Comparison of the immunological memory after DNA vaccination and protein vaccination against anthrax in sheep

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Abstract
Currently available live spore vaccines against anthrax in animals have many drawbacks, one of which is their presumed inability to induce a long lasting immunity. In the present study we compared the immunological memory after a protein vaccination with DNA vaccinations in sheep. The antigen used was the protective antigen (PA83) of Bacillus anthracis. Sheep were vaccinated three times with either PA83 plus alhydrogel, or with one of four different plasmid DNA formulations, which all encoded either the full-length PA83 or its domain 4. Two pDNA formulations included Vaxfectin™ adjuvant, the other two were injected in PBS without adjuvant. Initially, the antibody titres of protein vaccinated sheep were significantly higher than the titres of pDNA vaccinated sheep. After 5 months, however, the antibody titres of protein vaccinated sheep had dropped remarkably, while the titres of all four pDNA vaccinated groups were either stable or had increased. Humoral responses of sheep immunised with pDNA formulated with Vaxfectin™ adjuvant were higher than the responses of the corresponding groups that received pDNA in PBS only.

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1. Introduction
Animal vaccines against anthrax are typically live spore vaccines, which have a number of disadvantages. They retain residual virulence, their efficacy and unwanted side effects can be greatly influenced by small variations during production, and the duration of protectivity is limited, which makes yearly booster vaccinations a requirement [1]. Prospective new vaccines should thus be safe, well defined, and able to induce a long-lasting immunity. The protective antigen (PA83) of Bacillus anthracis is known to confer immunity against anthrax. In the present study we compared the long-term changes of antigen-specific titres in sheep vaccinated with either recombinant PA83 plus adjuvant, or immunised with various plasmid DNA formulations. Three different plasmids were used for immunisation: the first encoded the full length PA83, fused to a secretion signal for the eucaryotic target cell (pSecTag PA83). The second plasmid, also encoding PA83, had a targeting and an anchor sequence for the endoplasmic reticulum (pCMV/ER PA83). The third plasmid encoded only domain 4 of PA83, with a sequence optimised for mouse codon usage and fused to parts of the mouse invariant chain for improved MHC II presentation (pVAX D4 ivc). The domain 4 is responsible for host cell binding of the anthrax toxin complex, and therefore, essential for toxin activity. Antibodies raised against this binding domain are thought to have a great potential to neutralize anthrax toxin [2].

2. Materials and methods
2.1. Plasmids
The plasmids used for vaccination were pSecTag PA83 and pCMV/ER PA83, both encoding the full-length B. anthracis PA [3], and the plasmid pVAXD4ivc. The plasmid pVAXD4ivc was cloned as follows: the DNA sequence encoding domain 4 of PA (amino acid 596–735) was resynthesised with eucaryotic codon usage (Medigenomix, Munich, Germany). This DNA fragment was recovered as a restric-
tion fragment. The DNA sequence of the mouse invariant chain was provided by Dr. N. Koch, University of Bonn. Its MHC II targeting sequence, as well as the sequence for the transmembrane anchor were amplified using the primers 5′-AAGCTAGCATGGATGACCAACGCGACCTC-3′ and 5′-TATAAAGCTCATGGTGAAGGCTTCCAGGTC-3′, and then ligated to the N-terminal end of the domain 4 sequence. This fusion construct was cloned into the vector pVAX (Invitrogen, Karlsruhe, Germany). The resulting plasmid pVAXd4ivc leads to improved MHC II presentation of the encoded antigen. Plasmid DNA used for immunisation was purified using endotoxin-free isolation kits (Macherey-Nagel, Dueren, Germany). The endotoxin content of the preparations was tested using a Limulus amoebocyte-lysate assay (Charles River Germany, Sulzfeld, Germany).

2.2. Immunisations

Each DNA immunisation included the PA-encoding plasmid with the secretion signal (pSecTag PA83) in combination with either the ER-targeted construct (pCMV/ER PA83), or the domain 4 with the MHC II targeting signal (pVAXd4ivc). All sheep were age-matched, female Merino, immunised intramuscularly three times in week 0, 3, and 7. In the protein vaccine group each animal received 25 μg rPA83 adsorbed to Alhydrogel (Brenntag Biosector, Frederikssund, Denmark) per immunisation. All DNA vaccine groups received a total of 1 mg of above mentioned plasmid combinations, either complexed with 0.75 μmol of a cationic lipid adjuvant (Vaxfectin™ adjuvant, by courtesy of Vical, San Diego, CA) or just naked pDNA in PBS. Sheep were bled in week 3, 7, and 11, and additionally 5 months, and 11 months after the last immunisation.

2.3. Immune assays

Individual sheep serum samples were analysed for PA-specific immunoglobulin G (IgG). ELISA endpoint titres were determined as previously described [3] with a polyclonal rabbit anti-sheep IgG-peroxidase conjugate (Acris Antibodies, Hiddenhausen, Germany) as the secondary antibody. Eleven months after the final immunisation, lymphocytes were isolated from each sheep and stimulated in vitro with either rPA83 or ConA (positive controls). The cells were plated into round-bottom 96-well plates at a density of approximately 1 x 10⁶ cells per well. The medium used consisted of DMEM RPMI 1640 (mixed 1:1) with 10% fetal calf serum, 50 μM β-mercaptoethanol, 50 μU/ml penicillin, 100 μg/ml streptomycin, and 0.2 μg/ml amphotericin B (all from Biochrom, Berlin, Germany). Either 1 μg/ml rPA83 or 0.5 μg/ml well ConA (Sigma, Taufkirchen, Germany) were added and the lymphocytes incubated at 37°C and 5% CO₂. Cells receiving medium only served as negative controls. Each treatment was done in quadruplicates. After 72 h of in vitro stimulation we determined lymphocyte proliferation using a 5-bromo-2′-deoxy-uridine labelling and detection kit (Roche, Mannheim, Germany). ConA stimulation was assumed to represent maximum activation (100%), whereas cells with only media represented no activation. Media control values were subtracted from all data and the values for cells stimulated with PA83 were then expressed relative to ConA stimulation.

3. Results and discussion

After the first immunisation the humoral immune response of the protein vaccinated sheep was significantly higher than the DNA vaccinated groups. It continued to be higher until at least week 11 (see Fig. 1). However, 5 months after the final immunisation the titres of the protein-vaccinated group had dropped considerably, and after 11 months the titres were even lower than the most effective DNA vaccine group. The antigen-specific titres of DNA vaccinated groups did not reach the peak level of the titres of the protein vaccinated group, however, they were much more stable (see Fig. 1; 5 and 11 month data). Humoral immune responses in sheep vaccinated with pDNA formulated with Vaxfectin™ adjuvant were higher than the corresponding groups that received pDNA in PBS. The plasmid combination including the optimised sequence of domain 4, fused to the targeting signal containing part of the invariant chain, led to higher PA83-specific titres determined by ELISA. Each bar represents the geometric mean titre of individual sera from a group (n = 3; error bars= S.E.). After 11 months 3 age-matched naive sheep were also bled, to serve as negative controls.

Fig. 1. ELISA titres of individual sheep serum samples. All sheep were bled several weeks after each of the three vaccinations (week 3, 7, and 11) and additionally 5 months, and 11 months after the final immunisation. Antigen (PA83)-specific titres were determined by ELISA. Each bar represents the geometric mean titre of individual sera from a group (n = 3; error bars= S.E.). After 11 months 3 age-matched naive sheep were also bled, to serve as negative controls.
specific titres than the plasmid combination with the two full-length PA83 encoding plasmids. Despite the fact that the humoral responses of 3 of the DNA vaccine groups were back to non-immunised levels after 11 months (Fig. 1), all 5 vaccine groups did show antigen-specific lymphocyte proliferation at this time (see Fig. 2). Lymphocytes of naïve control sheep did not proliferate in response to rPA83. There were neither significant differences in the cellular immune responses between the different treatment groups, nor did the ELISA titres of a group correlate with the magnitude of the cellular response.

DNA immunisation against anthrax has previously been successfully tested in mice and rabbits [3–6], however, this is the first report of immunogenicity of a DNA anthrax vaccine in a large animal model such as the sheep.

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References