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Influenza virus-like particles as an antigen-carrier platform for the ESAT-6 epitope of *Mycobacterium tuberculosis*

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Abstract

Various virus-like particles (VLPs) have been shown to induce cytotoxic T-cell immune response as well as B-cell immune response. This makes VLPs promising candidates for antigen-carrier platforms for various epitopes. Influenza A VLPs were produced displaying a 20 amino acid sequence from *Mycobacterium tuberculosis* early secretory antigenic target 6 protein (ESAT-6). As this sequence is known to comprise a potent T-cell epitope it was chosen as a model for a foreign epitope to be presented on an influenza VLP scaffold. The ESAT-6 epitope was engineered into the antigenic region B of the influenza hemagglutinin (HA) from strain A/New Caledonia/20/99. VLPs were expressed in insect cells and subjected to immunization studies in mice. High serum antibody titers detected against recombinant ESAT-6 demonstrated the feasibility of influenza A VLPs serving as an efficient platform for epitope presentation.

Keywords

Influenza A; Virus-like particles; *Mycobacterium tuberculosis*; Surface display; Baculovirus expression system

1. Introduction

The most common expression systems for surface display include *E. coli* filamentous phages, *E. coli*, yeast and the baculovirus insect cell system (Arap, 2005; Becker et al., 2004; Ernst et al., 1998; Grabherr et al., 1997; Lee et al., 2003; Mäkelä and Oker-Blom, 2008; Pepper et al., 2008). These systems exploit the fact that replication competent organisms are able to display foreign peptides or proteins of interest on their surface making them accessible to protein interaction studies. Library screening strategies and in vitro evolution concepts have been described and applied successfully (Hosse et al., 2006; Lee et

al., 2003; Mäkelä and Oker-Blom, 2008; Pepper et al., 2008; Rothe et al., 2006a,b). Surface display of proteins or peptides usually serves the purpose of interacting with a substrate or target protein, and the applications thereof are manifold. For vaccine design based on the mere presentation of the antigen to the immune system, replication competence of the particle is not required, and sometimes even unwanted. Sub-unit vaccines and virus-like particles (VLPs) provide an attractive alternative to whole virus vaccines as they are safe and easy to produce on a large scale. They consist of viral structural proteins that, when over-expressed, spontaneously self-assemble into particles. To date, different VLPs, including hepatitis B VLPs, human papilloma VLPs, HI-VLPs, parvovirus-like particles, and SV40-like particles have been shown to display foreign epitopes and peptides efficiently on their surface (Amexis and Young, 2006; Dale et al., 2002; Deml et al., 2005; Doan et al., 2005; Jegerlehner et al., 2002; Notka et al., 1999; Pumpens and Grens, 2001; Sedlik et al., 1997; Slupetzky et al., 2001; Takahashi et al., 2008). Others have shown the presentation of chemical linked antigens on rabbit haemorrhagic disease VLP surface and the incorporation of whole bacterial proteins into influenza VLPs (Peacey et al., 2007; Wang et al., 2008). A strategy to incorporate relevant epitopes of, e.g. bacterial pathogens into the surface of highly antigenic nano-particles is attractive, as it allows for designing stable, safe and effective vaccines (Ludwig and Wagner, 2007; Ruedl et al., 2005). Bacterial surface proteins are sometimes difficult to produce as they often derive from gram-positive bacteria and tend to form inclusion bodies in *E. coli* (Straume et al., 2006). However, once antigenic sites become known, specific epitopes or domains can be presented to the immune system in the form of a synthetic peptide or in context with antigenic proteins or particles.

In the approach described below influenza A VLPs were chosen as the scaffold, a tuberculosis specific antigenic epitope to be displayed, and the baculovirus insect cell expression system for production. Influenza A VLPs have been generated previously in various expression systems such as mammalian cells, plants and insect cells by co-expression of hemagglutinin (HA), matrix protein 1 (M1), matrix protein 2 (M2) and neuraminidase (NA), HA, M1 and NA, or just HA and M1 (Bright et al., 2007; Cox, 2008; D'Aoust et al., 2008; Galarza et al., 2005; Krammer et al., 2010; Latham and Galarza, 2001; Mahmood et al., 2008; Perrone et al., 2009; Pushko et al., 2005; Quan et al., 2007, 2008; Ross et al., 2009; Szécsi et al., 2006). HA and NA are surface glycoproteins and major antigens of influenza virus which are incorporated into the viral membrane and that are responsible for the infection of host cells and the release of progeny viruses, respectively (Nayak et al., 2009). The M1 protein covers the inner surface of the viral membrane and has been shown to be crucial for the formation of virus particles in insect cells (Gómez-Puertas et al., 2000). In order to present a foreign epitope on the surface of influenza A VLPs, the corresponding peptide was incorporated into the HA. This approach has been demonstrated previously to result in well-exposed peptides, when the insertion was placed into the so-called antigenic region B (Ernst et al., 1998; Li et al., 1993; Muster et al., 1994). The epitope of choice for this study was the N-terminal region (amino acid 1-20) of the early secretory antigenic target 6 protein (ESAT-6), a 6 kDa protein secreted by *Mycobacterium tuberculosis*. Tuberculosis remains one of the most important infectious diseases of humans and animals. The current vaccine, *Mycobacterium bovis* Bacille Calmette-Gueïrin (BCG) has variable efficacy in humans and cattle, and as a result, there is urgent need for a new

vaccine (Fine, 1995). Recently, *M. tuberculosis* specific antigens, such as ESAT-6, have been applied successfully in clinical studies, e.g. as a DNA vaccine or in liposomal formulations (Kirby et al., 2008; Liang et al., 2008; Xu et al., 2008). Further, recombinant live attenuated influenza A viruses expressing ESAT-6 were able to induce immune protection in mice very efficiently (Sereinig et al., 2006). ESAT-6 is a known antigenic target as it comprises the H-2^b-restricted CD4⁺ T-cell epitope (Brandt et al., 1996). Hence, VLPs displaying the ESAT-6 epitope on their surface are expected to elicit an immune response against tuberculosis in vaccinees. Insect cells as expression system, in particular BTI-TN5B1-4 cells (High Five™) and Sf9 cells, have been proven to be efficient and feasible for the production of therapeutic proteins, VLPs and gene therapy using, for example, adeno-associated virus (Cox, 2008; Dormond et al., 2009; Fotouhi et al., 2008; Treanor et al., 2007; Wu et al., 2008). The introduction into the market of a human papillomavirus vaccine (Cervarix™) demonstrated the value of insect cell derived vaccines and therapy (Palmer et al., 2009).

2. Materials and methods

2.1. Cells and viruses

Spodoptera frugiperda Sf9 cells (ATCC# CRL-1711) were maintained as adherent cultures in Roux flasks in modified IPL-41 media (supplemented with lipid mixture (Sigma, St. Louis, USA) and Yeast Extract (Sigma)) and 3% FCS at 27 °C (Wickham et al., 1992). *Trichoplusia ni* BTI-TN5B1-4 cells (ATCC# CRL-10859) were maintained in Erlenmeyer flasks in serum free modified IPL-41 media at 27 °C shaking at 100 rpm (Hink, 1970; Wickham et al., 1992). Influenza A/New Caledonia/20/1999 was grown on Vero cells (ATCC# CCL-81) in Dulbecco's modified Eagles medium/Hams F12 medium.

2.2. Cloning and recombinant baculovirus generation

The A/New Caledonia/20/1999 HA (GenBank: CY031336.1) was obtained by TRIZOL™ extraction (Invitrogen, Carlsbad, USA) and reverse transcription from the supernatant of infected Vero cells. The sequence was amplified by PCR by the use of primers 5'atgatgatggatccATGAAAGCAAACACTACTGGTCCTGTTATGTACATTTACAG and 3'atgatgatgctcgagttaTCAGATGCATATTCTACACTGCAAAGACCCATTG, the product was digested by BamHI and XhoI restriction endonucleases and ligated into BamHI and XhoI digested pBacPAK8 (Clontech, CA, USA) resulting in pBacPAK8-HA.

The sequence coding for the first 20 amino acids of the ESAT-6 protein (NCBI Reference Sequence: ZP_03534457.1) was engineered into the HA gene sequence after nucleotide position 513 (equivalent to insertion behind amino acid position 171, Fig. 1) by inverse PCR of pBacPAK8-HA using primers 5'-

ATCGAGGCCGCGGCAAGCGCAATCCAGGGAGGTTTGTACCCAAACCTGAGCAA
GTCTTATGTAAAC and 3'-

ACCCGCGAAATTCCACTGCTGCTCTGTCATATTCTTCCCCGTCAGCCATAGCAAA
TTTCTG. The PCR product was self-ligated and transformed into competent *E. coli* JM109 bacteria resulting in pBacPAK8-ESAT6-HA.

The M1 gene from strain A/Udorn/307/1972 (H3N2) (GenBank: DQ508932.1) was synthesized by Sloning (Puchheim, Germany). The synthesis vector pPCR-M1 was digested by the restriction endonucleases XhoI and NotI. The M1 fragment was ligated into an XhoI and NotI digested pBacPAK8 resulting in pBacPAK8-UM. The green fluorescent protein (GFP) gene was ligated into a HindIII and XhoI digested pBac-5 (Merck, Darmstadt, Germany) resulting in pBac-5-GFP.

Recombinant baculoviruses were generated by co-transfection of pBacPAK8-HA, pBacPAK8-ESAT6-HA, pBacPAK8-UM or pBac-5-GFP together with Baculogold AcNPV linearized DNA (BD Bioscience, San Jose, USA) into Sf9 cells by using Cellfectin (Invitrogen) according to the manufacturer's instructions.

2.3. Production and purification of VLPs

BTI-TN5B1-4 cells were co-infected with recombinant baculovirus (rBV) expressing M1 and ESAT6-HA or HA at a multiplicity of infection (MOI) of 10 and a cell density of 1×10^6 cells/ml in Erlenmeyer flasks (Krammer et al., 2010). Cells were harvested 72 h post-infection and separated from supernatant by low speed centrifugation for 10 min at $2000 \times g$ at room temperature. Supernatant were tested for VLP content by hemagglutination assay and for baculovirus titer by plaque assay on Sf9 cells (King et al., 2007). VLPs were pelleted by ultracentrifugation at $136,000 \times g$ at 20 °C for 90 min. Pellets were resuspended in phosphate-buffered saline (PBS) for 1 h at room temperature. VLPs were further purified by discontinuous sucrose density gradient centrifugation (20–30–40–50–60%) at $190,000 \times g$ at 4 °C for 16 h. Gradients were split into fractions which were analysed by SDS-PAGE, Western Blot and protein quantitation with Bradford reagent. The three fractions containing most of the VLP material were diluted with PBS and were pelleted by ultracentrifugation at 4 °C at $136,000 \times g$ for 90 min. The pellets were resuspended in SPGN buffer (6%, w/v, sucrose, 3.8 mM KH₂PO₄, 7.2 mM K₂HPO₄, 4.9 mM L-glutamic acid, 75 mM NaCl, pH 7.5) and tested for VLP content by hemagglutination assay and for baculovirus titer by plaque assay on Sf9 cells. An rBV expressing GFP as negative control for animal testing was produced by three-step ultracentrifugation from similar gradient fractions in exactly the same way as VLPs.

2.4. SDS-PAGE and Western blots

Sucrose gradient fraction, infection supernatants and vaccination preparation were examined by SDS-PAGE and Coomassie staining (Laemmli, 1970). Western Blot analysis was performed using anti-M1 (#290, Institute of Virology, Bratislava), anti-ESAT-6 (HYB 076-08, Santa Cruz Biotech, Santa Cruz, California), anti-HA (#3AH1 MAb ln134, HyTest, Turku, Finland) antibodies and a secondary anti-mouse IgG alkaline phosphatase labelled goat antibody (Sigma, #A1047). Quantitation by Coomassie stained SDS-PAGE was undertaken using standard curves with bovine serum albumin (BSA) of known concentration and visual estimation.

2.5. Transmission electron microscopy

Sucrose gradient fraction 7 (Fig. 2) was used for electron microscopy. ESAT-VLPs as well as control VLPs (as described above) were adsorbed for 1 min on copper grids, washed with

PBS containing 1% BSA and stained for 30 min with 10 µg/ml anti-ESAT-6 monoclonal mouse antibody (HYB 076-08, Santa Cruz Biotech). Samples were washed intensively with PBS containing 1% BSA and incubated with 10 µg/ml secondary anti-mouse IgG gold (5 nm) labelled antibody (Sigma) for 30 min. Negative staining was performed using uranyl acetate (pH4.5) (Sarkar et al., 1975). Samples were examined using a transmission electron microscope at various magnifications.

2.6. Hemagglutination assay

VLPs were diluted serially twofold in PBS and incubated at room temperature for 2 h with 50 µl of 1.25% human red blood cells (RBC, blood group 0). Hemagglutination was estimated visually. The hemagglutination titer designated as hemagglutination units (HAU) was determined by the reciprocal of the highest dilution without complete sedimentation of RBCs.

2.7. Animals and vaccination

BTI-TN5B1-4 derived VLPs were pooled from sucrose gradient fractions, diluted in PBS and pelleted by ultracentrifugation at $136,000 \times g$ at 4 °C. Pellets were resuspended in SPGN buffer (6%, w/v, sucrose, 3.8 mM KH₂PO₄, 7.2 mM K₂HPO₄, 4.9 mM L-glutamic acid, 75 mM NaCl, pH 7.5).

Six-week-old female BALB/c mice were used for immunization. Mice (3–4 mice per group) were vaccinated with purified ESAT-VLPs (3 µg ESAT6-HA), with GFP expressing rBV (~100 ng, the same amount as the BV background found in the VLP preparation) and with SPGN, respectively, by intraperitoneal injection at week 0. Mice were re-inoculated on day 25 with VLPs carrying total 3 µg of ESAT6-HA whereas the negative control animals received baculovirus preparation or SPGN, respectively. On day 0, day 25 and on day 47 blood was collected from anesthetized mice via the orbit and transferred to a microfuge tube. Tubes were centrifuged at $1500 \times g$ and sera was removed and frozen at –20 °C. Serological tests of mice were approved by the Austrian Federal Ministry of Science and Research according to Austrian law (Zl. 2009/10116).

2.8. Evaluation of humoral immune response

ESAT-6 specific serum antibodies were determined by enzyme-linked immunosorbent assay (ELISA). 96-well microtiter plates (Nunc-Immuno Plate Maxisorp) were coated with 2 µg/ml of rESAT-6 (Fusion Antibodies, Belfast, North Ireland) in coating buffer for 2 h at room temperature. Plates were blocked with PBS containing 3% BSA and 0.005% Tween 20 (room temperature, 2 h) and then incubated with serial twofold dilutions of each 1:800 pre-diluted serum samples (room temperature, 2 h). Afterwards plates were washed with PBS containing 0.005% Tween 20 and incubated with peroxidase conjugated anti-mouse IgG antibody (Sigma, #A3673) in PBS containing 1% BSA and 0.005% Tween 20 (room temperature, 1 h). Unbound antibody was removed and plates were washed. Samples were incubated with substrate (o-phenylene diamine and H₂O₂), reaction was stopped using H₂SO₄ and the colorimetric change was measured at 492 nm.

3. Results

3.1. Generation and purification of influenza VLPs

Influenza A VLPs were generated by co-infection of BTI-TN5B1-4 cells with baculoviruses expressing M1 (A/Udm/307/1972) and either ESAT6-HA or wild type HA (A/New Caledonia/20/1999). In contrast to the wild type HA, ESAT6-HA contained additional 20 amino acids within the antigenic site B, at position 171 (Fig. 1). VLPs were detected and quantified in the supernatant by hemagglutination assay while the concentration of baculoviruses was measured by plaque assay. Supernatants from co-expression of M1 and ESAT6-HA showed baculovirus titers of $\sim 1 \times 10^6$ pfu/ml and a biological activity of 4 HAU per 50 μ l, whereas supernatants from M1 and wild type HA co-expressions with comparable VLP concentrations showed 16 HAU. VLPs were harvested from supernatants and after purification by sucrose density gradient centrifugation analysed by Western blot. The ESAT6-HA was detected as a protein of approximately 85 kDa and the M1 of 29 kDa. In ESAT-VLPs the M1 was present in fractions 3–11 of the sucrose gradient and in the pellet (Fig. 2). The highest concentrations of HA and therefore, of VLPs were found in fraction 6, 7 and 8 (~ 35 – 45% sucrose). The yield of VLPs based on HA content was found to be approximately 5 μ g per ml culture, which is comparable to yields for recombinant HA production (~ 6 μ g/ml) and wild type influenza A VLPs in insect cells (Krammer et al., 2010; Wang et al., 2006). For comparison of ESAT6-HA and wild type HA in terms of concentration and size, two separate Western blot analyses were carried out (Fig. 3A and B). The anti-ESAT-6 primary antibody detected exclusively ESAT6-HA (Fig. 3A) whereas the anti-HA primary antibody was specific for both proteins (Fig. 3B). A significant difference in molecular weight could be observed, wild type HA was detected slightly above 70 kDa whereas ESAT6-HA was detected at approximately 85 kDa (Fig. 3). The calculated molecular protein mass of wild type HA is 63 kDa, while the mass of ESAT6-HA is 65 kDa. It is therefore concluded that both proteins were highly glycosylated. A, theoretical additional glycosylation site in ESAT6-HA, located at amino acid position 171 may have accounted for the increase in molecular weight.

3.2. Display of ESAT-6 epitope on the surface of influenza A VLPs

In order to confirm the presence and morphology as well as the efficient display of ESAT-6 epitopes, ESAT-VLPs as well as wild type HA VLPs from gradient fraction 7 were stained with 5 nm gold labelled antibodies directed against the primary anti-ESAT-6 anti-body and analysed by electron microscopy. Both, ESAT-VLPs and wild type HA VLPs appeared spheric and partially polymorphic. Both VLPs measured 80–200 nm in diameter which is consistent with the morphology of wild type influenza A virions (Harris et al., 2006). Fig. 4 is representative for the pictures seen by electron microscopy, showing gold labelled ESAT-VLPs and wild type HA VLPs. Gold particles were detected exclusively on the surface of ESAT-VLPs but not on corresponding wild type HA VLPs.

3.3. Analysis of immune response

Immunogenicity of VLPs was examined in BALB/c mice (6 weeks old) by intraperitoneal injection of purified antigen (VLPs carrying total 3 μ g ESAT6-HA, 4 animals), of purified GFP expressing rBV (~ 100 ng, 4 animals) and of SPGN (3 animals) on day 0 and day 25.

Pre-immune sera and post-vaccination antisera 25 days after the first vaccination and 22 days after the boost were evaluated for the induction of specific immune response by ELISA. Four out of four mice sero-converted after the first immunization and responses increased significantly after boosting. Sera from pre-immunized mice and from the negative controls showed no immune response at all (Fig. 5). IgG levels specific against the ESAT-6 protein (rESAT-6) expressed recombinantly were tested by ELISA. ELISA titers of 1:800 pre-diluted sera (OD 492 nm value) ranged from 0.064 to 0.105 in immunized mice whereas sera from both negative control animals showed only background reactivity (0.001–0.003). After boosting, a significant rise in titers was observed, reaching OD 492 nm values between 0.207 and 0.424. Again, both negative controls showed only background reactivity (0.001–0.004) (Fig. 5).

4. Discussion

Presentation of foreign epitopes on the surface of VLPs has been shown previously to be an effective strategy for vaccine design, especially when it comes to bacterial antigens (Amexis and Young, 2006; Dale et al., 2002; Deml et al., 2005; Doan et al., 2005; Ernst et al., 1998; Jegerlehner et al., 2002; Notka et al., 1999; Peacey et al., 2007; Pumpens and Grens, 2001; Sedlik et al., 1997; Takahashi et al., 2008; Wang et al., 2008). Influenza A VLPs as an antigenic scaffold offer the advantage of accessible antigenic sites within the envelope protein HA, easy production by co-expression of 2–4 proteins in insect cells, safety, stability and the possibility to use up to 16 different HA subtypes as scaffold.

For this study, the N-terminal region of the ESAT-6 peptide of *M. tuberculosis* was chosen. Twenty amino acids of the ESAT-6 protein (amino acid 1–20) were engineered into influenza A HA of strain A/New Caledonia/20/99 and influenza A VLPs were generated. Although the ESAT-6 epitope is a T-cell epitope, which is often located at the inner unexposed side of the antigen and thus only becomes accessible to the T-cell receptors after proteolytic processing, data suggest that exposure to ESAT-6 protein only leads to efficient antibody response (Kirby et al., 2008). Also, B-cell epitopes, e.g. from surface proteins of Gram-positive bacteria are additional candidates for display on the surface of influenza A VLPs.

The structure of HA comprises three monomers which are incorporated into the viral membrane as trimers. Four major antigenic sites have been located on the HA monomer: Site A, B, C and D. Site B contains a loop structure which comprises residues of an α -helix combined with several residues of the pocket responsible for sialic acid binding (Wiley et al., 1981). This antigenic site B was shown previously to tolerate foreign epitopes, hence it can be used for altering the viral surface (Li et al., 1993; Muster et al., 1994). Based on these data, the amino acid position 171 of HA within the B-loop region was chosen for insertion (Fig. 1). For assembly of particles, ESAT6-HA and M1 were co-expressed in insect cells by co-infection with two recombinant baculoviruses (Figs. 2 and 3). In the past, a 9 amino acid epitope derived from HIV-1 glycoprotein 41 was displayed, using the same site of insertion within the HA (Ernst et al., 1998). The presentation of this epitope was improved further by changing three adjacent amino acids using a library approach. Such fine tuning will have to be done for every peptide individually, due to differences in structure and sequence. The 20

amino acid ESAT-6 epitope did impair partially the function of HA in terms of hemagglutination activity, but did not alter the HA function in regards to particle assembly. Electron microscopy of purified VLPs showed the typical size and shape of influenza A virions. In addition, the presentation of the ESAT-6 epitope on the surface of the VLPs was confirmed by immunogold staining (Fig. 4). Immunization of BALB/c mice with ESAT-VLPs rose high titers of IgG against a recombinant version of the ESAT-6 protein (Fig. 5). Antibody levels were comparable with results of previous studies with an ESAT-6 expressing live *Salmonella* vaccine strain and studies based on DNA vaccines comprising mycobacterial epitopes (Gao et al., 2009; Hall et al., 2009; Xu et al., 2008).

In conclusion, it was shown that influenza VLPs that consist of the two influenza A virus proteins HA and M1 are feasible for the display of foreign epitopes on their surface. It was demonstrated that the baculovirus expression system is adequate to generate them, since VLPs were produced in high yields (~5 mg per litre) using three non-optimized ultracentrifugation steps. For future production schemes, it is expected that other purification methods, e.g. those based on polymethacrylate monoliths will be used to optimize purity and recovery rate (Jungbauer and Hahn, 2008). Since a great variety of HAs of other influenza A strains are available, the choice of applicable scaffolds will depend on immunological aspects as well as stability and purification factors. For the scaffold described in this study, immunological studies in mice demonstrated that the presented epitope was recognized by the immune system of the vaccinee. Due to their efficacy and their safety, influenza A VLPs with altered surfaces represent a very promising tool for antigen presentation for future vaccine designs. This strategy provides an appealing alternative to the existing display and expression platforms. Since influenza VLP vaccines are undergoing currently clinical trials and may enter the market within a few years, presentation of foreign epitopes on their surface clearly demonstrates an additional application of this technology (Kang et al., 2009).

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HA	151	SCSHNGKSSFYRNLLWLTGKN-----GLYPNLSKS	180
ESAT6-HA	151	SCSHNGKSSFYRNLLWLTGKN <u>MTE</u> QQWNFAGIEAAASAIQGGLYPNLSKS	200

Fig. 1.

Insertion site of the 20 amino acid N-terminal sequence from *Mycobacterium tuberculosis* ESAT-6 protein in the B-loop region of influenza HA. The insertion site is amino acid 171 (nucleotide position 513) creating a putative additional N-glycosylation site indicated by underscore.

Fig. 2.

Western blot of ESAT-VLPs in a discontinuous sucrose density gradient (20–60% sucrose). The blot was developed using a monoclonal anti-M1 and monoclonal anti-ESAT-6 antibody and a monoclonal alkaline phosphatase conjugated anti-mouse IgG antibody, respectively. Sucrose concentration increases from lane 1 to 11, P indicates the pellet. Positions of ESAT6-HA and M1 are indicated.

Fig. 3.

Comparison of ESAT6-HA and wild type HA by Western Blot. Lane 1 indicates wild type HA, lane 2 indicates ESAT6-HA. Anti-ESAT-6 primary antibody (A) detected exclusively ESAT6-HA whereas anti-HA primary antibody (B) detected both proteins.

Fig. 4.

Transmission electron micrographs of immunogold labelled and negatively stained ESAT-VLPs and wild type HA VLPs. VLPs ranged in the size between 200 nm (A) and 80 (B).

Anti-ESAT-6 primary antibodies detected ESAT-6 epitopes exclusively on ESAT-VLPs (A and B), binding to wild type HA VLPs could not be observed (C). This figure is representative for the pictures seen by electron microscopy.

Fig. 5.

ESAT-6 specific total serum IgG responses. Day 0, 25 (post-immunized) and 47 (22 days post-boost) serum IgG antibody responses were assessed by ELISA against rESAT-6 (Fusion Antibodies). Sera were 1:800 pre-diluted. Negative controls (neg. contr.) and baculovirus controls (BV) showed only background levels of specific anti-ESAT-6 IgG at all time points, whereas ESAT-VLPs raised high levels of specific anti-ESAT-6 antibodies. Optical densities were read at 492 nm and results are expressed as arithmetic mean titer.