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Title: STABLE, SPRAY DRIED, IMMUNOGENIC, VIRAL COMPOSITIONS

Abstract: Viruses, and particularly genetically engineered, replication deficient- viruses such as adenoviruses, poxviruses, MVA viruses, and baculoviruses which encode one or more antigens of interest, such as TB, malarial, and HIV antigens, are spray dried with a mannitol-cyclodextrin-tri-ehalose-dextran (MCTD) to form a powder where the viability of the viruses are maintained at a suitable level for mass vaccinations after spray drying, and, where the viability of the viruses are maintained at suitable level over a period of storage time, even in the presence of humidity.
SUMMARY

The invention includes a method for stabilizing viruses in a spray dry powder, as well as to ati immunogenic composition containing a vims in a manuitol-cyclodextrin-trehalose-dextran (MCTD) spray dried powder. Experiments have demonstrated that viruses survive spray drying and subsequent storage in powder foπ with MC-TD at a rate which makes them viable as dry powder immunogenic compositions suitable for mass vaccinations.

DESCRIPTION OF THE DRAWINGS

Figure 1. The vims panicle size distribution of rAd35.

The size of the rAd35 vaccine vims was measured by CPS Disc centrifuge-. 8% and 24% sucrose in sample buffer were used for gradient solutions.

Figure 2A. The glass transition temperature \( T_g \) of MCTD powder sample

Figure 2B. The glass transition temperature \( T_g \) of trehalose powder sample

For Figures 2A-B, the glass transition temperature \( T_g \) was determined by a DSC 82. V. The cover of the crucible was punched with a small hole before analysis. The sample (about 10mg) was heated from 25\(^\circ\)C to 170\(^\circ\)T with a scanning rate of 1CUFC\(\^n\)m n. The sample cell was purged with nitrogen gas at H\(\^\)Oml/mii

Figure 3. The particle distribution of MCTD powder

Particle size distributions were measured by laser diffraction (Masters\(\^\)er 2000). The poiydispersity of the powder was expressed by the span. Span \( = \frac{D_{10} - D_{90}}{D_{50}} \), where \( D_{10} \), \( D_{90} \), \( D_{50} \) are the equivalent volume diameters at 90, 10 and 50% cumulative volume, respectively.

Figure 4A. Microscope image (\( \times \)1000) of particle generated from mannitol based formulation

The powder was re-suspended in anhydrous mef.ha.uol at 2\(\mu\)g.mg

Figure 4B. Microscope image (\( \times \)1000) of particle generated from trehalose formulation

The powder was re-suspended in Halocarbon 0.5 oil at 20mg/mL.

Figure 5. Increase in water content of dry powder formulations exposed to 70% relative humidity.
Figure 6. The TCID$_{50}$ recovery (log loss) of rAd35 in different formulations during spray drying process.

The TCID$_{50}$ recovery is expressed as the infectivity loss of rAd35 between pre spray drying and post spray drying samples. Titer changes are expressed in log loss per gram solid or powder. Man = mannitol; ManLeu = mannitol mixed with leucine; Tre = trehalose; Leu = leucine; lVlanSuc = mannitol mixed with sucrose; ManPBS = mannitol in PBS buffer; ManPVP = mannitol mixed with PVP; MCTD = mannitol-cyclodextrin-trehalose-dextran; ManIno = mannitol mixed with inositol; MTDT = mannitol-trehalose-dextran-twee% 8K

Figure 1. The stability of rAd35 spray drying samples at 25°C and 37°C.

The stability study of rAd35 spray lot with the candidate MCTD, was conducted for 1 month at both 25°C and 37°C. The change in virus activity is expressed as log loss of virus infectivity by the TOD$_{50}$ test. Post-SD = post spray drying.

DESCRIPTION

Viruses are spray dried with a formulation of mannitol-cyclodextrin-trehalose-dextran (MCTD) to produce an immunogenic, spray dried, powder composition that is suitable for delivery by inhalation or other routes (e.g., oral parenteral, intradermal, sublingual, etc.). The constituents of the MCTD formulation for spray drying axe as follows;

M) Mannitol, present at 10-150 mg/ml, and more preferably at 50-100 mg/ml
C) Cydodextrin, present at 0.1-10 mg/ml, and more preferably 0.2-1 mg/ml

Preferably the cyclodextrin is 8-Cyclodextrin; however, 6- or 7-Cyclodextrin can be used, and mixtures of cyclodextrins may also be used.

T) Trehalose, present at 0.2-30 mg/ml, and more preferably at 0.5-5 mg/ml
D) Dextran, present at 0.1-30 mg/ml, and more particularly 0.5-5 mg/ml

Preferably the molecular weight of the dextran is from 25K to 500K, and more preferably from 40K to 90K.

The MCTD formulation can include buffering agents (e.g., L-histidine at 1-2M (preferably S-iQniM) and other stabilizers and excipients.
An exemplary contents of the formulation pre-spray drying is 100 mg/mL maimiitoi, 0.2 mg/mL, cyclodextrin, 0.78 mg/mL (SmM) histidine, 2 mg/mL trehalose and 1 mg/mL dextran. The percent of active reagent (i.e., the virus (which can take the form of a genetically engineered viral vaccine vector)) in a pre-spray drying formulation would range from 1E4/Z/mL to 1E3/V/mL, and often from 1E5/µL to 1E6/µL (the content of virus being dosage related and dependent on choice of virus), and in a the final powder vaccine: the range is from virus© particle of 1E4/µg to 1E5/µg.

As discussed in the Example below, a powder vaccine intended for aerosol delivery was formulated by spray drying rAd35 with a plurality of TB antigens with the aforementioned MCTD rααanitol-based stabilizers. Thermodynamic properties, water absorption, particle size distribution and morphology of the powders were evaluated and the virus survival during spray drying and storage was determined by medium Tissue Culture Infectious Dose (TCIDso). The MCTD maimiitoi-based powder had a narrow size distribution with a median volume diameter around 3.2 – 3.5 µm (suitable for human pulmonary vaccination of human) and good aerosolization characteristics. The spray dry powders generated from MCTD maimiitoi-based formulations were hydrophobic, which benefits virus survival during both production and storage at 4, 25 and 37°C as compared to the hygroscopic formulations (trehalose, sucrose, dextran, PVP, leucine). The results in the Bxanipiedemoisitraies that it is possible to produce, in a one-step spray drying process, a stable dry powder formulation of, e.g., a TB vaccine, suitable for mass vaccination.

While the spray dried vinis-MCTD powder is designed for use by inhalation, it should be understood that the powder can be combined with excipients for delivery by oral, parenteral, intradermal, and other routes. Suitable excipients are, for example, water, saline, dextrose, raffmose, glycerol, ethanol and the like, or combinations thereof. In addition, the composition may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, and the like. The vaccine preparations of the present invention (i.e., the immunogenic compositions) may further comprise an adjuvant, suitable examples of which include but are not limited to Seppic, QuU A, Alhydrogel, etc.

If it is desired to administer an oral form of the composition, various thickeners, flavorings, diluents, emulsifiers, dispersing aids or hinderers and the like may be added.
The composition of the present invention may contain any such additional ingredients so as to provide the composition in a form suitable for administration. The final amount of virus in the formulations may vary. However, in general, the amount in the formulations will be from about 0.1-99 percent. Further, the preparations of the present invention may contain a single type of virus or more than one type of virus (e.g., for vaccinating against multiple diseases simultaneously).

In the case of vaccine preparations, the present invention also provides methods of eliciting an immune response to antigens encoded by a genetically engineered vaccine vector, and methods of vaccinating a mammal against diseases or conditions associated with such antigens. By eliciting an immune response, we mean that administration of the vaccine preparation (i.e., immunogenic composition) of the present invention causes the synthesis in the subjects, post administration, of specific antibodies (at a titer in the range of 1 to 1 x 10^3, preferably 1 x 10^3 more preferable in the range of about 1 x 10^3 to about 1 x 10^6, and most preferably greater than 1 x 10^5) and/or cellular proliferation, as measured, e.g. by 3H thymidine incorporation. The methods involve administering a composition comprising the virus-MCTD spray dried powder in a pharmacologically acceptable carrier to a mammal (e.g., air or a propelteni in the case of an inhalable formulation). It will be recognized that the virus-MCTD powder can be formulated into vaccine preparations that may be administered by any of the many suitable means which are well known to those of skill in the art, including but not limited to by injection, orally, intranasal! by ingestion of a food product containing the virus, etc. The targeted host is generally a mammal, and may be a human, although this need not always be the case, as veterinary applications are also contemplated.

The viruses employed in the practice of the invention can be simple attenuated viruses suitable for use as a vaccine such as poliovirus, rotaviruses, orthomyxoviruses such as influenza viruses, retroviruses such as RSV, poxviruses such as vaccinia, paroviruses such as adeno associated viruses, papillomaviridae such as HPV, herpesviruses such as EBV, CMV or herpes simplex virus, lenti viruses such as HIV-I and HIV-2, etc. Preferably, the viruses are non-replicating or are replication deficient (i.e., do not replicate or replicate at a low rate).
However, the invention has particular application to live, attenuated, recombinant viral vaccine vectors based on, for example, adenoviruses, poxviruses, modified vaccinia Ankara (MVA) viruses, baculoviruses, recombinant vesicular stomatitis viruses (CVSV), etc. The viral vaccine vector can take a variety of different forms, and will preferably be genetically engineered to encode one or more genes of interest, i.e., passenger genes or transgenes. The passenger genes are typically heterologous transgenes ("foreign" genes) that originate from another organism, such as another virus, a bacteria or other pathogen, and may be from any organism. "Passenger gene" is intended to refer not only to entire "genes" but to any sequence that encodes a peptide, polypeptide, protein, or nucleic acid of interest, i.e., as entire "gene" per se may not be included, but rather the portion of a gene that encodes a polypeptide or peptide of interest e.g., an antigenic peptide. Further, various other constructions may be encoded by passenger genes, e.g., chimeric proteins, or various mutant (either naturally occurring or genetically engineered) forms of an amino acid sequence in addition, totally artificial amino acid sequences that do not appeal in nature may also be encoded. The viral vaccine vector is genetically engineered to contain one or more of such "passenger genes", and may also encode multiple copies of individual passenger genes. The viral vaccine vector functions as a vector to carry the passenger genes and/or genes encoding suppression factors or other factors into host cells that are invaded by the viral vaccine vector, where the gene products are expressed, i.e., the gene sequences are expressible and transcription and/or translation of the gene products occurs within the host cell that is invaded by the bacterium. The sequences encoding the passenger genes and the genes encoding the suppression factors are operatively (operably) linked to expression control sequences, particularly expression control sequences that allow expression within the eukaryotic host cell. In some embodiments, if multiple passenger genes are encoded, each will have its own expression control system. In other embodiments, one expression control system will serve to drive expression of more than one passenger gene, e.g., as a single transcript with a plurality of gene sequences. Similarly, if multiple suppression factors are encoded in a viral vaccine vector, the transcription of each may be separately controlled, or multiple sequences may be under the control of one expression control sequence.
In particular, such passenger genes may encode one or more peptides or proteins that are antigens, and to which it is desired to elicit an immune response. Those of skill in the art will recognize that a wide variety of such antigens exists, including but not limited to those associated with infectious agents such as various viruses, bacteria, and fungi, etc. The viral pathogens, from which the viral antigens are derived, include, but are not limited to, Orthomyxoviruses, such as influenza virus (Taxonomy ID: 59771; Retroviruses, such as RSV, HTLV-I (Taxonomy ID: 39015), and HTLV-T1 (Taxonomy ID: 1909), Papillomaviridae such as RPV (Taxonomy ID: 337043), Herpesviruses such as EBV Taxonomy ID: 10295); CMV (Taxonomy ID: 1035S) or herpes simplex virus (ATCC #: VR-148?); Lentiviruses, such as HIV-I (Taxonomy ID: 12721) and HIV-2 (Taxonomy ID: 11709); Rhabdoviruses, such as rabies; Picornovirus, such as Poliovirus (Taxonomy ID: 12080); Poxviruses, such as vaccinia (Taxonomy ID: 10245); Rotavirus (Taxonomy ID: 10912); and Paroviruses, such as adeno-associated virus I (Taxonomy ID: 85106).

Examples of viral antigens can be found in the group including but not limited to the human immunodeficiency virus antigens NeH National Institute of Allergy and Infectious Disease BiV Repository Cat. # 183; Genbaak accession # AF23827S), Gag, Env (National Institute of Allergy and Infectious Disease HIV Repository Cat. # 2433; Genbank accession # U39362), Tat (National Institute of Allergy and Infectious Disease HIV Repository Cat. # S27; Genbank accession # M13137), mutant derivatives of Tat, such as Tat-3 i-45 (Agwale et al., Proc. Natl Acad. Sci. USA 99:10037; 2002), Rev (National Institute of Allergy and Infectious Disease HIV Repository Cat. # 2088; Genbank accession # U 4572), and Pol (National Institute of Allergy and Infectious Disease HIV Repository Cat. # 238; Genbank accession # AJ23756S) and T and B cell epitopes of gpl20 fHank ε and McMichael, AIDS Immunol Lett. 66:177; 1999); (Hanke, et al. Vaccine, 17:5S9; 1999); (Talker et al., J. /mmuHoL 142:3612 3619; 1989) chimeric derivatives of HIV-I bαv and gpl2αX such as but not restricted to fusion between gpl20 and CD4 (Fouls et al., J. Virol 2000, 74; 11427-1 1436; 2000); truncated or modified derivatives of HIV-I env, such as but not restricted to gp14U (Stamatos et al. J Virol, 72:9656-9667; W 88) or derivatives of HTV-I Env and/or gp40 thereof fBinJey, et al., J Virol, 76:2606-2616; 2002); (Sanders, et al, J Virol 74:5091-5100 (2000); (Bmley, et al
J Virol, 74:627-643; 2000), the hepatitis B surface antigen (GenBank accession # AF043578); (Wo et al., Proc. Nail Acad. Sci., USA, 86:4726 4730; 1989); rotavirus antigens, such as VP4 (Genbank accession # AJ293721); (Mackow et al., Proc. Nad Acad. Sci., USA, 87:51 S 522; 1990) and VP? (GenBank accession # AY003871); (Green et al., Virol, 62:1819 1823; 1985); influenza virus antigens such as hemagglutinin or (GenBank accession # AJ404627); (Pacaktrac and Robinson, Virology, 257:406; 1999); nucleoprotein (GenBank accession # AJ289872); (Lin et al., Proc. Nad Acad. Set., 91: 9654-9658; 2000) herpes simplex virus antigens such as thymidine kinase (Genbank accession # AB047378; (Whitly et al. In: New Generation Vaccines, pages 825-854).

The bacterial pathogens, from which the bacterial antigens are derived, include but are not limited to: Mycobacterium spp., Helicobacter pylori, Salmonella spp., Shigella spp., E. coli, Rickettsia spp., Listeria spp., Legionella pneumophila, Pseudomonas spp., Vibrio spp., Bacillus ankracis and Borelia burgdorferi. In particular, Mycobacterium tuberculosis antigens of interest include but are not limited to Rv0079, RvOlO1, RvOl25, RvO1TO, RvO19Sc, RvO21I, RvO227c, RvO243, RvO251c, RvO252, Rv0283, RvO284, RvO255, RvO256, RvO287, RvO288, RvO290, RvO29, RvO35O, RvO351, RvO383c, RvO384c, RvO38Sc, RvO467, RvO468, RvO503c, RvO569, RvO572c, RvO574c, RvO588, RvO628c, RvO685, RvO754, RvO798c, RvO824c, RvO847, RvO867c, RvO885, Rv1006, Rv1009, Rv1057, Rv1094, Rv1124, Rv1130, Rv1131, Rv1169c, Rv1174c, Rv1182, Rv1186, Rv1187, Rv1188, Rv1196, Rv1221, Rv1347c, Rv1348, Rv1349, Rv1411c, Rv1436, Rv1461, Rv1462, Rv1464, Rv1465, Rv1466, Rv1477, Rv1478, Rv1594, Rv1636, Rv1733c, Rv1734c, Rv1735c, Rv1736c, Rv1737c, Rv1738, Rv1793, Rv1812c, Rv1813c, Rv1876, Rv1884c, Rv1908c, Rv1926c, Rv198Oc, Rv1986, Rv1996, Rv1997, Rv1998c, Rv2004c, Rv100OSc, Rv2006, Rv2007c, Rv2008c, Rv201 Ic, Rv2028c, Rv2029c, Rv2030c, Rv2031c, Rv2032, Rv2110c, Rv2123, Rv2140c, Rv2182c, Rv2224c, Rv2224, Rv2245, Rv2246, Rv2251, Rv2377c, Rv2378c, Rv23SOc, Rv2381c, Rv2382c, Rv2383c, Rv2388c, Rv2428, Rv2429, Rv2430c, Rv2450c, Rv2457c, Rv2466c, Rv2510c, Rv2515c, Rv2516c, Rv2557, Rv2590, Rv2620c, Rv262Ic, Rv2622, Rv2623, Rv2625c, Rv2626c, Rv2627c, Rv2628, Rv2629, Rv2657c, Rv2659c, Rv2660, Rv2744c, Rv2780, Rv2833c, Rv2856, Rv2869c, Rv2875, Rv2930, Rv2999, Rv3126c, Rv3127, Rv3129, Rv3130c, Rv3131, Rv3132c, Rv3133c, Rv3134c,

The parasitic pathogens, from which the parasitic antigens are derived, include but are not limited to; *Plasmodium spp.*, such as *Plasmodium falciparum* (ATCC#: 30145); *Trypanosome spp.*, such as *Trypanosoma cruzi* (ATCC#: 5079?); *Giardia spp.*, such as *Giardia intestinalis* (ATGCC#: 308S8D); *Boophilus spp.*, *Babesia spp.*, such as *Babesia microti* (ATCC#: 10221); *Entamoeba spp.*, such as *Entamoeba histolytica* (ATCC#: 30015); *Eimeria spp.*, such as *Eimeria maxima* (ATCC#: 40357); *Lishmania spp.* (Taxonomy ID: 3856S); *Schistosome spp.*, *Brugia spp.*, *Fascida spp.*, *Dirofilaria spp.*, *Wuchereria spp.*, and *Onchocerca spp.* (See also International patent application

The viral vaccine vector is also be genetically engineered to express nucleic acid sequences that encode one or more proteins that interfere with mammalian host cell type I interferon (IFN) responses. Examples of proteins that modulate type I IFN response include non-stetural protein I (NSP-1) from rotavirus, NSI protein from *influenza* virus, and C12R from ectromelia virus. In addition, other suitable IFN modulating proteins include but are not limited to: Ebola VP35 (The Ebola virus VP35 protein functions as a type I IFN antagonist. CF Baskr, X Wang, E Mulilbetger, V Vokhllov, *Proceedings of the National Academy of Sciences*, 2000. National Acad Sciences); Vaccinia BISR (Waibier et al at *Journal of Virology*. 2009 Feb;83(4):15 61-7n; rabies phosphoprotein P (Krzysxtof Brzózka. et ai. *Journal of Virology*, March 2006, p. 2&75-26S3, Vol. 80, No. 6); lymphocytic choriomeningitis virus (LCMV) nucleocprotein (Martinez-Sobrido Luis et al *Journal of Virology* 2006;8U(8):9 192-9); and Hepatitis C virus (HCV) protease NS3/4A (Xiao-Dong JJ, et al *Proc Natl Acad Sci USA*, 2005).
December 6; 102(49): 1771-17722}. In addition, Weber and Hal'ier (Biochemie 89, 2007, 836-S42) describe other examples of suitable proteins such as the E3L protein of poxviruses, the sigma3 protein of reoviruses, the US1 protein of herpes simplex virus, and murine cytomegalovirus proteins m142 and m143.

Alternatively, it may be desired to elicit an immune response to antigens that are not associated with infectious agents, for example, antigens associated with cancer cells, Alzheimer's disease, Type 1 diabetes, heart disease, Crohn's disease, multiple sclerosis, etc. The viral vaccine vectors of the present invention may also be genetically engineered with one or more passenger genes encoding for these types of antigens.

In addition, the passenger genes that are earned by the viral vaccine vector need not encode antigens, but may encode any peptide or protein of interest. For example, the methods of the invention can be used for the delivery of passenger molecules for correction of hereditary disorders, e.g. the vectors may be used for gene therapy. Such genes would include, for example, replacement of defective genes such as the cystic fibrosis transmembrane conductance regulator (CFTR) gene for cystic fibrosis; or the introduction of new genes such as the inegrase antisense gene for the treatment of HIV; or genes to enhance Type 1 T cell responses such as interleukin-2 (IL-2); or genes to modulate the expression of certain receptors, metabolites or hormones such as cholesterol and cholesterol receptors or insulin and insulin receptors; or genes encoding products that can kill cancer cells such as mraor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL); or a naturally occurring protein osteoprotegerin (OPG) that inhibits bone resorption; or to efficiently express complete-length humanized antibodies, for example, humanized monoclonal antibody that acts on the HER1/neu (erbB2) receptor on cancer cells.

In addition, the passenger genes may encode inhibitory RNAs such as "small inhibitory" siRNAs. As is known in the art, such RNAs are complementary to an mRNA of interest and bind to and prevent translation of the mRNA, e.g. as a means of preventing the expression of a gene product.

Preferably, the viral vaccine vector will be genetically engineered to express one or more proteins (i.e., antigens) or a fusion protein of multiple antigens which, when administered to a subject (e.g., a human or other animal (e.g., mammal), will result in an
immune response to one or more infections, e.g., tuberculosis (TB), materia, HIV, dengue fever, etc.

The construction of a vaccine vector is well known in the art. Gencaliv, the genes that are placed into the viral vector vector or Ma genetic engineering are under control of an expression sequence such as a promoter, internal ribosomal entry site (IRES) various enhancer sequences, etc. Such sequences and promoters maybe unnatural or within the virus vector or within the virus. The sequences of interest placed at a location such that their expression is required by the virus vector sequences. Alternatively, promoters from organisms other than viral vaccine vectors (e.g., adenovirus) may be cloned into the virus, together with the genes of interest. Exemplary promoters that may be utilized in the practice of the invention include but are not limited to various animal, prokaryotic or eukaryotic promoters, e.g., cytomegalovirus (CMV) promoters, e.g., cauliflower mosaic virus promoters influenza and HAV dual promoters, heat shock promoter, e.g., hsp(68) promoter and other promoters from animal, e.g., tuberculosis, etc. of these, both constitutive and inducible promoters can be utilized.

ΓX ΛΛRΠΕ

Results

Virus Panicle

Vot the experiments a recombinant adenovirus (rAd35) panicle expressing a plurality of IB antigen was used. rAd35 induces low levels of neutralizing antibodies in non-human primates which are an important model for preclinical vaccine trials since these are suscep-vible to influenza and develop clinical features which are similar to those in human (e.g., Nand a et al. J Infect Dis 182:1416-1416 and LecMurra). The (2000) CVm Pifeu Dis 30 Suppl 3 S210-212) As noted above, a variety of viruses can be used, and a variety of different antigens, lactosis, and other nucleotide sequences can be encoded into a variety of vectors such as rAd35. The main site tititigation peak of the tested rAd35 on the Disc centrifuge curve was around 7 nm. (e.g., see 1) The tititigation peak of vims was of 65 nm. The 60,000 peaks did not shift even when the virus bulk Bakteum was concentrated (data not shown).
**Thermodynamic Properties**

Glass transition temperatures of the drying powders were determined after production and after storage at high humidity. The mannitol based powder (MCTD) had the highest \( T_g \) value of 97.09°C, with a melting point of 166.53°C for the crystalline mannitol component (Figure 2A). The \( T_g \) of trehalose powder after spray drying was 5G.5T (Figure 2B), Mannitol alone did not have a \( T_g \), and Mannitol with PVP powder had a \( T_g \) of 85.78°C. The thermodynamic curves and glass transition temperatures of representative spray powders are shown in Table 1.

Table 1. The characteristics of different spray drying powders

<table>
<thead>
<tr>
<th>Formula</th>
<th>( T_g )</th>
<th>Yield (^{(1)})</th>
<th>( d(0.5) ) (^{(2)})</th>
<th>Moisture content (^{(3)})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mannitol</td>
<td>-</td>
<td>30.0</td>
<td>3.1</td>
<td>1.23</td>
</tr>
<tr>
<td>Mannitol with PV?</td>
<td>85-78</td>
<td>19.9</td>
<td>7.0</td>
<td>1.94</td>
</tr>
<tr>
<td>Trehalose</td>
<td>50.55</td>
<td>6.0</td>
<td>2.6</td>
<td>5.40</td>
</tr>
<tr>
<td>Leucine</td>
<td>97.09</td>
<td>35.3</td>
<td>3.2</td>
<td>1.45</td>
</tr>
</tbody>
</table>

\(^{(1)}\) Powder yield was calculated by the wt/wt of pre and post spray drying solid. The total amount of pre-spray drying solid was determined from iyophiikation weight of pre-spray drying mixture.

\(^{(2)}\) \( d(0.5) \) is particle size at \( D(v,50) \), which is the equivalent volume diameter at 50% cumulative volume. The particle size of the powders was described by the volume median diameter (VMD).

\(^{(3)}\) The residual moisture contents were evaluated by a Karl Fischer (Radiometer Analytical) in a dry box, the resulting water percents were expressed based on wt/wt.

**Particle Size Distribution and Polydispersity**

Spray drying the formulation of MCTD resulted in fine powder with an average particle size range of \( D(v,50) = 3.2 \rightarrow 3.5 \mu m \). Combined with \( D(v,0.1) \) and \( D(v, 0.9) \), the span was around 1.5 \( \mu m \). The percent of inhalable particles [IP, \( 1 \mu m < IP < 5 \mu m \)] could reach to 72.6% of the total particles, and most powders are spherical (Figures 3 and 4A) (it being recognized that the powder can be provided to a subject by routes other than inhalation). The spray dried powder using the MCTD formation did not have either
different distribution or thermodynamic properties in rAB35 and placebo tests (data not shown). The Mannitol with PVP formulation had a dry powder size of $D(\nu, 50) = 7.0 \, \mu m$ and the trehalose product had a $D(\nu, 50) = 2.6 \, \mu m$ (Table 1). Although the trehalose formulation had a smaller particle size after spray drying (Table 1 and Figure 4B), the trehalose powder clumped easily after exposure to high humidity, and panicle size could increase significantly which was not suitable for laser diffraction measurement. MCTD, however, did not have any detectable decrease in IF < 5 \, \mu m, even after storage at 37°C for 2S days (results were the same as shown in Figure T).

**Moisture Content and Shifting of Glass Transition Temperature**

To compare the moisture content variations under high humidity (70%) of different formulations, we selected four representative powders prepared from placebo formolaiotis under the same processing conditions. The water contents of post spray drying powders were; Mannitol 6.28%; Mannitol with PVP 1.94%; Trehalose 5.40%; and MCTD 1.45% (Table 3). The water absorption tendency was different between the trehalose and mannitol-based formulations (Figure 5). After 2hr in 70% humidity, the water content of trehalose powder increased 61% (from 5.40 to 8.70%. wt/wt), and after 4hr, trehalose powder absorbed 37% more water. The total weight of water increased 120.9% (from 5.40 to 11.93%). The moisture saturation stage occurred after 4hr for trehalose powder. The mannitol based formulations (Mannitol. Mannitol with PVP and MCTT) had high resistance to water absorption. MatioUot alone did not have any increase in moisture content during 6 hr under high humidity exposure, and MCTD powder only increased from 1.45 to 1.80% in water content during this period.

After exposure at high humidity of 70%, the Tg of mannitol based powder (MCID) did not show significant change within 6 hr. Trehalose powder, however, showed apparent difference in thermodynamic properties; first, after 2 hr exposure at high humidity, its Tg shifted down to 38.76°C then, after 4 and 6 hr the melting point was reduced to around 102°C (Figure 2R), and the exposed powder appeared crystallized.

**Recovery and Stability of Different Spraying Lots**

The effect of spray drying on the infectivity of rAd35 in 10 different formulations is shown in Figure 6, where the Liter before drying is compared with the titer after spray drying. The titer changes were expressed in log loss per gram solid or powder. The total
amount of pre-spray drying solid was from the weight of lyophiluation of the pre-spray drying mixture.

All formulations except MCTD suffered at least a 1.5 (maximum 4.9) log loss of the viral activity. For the MCIT formulation, the loss in virus titer after spray drying was only 0.83 log. The largest decreases in virus activity by median Tissue Culture Infective Dose (TCID50) test were for the trehalose and leucine formulations. Although adding sucrose, inositol, or PVP, or adding PBS buffer could increase the survival of virus during the spray drying process, the TCID50 of live rAd35 was still over 1 log decreased at the end of preparation. Most formulations also resulted in low yield at less than 10%, while mannitol-foased formulations could reach more than 30% solid yield at the end of spray drying processing (partial data shown in Table 1).

The stability study of rAcB5 spray samples using the MCTD formulation showed that they could be stored at 4 and 25°C for 12 months without significant change in TCID50 titer. After storage at 37°C for five weeks, the loss of virus activity was only 0.2 log (Figure 7).

Discussion


The glass transition temperature of the dry formulations is also strongly dependent on water content; just a few percent increase in the water content of sugar-based formulations can decrease the Tg by several tens of degrees Celsius (see, Corbaniea EA, et al. (2007) Vaccine 25:8306-8317). Higher moisture content also results in decreased viral stability (see, Burger JL, et al. (2008) JAerm Med Pulmαn Drug Deli 2 1: 25-34, and Corbaniea EA, et al. (2007) Vaccine 25:8306-8317). Immobilization of the labile materials in amorphous glass is believed to be advantageous to maintain the activity of the incorporated molecules (see, Imamura K et al, (2001) J Pharm Sci
90:1955-1963). The resistance to crystallization can be evaluated by measuring the glass transition temperature, which is the temperature at which the transition from the glassy to the rubbery state or from a low molecular mobility to a high molecular mobility (and therefore, higher risk of crystallisation) occurs. PYF and albumins are known to increase the glass transition temperature, which means that the formulations can be exposed to higher ambient temperatures before the glass transition occurs (see, Corbaniea EA, et al. (2007) Vaccine 25:8306-8317; Mahiin D, et al. (2006) Int JPkarm 321:78-85; and Zhang J, et al. (2001) J Pharm Sci 90:1375-1385). However, PVP as a stabilizer in the tested formulation did not appear to prevent loss of virus activity during the spray drying process.

Dextran has also been shown to prevent crystallization of spray-dried and freeze-dried excipients. Therefore, the mannitol-based formulation used in the present study, MCTD, includes two kinds of dextran as components. This formulation could increase the glass transition temperature of trehalose from 50.55 to 97.09°C. The trehalose also generates a dry powder that inhibits re-crystallisation of stabilizing sugars, preventing activation of incorporated labile materials, and its glass transition temperature does not decrease during storage at high humidity. Equally important as low hygroscopicity in formulation selection, since water molecules are known to increase the molecular mobility, is a high and non-shifting glass transition temperature during storage. With glass transition temperature of die formulations occurring at about 50°C and higher, the powders and microparticles should be physically stable at temperatures up to about 4°C as long as the powders are protected from moisture ingress. As mentioned above, the MCTD formulation showed no detectable decrease in IP < 5 urn after storage at 37°C for 28 days. The higher $T_g$ values measured for this formulation suggest that enhanced long-term thermostability may be possible.

These results show that MCHTJ is a good candidate for both live virus and placebo selections. M(TITJ is not only conducive to forming easily dispersed microparticles in dry processing, but also appears to be a good stabilizer formulation for the rA<35 vaccine vector vims. Combinations of small and high molecular weight sugar stabilizers help achieve optimized viral processing and storage stability, while mitigating the negative particle forming properties of trehalose. The other tested formulations did not retain
activity as well as the MCTD formulation during the spray drying process, or at 37°C in the 1-month stability test.

Materials and Methods

**Chemicals**

Leucine was bought from Spectrum, Gardena, CA; inositol, sucrose, hisidme, Trehalose were from J.T. Baker, PhilHpsburg, NJ; Dextran (M. W. 60,000 - 90,000) was from MP Biomedicals, Solon, OH; β-Cyclodextrin was from TCI-GR, Kita-Ku, Tokyo, Japan; polyvinylpyrrolidone (PVP, M.W. 8,000, K16-18) was from ACROS, NJ; inositol was bought from EMD, Gibbstown, NJ.

**CPS Disc Centrifuge**

The size of rAd35 vaccine virus was measured by CPS Disc centrifuge (CPS instruments, inc., Suian, Florida). Sucrose (8% and 24%) in sample buffer was used for gradient solutions. CPSV95 software was set up for data collection, analysis and process control. The maximum speed was selected at 24000 rpm. The total injection volume for each analysis was 100 µL. A solution of PVC (20%, v/v) 0-377 Micro Calibration Standard was used for calibration.

**Spray Drying**

The spray drying powders were generated by a Buchi Mini Spray Dryer B-290. Nkroge was used as drying and atomizing gas. Ten different feed solutions were prepared: manntol; trehalose; leucine; manntol mixed with leucine, sucrose, PVP or inositol; raannitol in PBS buffer; manmto!cyclodexmn-trehalos e-dextran (MCTD), and manntol-trehalose-dextran-tveeri 80 (MTDT). Except leucine using 0,5%, the formulations were based on 10% solid of total volume. Formulations with the same concentrations without rAd35 were used for placebo tests. The inlet temperature was set a 65 to 125°C and the drying gas (low rate at 439 to 538 J/h) resulting in an outlet temperature of 34 to 50°C. The aspirator rate was 35 mA/′. The spray drying process and subsequent powder aqquoting were executed in a BioProtect II hood (The Baker Co.). To minimize both environmental microbial contamination to the powder and small powder particles released to environment, the spray dryer was assembled with a PTFE outlet filter and a 0.2-/tn EMFLON Filter (Pall Life Sciences, USA) fitted to the compressed air line.

**Differential Scanning Calorimeiry (DSC)**
The thermodynamic behavior of the powders was determined on a DSC 823* (METTLER TOLEDO, Switzerland). The cover of the crucible containing the powder sample was punched with a small hole before analysis. The sample (around 10 mg powder) was heated from 25°C to 170°C with a scanning rate of 10,0°C/min. The sample cell was purged with a nitrogen gas of 10.0 mL/min. The glass transition temperature ($T_g$) was recognized on the reversing heat flow curve as an endothermic shift of the baseline and determined as the midpoint of this transition by a STAR® SW9.01 software (METTLER).

**Particle Size Distribution, Polydispersity and Morphology**

Particle size distributions were measured by laser diffraction (Mastersizer 2000, Malvem, Worcs, UK). The polydispersity of the powder was expressed by the span. Span = 10HV,90V-D(V,10)/D(V,50), where D(V,90), D(V,50) and D(V,5) are the equivalent volume diameters at 90, 50 and 50% cumulative volume, respectively. The particle size of the primary powders was described by the volume median diameter (VMD), which is related to the mass median diameter (MMD) by the density of the particles (assuming a size independent density for the particles). A microscope (Axioskop 40, Zeiss) was used to examine particle morphology of spray-dried powders. The niarmitol powders were re-suspended in anhydrous methanol at around 20 mg/mL. A drop of this suspension was placed on a clean microscope slide. After 2 minutes, the slide was examined with the oil immersion objective (×100) and a × 40 ocular. Re-suspension of trehalose powder in methanol was found to be impractical because of high solubility. For trehalose, the method of Tracy et al. (Tracy PH, Hetrick JH, Karenke WA (1951) J. Dairy Set 34 (6):53-56) was modified as follows: about 20 mg of powder was mixed with 1 mL of Halocarbon 0.8 oil, and a drop of the suspension was examined with a cover slip.

**TCID$_{50}$ Assay**

Ad.$\Delta$5 virus titers in the original feed solutions and in the corresponding powders were determined by titration in TCID$_{50}$ tests. The TCLO titer value (tissue culture infectious dose) was determined by the greatest dilution at which cytopathic effects (CCPH) were observed on human embryonic retinoblast cells (HER-911 cells) in a TCID$_{50}$ assay. Briefly, the 911 cells were cultured in a 75 cm$^2$ flask containing Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and antibiotics.
{penicillin aad streptomycin). When cells were confluent, cells were detached using Trypsin-EDTA solution and the cell concentration was adjusted to 4 x 10^5 cells/mL. Cell suspension (1(K) μL) was seeded in one or two 96-well flat bottom tissue culture plates and incubated at 37°C with 10% CO₂ for 4 hr. After cells were attached the media was removed and 160 μL of medium was dispensed into all the wells. Then 40 μL each of pre-diluted vims was added to 8 wells in the first column and subsequently 5-fold serial dilution was performed in the plates for dilutions ranging from 10⁻¹ to 10⁻¹⁰ or TCID₅₀ to 10⁻¹⁰⁻¹⁷, depending on the expected titer value, and the plates were incubated for 14 days. CPE was scored on day 14 and the vims titer was determined employing the Spearman-Karber formula as 6 llows.

$$\log_{10} \text{TCID}_5 \text{su} / 100 \text{ML} = X_0 / \log_{10} \text{frt/2} + (\sum \gamma) / n$$

Where $\log_{10}$ is the log of the reciprocal of the highest dilution at which all testing columns are CPE positive, 'f' represent the logjo value of the dilution factor ($d = 0.699$ for 5-fold dilution factor) and 'n' is the number of wells for each dilution. ‘$\sum \gamma$’ is the sum of all wells that give CPE, from the dilution ‘$X_0$’, including CPE of dilution ‘$X_0$’. The result of the titer value was adjusted for initial dilutions and reported as TCID₅₀/mL.

**Karl Fischer Titration**

The residual moisture content after spray drying and the water content after 2 to 6 hr storage in a humidity box (70%, humidity detector from VWR, USA), were evaluated by a THvISSO Karl Fischer (Radiometer Analytical) in a dry box. The samples C.t30mg) were resuspended in absolute, dry methane*) (Phillipshurg, NJ). After background standby, the titration started automatically. During this titration, water molecules react stoichiometrically with the AQUA STAR α CombiTitrant S (BMD) reagent; subsequently, the volume of CombiTitrant 5 used to reach the endpoint of titration is used to calculate the percentage of water present in the sample (ImL CombiTitrant 5 = 5 rag water). All titrations were performed in triplicate-
CLAIMS

1. An immunogenic composition comprising:
   one or more live viruses in a powder formulation spray dried from a composition
   comprising maiaiitol at 10-150 mg/mL eyelodextrin at 0.1-10 mg/mL trehalose at 0.2-30
   mg/mL and dextran at 0.1-30 mg/mL.

2. The immunogenic composition of claim 1 wherein said powder formulation is spray
   dried from a composition which comprises maraiitol at 50-100 μg/mL, eyelodextrin at
   0.2-1 mg/mL, trehalose at 0.5-5 mg/mL, and dextran at 0.5-5 mg/mL.

3. The immunogenic composition of claim 1 wherein said eyelodextrin includes one or
   more of α- or β- or γ-cyclodextrin.

4. The immunogenic composition of claim 1 wherein said dextran has a molecular weight
   ranging from 25K to 500K.

5. The immunogenic composition of claim 4 wherein said dextran has molecular weight
   ranging from 40K to 90K.

6. The immunogenic composition of claim 1 further comprising buffering agents.

7. The immunogenic composition of claim 1 wherein said one or more viruses includes at
   least one virus that is a genetically engineered viral vaccine vector encoding one or
   more passenger genes that are foreign to said genetically engineered viral vaccine vector.

8. The immunogenic composition of claim 7 wherein said viral vaccine vector is non-
   replicating or replication deficient.

9. The immunogenic composition of claim 7 wherein said viral vaccine vector is an
   adenovirus.
10. The immunogenic composition of claim 7 wherein said viral vaccine vector includes nucleic acid sequences that encode one or more proteins that interfere with mammalian host cell type \( \alpha \) interferon (IFN) responses.

11. The immunogenic composition of claim 7 wherein said viral vaccine vector expresses one or more viral, bacterial or parasitic antigens from said one or more passenger genes.

12. The immunogenic composition of claim 7 wherein said one or more passenger genes express one or more tuberculosis antigens.

13. The immunogenic composition of claim 7 wherein said one or more passenger genes express one or more malarial antigens.

14. The immunogenic composition of claim 7 wherein said one or more passenger genes express one or more HIV antigens.

15. The immunogenic composition of claim 1 wherein said powder formulation has a median volume diameter of 3.2-3.5 \( \mu \)m.

16. A method of forming an immunogenic composition, comprising:
   spray drying one or more live viruses from a composition comprising maaoitol at 10450 \( \text{mg/ml} \), eyck\( \text{dext} \)\( \text{f} \)\( \text{n} \) at 0.1-10 \( \text{mg/ml} \) trehalose at 0.2-30 \( \text{mg/ml} \), and dextran at 0.1-30 \( \text{mg/ml} \) to form a spray dried powder containing said one or more live viruses.

17. The method of claim 16 wherein said one or more viruses includes at least one \( \alpha \)-virus that is a genetically engineered viral vaccine vector encoding one or more passenger genes that are foreign to said genetically engineered viral vaccine vector.

18. The method of claim 17 wherein said viral vaccine vector is non-replicating or replication deficient.
19. The method of claim 17 wherein said viral vaccine vector is an adenovirus.

20. The method of claim 17 wherein said viral vaccine vector expresses one or more viral, bacterial or parasitic antigens from said one or more passenger genes.

21. The method of claim 17 wherein said one or more passenger genes express one or more tuberculosis antigens, malarial antigens, or HIV antigens.