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Co-expression of HIV-1 virus-like particles and granulocyte-macrophage colony stimulating factor by GEO-D03 DNA vaccine

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Here, we report on GEO-D03, a DNA vaccine that co-expresses non-infectious HIV-1 virus-like particles (VLPs) and the human cytokine, granulocyte-macrophage colony-stimulating factor (GM-CSF). The virus-like particles display the native gp160 form of the HIV-1 Envelope glycoprotein (Env) and are designed to elicit antibody against the natural form of Env on virus and virus-infected cells. The DNA-expressed HIV Gag, Pol and Env proteins also have the potential to elicit virus-specific CD4 and CD8 T cells. The purpose of the co-expressed GM-CSF is to target a cytokine that recruits, expands and differentiates macrophages and dendritic cells to the site of VLP expression. The GEO-D03 DNA vaccine is currently entered into human trials as a prime for a recombinant modified vaccinia Ankara (MVA) boost5 and is currently undergoing phase 2a testing in humans. The GEO-D03 DNA vaccine is a 9.5 kb plasmid DNA composed of a 2.9 kb expression vector termed pGA2 and a 6.6 kb vaccine insert termed JS7. The JS7 vaccine insert expresses the HIV-1 proteins Gag, protease (PR), reverse transcriptase (RT), gp160 Env, Tat, Vpu, and Rev from a single transcript that undergoes subgenomic splicing in mammalian cells.6 The subgenomic splicing is rev-dependent and uses the rev responsive element and splicing signals present in the wild type HIV-1 genome. The VLP have been rendered noninfectious by deletions as well as point mutations.6,7 The deleted regions include both long-terminal repeats, a portion of the 5’ sequences encoding encapsidation of viral RNA, and coding sequences for integrase, Vif, Vpr, and Nef. Point mutations reduce packaging of viral RNA and inactivate the viral PR, RT, strand transfer, and RNase H activities encoded in Pol. Expression of GM-CSF in JS7 in the position of Nef was achieved using a synthetic sequence and standard recombinant DNA technology.

Electron micrographs of 293T cells transiently transfected with GEO-D03 DNA revealed the anticipated expression of immature virus-like particles (Fig. 1). The VLPs were observed budding from cells and adjacent to cells. Immunogold staining for the presence of Env using a mixture of 4 rabbit monoclonals to gp120 revealed the presence of Env on the surface of the particles (see arrow heads, Fig. 1).

Like HIV-1 proviral DNA, the GEO-D03 DNA vaccine expresses multiple proteins from a single transcript through alternative splicing and alternate reading frames (Fig. 2A). Full-length RNA is spliced into two subgenomic classes of mRNAs: 4.0-kb, and 1.8-kb.8 Because of the presence of multiple closely spaced splice donor and acceptor sites, subgenomic mRNAs are grouped into size classes. Based on the deletions and putative splice junctions in the GEO-D03 sequence, we predicted full length mRNA at 7.3kb and subgenomic mRNAs at -3.4 kb, and -1.4 kb. If processed correctly, the full-length 7.3 kb mRNA expresses Gag-Pol, the 3.4 kb subclass mRNA expresses Vpu and Env, and the
of fragments of gag or JS7 DNA. Probes were generated through PCR amplification of RNA purified from HEK293T cells transfected with GEO-D03 mRNAs. Splicing patterns were determined in total RNA purified from HEK293T cells transfected with GEO-D03 consistent with the predicted splicing pattern where the 3' end of their mRNA. However, all of the subclasses of mRNA include GM-CSF 1.8 kb subclass mRNA expresses Tat, Rev, and GM-CSF. Furthermore, the blots show that GEO-D03, but not JS7, expresses full-length Gag and Env proteins (Fig. 3A).

Northern blot analyses revealed the correct transcription and splicing of the GEO-D03 message to create properly-processed mRNAs (Fig. 2B). Splicing patterns were determined in total RNA purified from HEK293T cells transfected with GEO-D03 or JS7 DNA. Probes were generated through PCR amplification of fragments of gag and gm-csf. On the Gag blot, the gag sequence was detected at approximately 7.3 kb in cells transfected with GEO-D03 and at approximately 6.9 kb in cells transfected with pGA2/JS7 (data not shown). This result is consistent with the predicted splicing patterns (Fig. 2A), in which the gag sequence is present in the full-length mRNA but spliced out of the 3.4- and 1.8-kb classes. On the GM-CSF blot, the GM-CSF sequence was detected in all three classes of mRNA from cells transfected with GEO-D03 consistent with the predicted splicing pattern (Fig. 2A), in which the gm-csf sequence is present in all classes of mRNA. The gm-csf sequence was not detected in RNA from cells transfected with JS7 DNA (Fig. 2B).

Western blots demonstrated that GEO-D03, like its precursor JS7, expresses full-length Gag and Env proteins (Fig. 3A and B). Furthermore, the blots show that GEO-D03, but not JS7, expresses full-length GM-CSF, which was glycosylated as expected (Fig. 3C). Western blot analyses were performed on lysates and supernatants of cells transfected with GEO-D03 DNA, JS7 DNA and mock-transfected cells. In the Gag blot, the full-length pr55 protein is clearly present in lysates and supernatants from cells transfected with pGA2/JS7 and GEO-D03 DNA vaccines. Similarly, the Env blot demonstrated expression of the full-length gp160 protein as well as cleavage of gp160 to the gp120 form. As expected, the gp120 band is more prominent in supernatants than in lysates, indicating greater processing of secreted protein. Migration patterns are similar from both plasmids; therefore, addition of the GM-CSF sequence to the JS7 plasmid did not affect the ability to express full-length Gag and Env proteins.

In the GEO-D03 lanes of the GM-CSF blot, expression bands are present at approximately 30 kDa in lysates and at approximately 36 kDa in supernatants, whereas the E. coli-derived GM-CSF has an approximate molecular weight of 17 kDa. The molecular weight of the E. coli-derived GM-CSF corresponds well with the predicted mass of 16.2 kDa based on its primary sequence. The much higher molecular weights of the proteins from transfected cells are due to extensive glycosylation of GM-CSF in mammalian cells. GM-CSF expression was not seen in lanes from cells transfected with pGA2/JS7.

In addition to the western blot analysis to confirm expression of full-length Gag, Env, and GM-CSF proteins, we performed ELISA analyses to quantify expression of these proteins (Fig. 4). The ELISA data demonstrate that GEO-D03 expresses Gag and Env proteins in quantities that are 60–70% of the quantities expressed by pGA2/JS7. We hypothesize that the lower expression of Gag and Env from GEO-D03 is due to the subgenomic splicing of its mRNA including the formation of the 1.4 kb GM-CSF mRNA. The data also indicate that GM-CSF is expressed at approximately 200 nanograms per 10^6 cells, per day, a level that has proven effective as an adjuvant for protection in our studies in macaques. As expected, the Gag and Env proteins were detected in lysates and supernatants from cells transfected with pGA2/JS7 and GEO-D03, whereas GM-CSF was detected only in lysates and supernatants from cells transfected with GEO-D03. While Env is present in greater quantities in lysates than in supernatants, Gag and GM-CSF were present at higher levels in supernatants than in lysates. The ratio of secreted-to-intracellular protein is especially high for GM-CSF. Exact molar quantities of the proteins cannot be calculated accurately due to the unknown affinity of the kit antibodies for the vaccine-expressed proteins; however, general conclusions may be drawn. The calculated quantity of gp120 Env protein was approximately four to five times as high as the calculated quantity of p24 Gag protein. Because of the higher molecular weight of Env relative to Gag, the molar ratio of Env to Gag is likely closer to one.

GEO-D03 vaccine DNA, used as a prime for an MVA boost, was first inoculated into humans on May 18th 2012, in a
dose-escalation study through the HIV Vaccine Trials Network. The doses of DNA are the same as those used in the dose escalation of its parent plasmid pGA2/JS2. The 1/10th dose is 0.3 mg, and full dose is 3 mg. All inoculations are intramuscular with needle and syringe.

In the non-human primate model, priming with an SIV prototype of the GEO-D03 DNA and boosting with MVA has proven particularly effective at eliciting protective immunity. With the GM-CSF co-expressing DNA prime, the vaccine achieved a 90% reduction in risk of infection with 70% of the macaques protected against 12 weekly rectal challenges. In the absence of the co-expressed GM-CSF, the vaccine achieved a 61% reduction in the relative risk of infection with 25% of the macaques protected against infection during the 12 weekly exposures. Co-expression of GM-CSF in the DNA prime enhanced neutralizing antibody titers, antibody-dependent cellular cytotoxicity and the avidity of Env-specific antibody. Use of GM-CSF also enhanced the frequency of detection of viral-specific IgA in rectal secretions. Of these parameters, the avidity of Env-specific antibody for the Env of the challenge virus, an overall measure of the quality of the Ab response for the Env of the challenge virus, correlated with prevention of infection \((r = 0.9, p = < 0.0001)\). Neutralizing antibody, antibody-dependent cellular cytotoxicity and viral-specific rectal IgA did not correlate with protection. However, the individual Envs used in these latter tests may not have accurately mimicked the Envs undergoing transmission.

We believe that GEO-D03 effectively primes protective immunity due largely to the presence of two features: the expression of VLP displaying full length Env and the co-expression of GM-CSF. Virus-like particles have been found effective as vaccines, presumably because VLP mimic viruses in their natural form. We also consider display of the native trimeric gp160 form of Env on the surface of the VLPs to be important. We have found that priming and boosting with MVA that expresses VLPs displaying a truncated trimeric gp150 Env elicits higher titer and higher avidity Ab than priming with DNA that expresses gp160 Env and boosting with MVA that expresses gp150 Env. Disappointingly, the avidity of this Ab did not correlate with prevention of infection, a phenomenon that we believe reflects the trimeric gp150 Env failing to elicit Ab against the native gp160 structure of Env. The C-terminal complete gp160 Env is toxic to cells and difficult to express in live recombinant vaccine vectors. However it is possible to express gp160 Env using recombinant DNA. Thus, the DNA prime affords a unique opportunity to express VLPs that display the complete gp160 form of Env.

We also consider the ability of GEO-D03 to co-express GM-CSF at the site where VLPs are being presented to the immune system important. GM-CSF stimulates the production and differentiation of monocytes into dendritic cells and macrophages. In turn, dendritic cells and macrophages initiate adaptive immune responses (for review, see 10). GM-CSF is licensed for human use for acceleration of white blood cell recovery following bone marrow transplantation, for treatment of fungal infections, for replacement of white blood cells following chemotherapy and, most recently, for use in a fusion protein as an adjuvant for a dendritic cell therapy for prostate cancer. GM-CSF has been broadly used in preclinical vaccine studies. It was the first reported “genetic adjuvant” for DNA vaccines. GM-CSF stood out in early preclinical screens of retroviral expressed cytokines.
as an adjuvant for cancer cell vaccines, and can serve as an effective adjuvant for immunizations with peptides.

There are also caveats for the use of GM-CSF as a vaccine adjuvant. The level of GM-CSF expression is important. If GM-CSF is present at excessive levels, it expands myeloid suppressor cells that dampen adaptive immune responses. The context in which GM-CSF is expressed is also important for eliciting protective immunity for immunodeficiency virus infections. GM-CSF expressed in recombinant vesicular stomatitis SIV vaccine and recombinant MVA vaccine simian immunodeficiency virus vaccines have not enhanced protection in preclinical macaque studies (ref. 19 and Amara and Robinson, unpublished observations).

In summary, we are pleased to report the construction and expression testing of GEO-D03, a DNA prime that co-expresses GM-CSF and virus-like particles. The goal of our recently initiated human trials is to determine whether priming with GEO-D03 DNA and boosting with MVA will achieve the excellent protection in humans that was achieved by its SIV-prototype in macaques.

Disclosure of Potential Conflicts of Interest
M.H, Y.X. and H.R. are employees of GeoVax, Inc., a company developing GEO-D03 as a prime for a commercial DNA/MVA HIV vaccine.

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