector, MVA, also encoding HIV-1 antigens. Already after the 1st MVA boost, antibody responses against Gag were observed in some individuals but no responses against Env were detected [54]. This current project focused on the ab responses after the second boost with MVA. One of the major aims of a HIV-1 vaccine is to induce B cells that produce broadly Nabs. This has been shown to be very difficult as the most efficient abs are directed only against a few conserved epitopes on the surface protein (Env) of the virus, and these epitopes are in various ways shielded from access by abs. It is thus important that a vaccine can induce Nabs against Env, but so far all vaccine-attempts have failed to achieve this [11, 58, 68]. Our vaccinees required two boosts of MVA to induce binding abs against Env and, as compared to what is observed in infected individuals, the titers were moderate. The presentation of HIV-1 is of course different in vaccinated and infected individuals and the amount of antigen present after vaccination is much less than during an infection. Vaccinated people receive HIV-1 DNA, which is transcribed and translated in our cells into proteins that is in turn introduced to our immune cells. One of the major problems with developing a vaccine against HIV-1 is the great variability of the virus. The viral enzyme RT lacks proof-reading functions and this results in extremely high variation in Env amino acid sequence, which in turn leads to escape from the immune system. However, the virus has the conserved regions in the envelope but these epitopes are very well concealed from the immune system by various mechanisms. The env protein is heavily glycosylated [66] and folds in a way which conformationally masks the potentially neutralization sensitive epitopes (conformational masking) [30]. This poses two problems, the first being how a vaccine construct should be designed in order to be able to induce abs against these epitopes and, secondly, even if you can induce the “right” ab with a vaccine the epitope on the real virus will be masked.

Both Gag and RT abs cannot neutralize the HIV-1 virus, but we analyzed the Gag and RT ab responses to analyze the immunogenicity of the vaccine. Gag-specific ab responses increased after, and were detected, in all volunteers following the 2nd boost of MVA. A few vaccinees also developed RT-specific ab responses, and interestingly, three of the vaccinees already had abs in the baseline sample. As the RT is relatively conserved between different retroviruses, this reactivity is most likely due to exposure to another retrovirus or from retrotransposons found in our own genome [5]. The reactivity could, however, be boosted by the HIV-1 vaccine.

Group specific responses did not relieve the best vaccination delivery method

The volunteers were vaccinated differently to investigate the optimal delivery strategy. Earlier results have shown that volunteers immunized with DNA ID were able to induce similar immune responses as those vaccinated IM. Addition of the adjuvant GM-CSF seemed to reduce the responses when delivered ID together with the vaccine. It has also been shown that the first MVA boost were more immunogenic when injected IM than ID [54]. Only 23 vaccinees received all immunization, resulting in too few individuals/group to make a proper comparison between groups. Thus, no solid conclusion can be made regarding the optimal route of delivery. However, the vaccinees in the group receiving both the DNA and MVA IM, without GM-CSF, seem to respond slightly better than the other, although the difference is not statistically significant.
Gp41 mapping

The gp160Env protein is divided into gp120 and gp41 after translation and both proteins contain several conserved regions that is potential targets for Nabs. In order to determine to which epitopes the abs in the vaccinees were directed, a peptide ELISA was performed using peptides covering the entire gp41. However, no reactivity in any of the samples could be detected against any of the peptides. The abs bound to the whole gp160 protein, but not to the 15 amino acid long sequences of the gp41 protein, possibly indicating that the majority of responses are directed against conformational epitopes and cannot bind to the linear epitopes represented by the peptides. We cannot be certain that this is the case but it is desirable to induce responses against conformational epitopes as this indicates that the abs will bind the native virus.

Future aspects

The volunteers will out of practical reasons not receive a 3rd MVA boost, but it would be interesting to see if such a boost could increase the titers.

In the HIVIS-study, both needle injection and the Biojector system have been used when delivering the vaccine. There will probably be a new trial set up further on using a different and new approach including a new delivery method. A HIVIS program is also running in Tanzania and this program includes 60 healthy uninfected volunteers. The program has been successful when it comes to immunogenicity and safety and a new trial with a larger group of volunteers will start during 2010 [2].

The experience of existing vaccines and extensive studies of the virus, suggests that an effective vaccine will have to induce both humoral and cellular immunity. When designing a vaccine that should elicit Nabs there are a few things that need to be considered: The abs seem to be conformational sensitive and the immunogens should consequently not consist of peptides. The immunogen should be constructed in a way that would induce abs against the conserved, immunogenic regions of Env. Moreover, it would be beneficial to induce abs that interfere with the required conformational changes in gp41 and thus prevent the fusion of virus and cellular membrane. Possibly, it is better to concentrate the humoral response against a few neutralizing epitopes rather than to use big molecules containing many epitopes.

In the wait for a vaccine, it is highly important that other preventive and therapeutic means are implemented. For instance, condom use, male circumcision [45], access to HAART, education, strengthening of women’s rights and the fight against stigmatisation of HIV-1 infected persons should be the top priorities in developing countries. These are matters that will be important for stopping the epidemic, even if a protective vaccine is developed.
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Andreas, my family and all my friends, I could not have done this work without your support!!!
REFERENCES


### Appendix 1

Table 2. Endpoint titers in volunteers at different time-points.

<table>
<thead>
<tr>
<th>Volunteer</th>
<th>Baseline (RT/Gag/Env)</th>
<th>2 w post 1 MVA (RT/Gag/Env)</th>
<th>40 w post 1 MVA (RT/Gag/Env)</th>
<th>2 w post 2 MVA (RT/Gag/Env)</th>
<th>4 w post 2 MVA (RT/Gag/Env)</th>
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