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Thermal stability of self-assembled peptide vaccine materials

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ABSTRACT

The majority of current vaccines depend on a continuous "cold chain" of storage and handling between 2 and 8 °C. Vaccines experiencing temperature excursions outside this range can suffer from reduced potency. This thermal sensitivity results in significant losses of vaccine material each year and risks the administration of vaccines with diminished protective ability, issues that are heightened in the developing world. Here, using peptide self-assemblies based on the fibril-forming peptide Q11 and containing the epitopes $OVA_{323-339}$ from ovalbumin or $ESAT6_{51-70}$ from *Mycobacterium tuberculosis*, the chemical, conformational, and immunological stability of supramolecular peptide materials were investigated. It was expected that these materials would exhibit advantageous thermal stability owing to their adjuvant-free and fully synthetic construction. Neither chemical nor conformational changes were observed for either peptide when stored at 45 °C for 7 days. $ESAT6_{51-70}$-Q11 was strongly immunogenic whether it was stored as a dry powder or as aqueous nanofibers, showing undiminished immunogenicity even when stored as long as six months at 45 °C. This result was in contrast to $ESAT6_{51-70}$ conjugated to a protein carrier and adjuvanted with alum, which demonstrated marked thermal sensitivity in these conditions. Antibody titers and affinities were undiminished in mice for $OVA_{323-339}$-Q11 if it was stored as assembled nanofibers, yet some diminishment was observed for material stored as a dry powder. The OVA study was done in a different mouse strain and with a different prime/boost regimen, and so it should not be compared directly with the study for the ESAT epitope. This work indicates that peptide self-assemblies can possess attractive thermal stability properties in the context of vaccine development.

Statement of Significance

Almost all current vaccines must be maintained within a tight and refrigerated temperature range, usually between 2 and 8 °C. This presents significant challenges for their distribution, especially in the developing world. Here we report on the surprisingly robust thermal stability of a self-assembled peptide vaccine. In particular a self-assembled peptide vaccine containing a tuberculosis epitope maintained all of its potency in mice when exposed to an extreme thermal treatment of six months at 45 °C. In a different mouse model, we investigated another model epitope and found some storage conditions where potency was diminished. Overall this study illustrates that some self-assembled peptide vaccines can have remarkable thermal stability.

1. Introduction

Almost all current vaccines must be maintained within a tight and refrigerated temperature range, usually between 2 and 8 °C. Any excursions outside this range can diminish a vaccine's efficacy or require that it be discarded. This challenge is presently addressed using the "cold chain" system of constant refrigeration from production to administration. This system, relying on refrigerated packaging, refrigerated shipping vehicles, clinics with reliable refrigerators, and well-trained staff, is highly effective when executed properly, but it is costly and prone to lapses in temperature control [1,2]. It is also at times difficult or impossible to implement, especially in the developing world or in locations without reliable electricity. Because of this, the introduction of new thermally stable vaccines that do not rely heavily on the cold chain has become an important goal [3,4].

Temperature excursions can damage vaccines in various different ways, depending on their construction and formulation. High

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temperatures can cause protein antigens to unfold in subunit vaccines, dissociate polysaccharides from protein carriers in conjugate vaccines, or reduce the viability and infectivity of live attenuated vaccines [3]. Different vaccines within each of these categories have shown variable sensitivities (reviewed in [3–5]), although live attenuated organisms tend to be especially problematic. For example, most measles vaccines based on live viruses can only withstand exposure to 37 °C for about 7 days [4]; some can lose potency by a factor of 10 after only 8 h at 37 °C [6]. Liquid-formulated oral poliovirus vaccines based on live attenuated virus are only stable at 37 °C for two days [3,5]. The live attenuated tuberculosis vaccine BCG loses greater than 20% effectiveness after one month at 37 °C and is unstable above 45 °C [3,5]. Vaccines not based on live organisms can fare better. For example, diphtheria and tetanus toxoids in liquid formulations can be stable for months at 37 °C, and because they are simple polypeptides, they only lose their potency when heated above temperatures at which secondary structure is lost [5]. Liquid formulations of hepatitis B vaccines, which are composed of recombinant purified hepatitis B surface antigen adsorbed onto aluminum salts, are also relatively stable. However, at 45 °C, even these relatively stable vaccines can lose potency over the course of a few weeks (for toxoids [5]) or days-to-weeks (for hepatitis B vaccines [7,8]). Further, toxoid vaccines and those based on recombinant protein antigens require adjuvants, and when they are formulated with alum adjuvant, they are sensitive to freezing and freeze–thaw cycles [3,5]. One study found more than a 60% reduction in potency of some components of alum-adjuvanted diphtheria–tetanus–pertussis (DTP) vaccines after only two freeze–thaw cycles [5]. For alum-adjuvanted vaccines, freeze–thaw cycles can induce permanent, irrecoverable changes in the association between alum particles and the antigen and lead to aggregation and precipitation, all of which diminishes efficacy [5,9]. In this way, the presence of adjuvant complicates a vaccine's thermostability.

Deviations above the safe temperature window can happen for a number of reasons, including faulty or poorly maintained refrigeration equipment, lack of refrigeration capacity, power outages caused by any number of events such as weather or loss of fuel, or errors in handling, especially during arrival procedures [5]. Inadvertent freezing can occur as a result of improperly controlled refrigeration or shipping on ice packs. Assessments made by the World Health Organization have highlighted each of these issues for improvement [5], as the consequences of such deviations, both above and below 2–8 °C can be significant. In the best cases, any temperature excursions are known and caught before the vaccine is administered, and the vaccine is discarded. This leads to significant wastage but does not put patients at risk. In worse cases, unknown temperature deviations result in diminished vaccines being administered to patients, putting them at risk for infection. Therefore, there is a significant need for vaccines that are stable to both freezing conditions and elevated temperatures, and thermostable vaccines are seeing active development [10,11].

Recent efforts include stabilizing vaccines within silk films [10], by mineralizing the surface of viral capsids [11], by lyophilization [12], by optimizing formulations and utilizing various stabilizing agents [3]. Here we report on the thermal stability characteristics of self-assembled peptide nanofiber vaccines, which are both self-adjuvanting and constructed from short, unfolded peptide epitopes. Short peptides containing fibrillizing domains such as Q11 (QQKFQFQFEQQ) [13–18] or KFE8 (FKFEFKFE) [17] can be appended with T or B cell epitopes; when they are mixed with physiologic buffers, media, or fluids, they self-assemble into nanofibers displaying the epitopes [19], and we have found that these nanofibers raise strong immune responses without supplemental adjuvants [13,15–17]. The modularity of the platform makes it simple to adjust the epitope content within the nanofibers, and we have observed phenotypic modulation of T cell subsets when the T and B cell epitope ratios are varied [15]. In this way, self-assembled peptide nanofibers are a promising platform for immunotherapy development owing to their adjuvant-free and tailorable properties.

In this study, we investigated the thermostability of self-assembled peptide nanofibers, hypothesizing that their purely synthetic design and lack of any adjuvant, proteins, or live organisms would make them advantageously thermostable compared to other more conventional vaccine platforms. We investigated two model epitopes, $OVA_{323-339}$ and an epitope corresponding to residues 51–70 of *Mycobacterium tuberculosis* 6 kDa early secretory antigenic target (ESAT-6), synthesizing self-assembled nanofibers from both using the Q11 assembly domain. Both epitopes are able to raise B cell and T cell responses [13,20]. Having both a B cell epitope and a T cell epitope is essential for Q11-based vaccines, as Q11 nanofibers lacking either do not raise strong antibody responses [15,17]. We investigated the thermostability of lyophilized peptides as well as aqueous suspensions of nanofibers, finding that some formulations were more thermostable than others, with the most thermostable (ESAT-Q11) being capable of withstanding elevated temperatures of 45 °C for as long as six months without diminishment of effectiveness.

2. Materials and methods

2.1. Peptides, vaccine preparations, and heat treatments

The peptides Q11 (Ac-QQKFQFQFEQQ-Am), pOVA (also known as $OVA_{323-339}$, H2N-ISQAVHAAHAEINEAGR-COOH), OVA-Q11 (H2N-ISQAVHAAHAEINEAGR-SGSG-QQKFQFQFEQQ-Am), pESAT (corresponding to residues 51–70 of the 6 kDa early secretory antigenic target of *M. tuberculosis*, H2N-YQGVQQKWDATATELNNALQ-Am), ESAT-Q11 (H2N-YQGVQQKWDATATELNNALQ-SGSG-QQKFQFQFEQQ-Am), and Cys-pESAT (H2N-CYQGVQQKWDATATELN NALQ-Am) were synthesized using standard Fmoc solid-phase chemistry, purified by HPLC, and lyophilized as previously reported [21,22]. Epitope peptides were appended to the Q11 assembly domain by a flexible Ser-Gly-Ser-Gly linker. Cys-pESAT was conjugated to keyhole limpet hemocyanin (KLH) using the Imject Maleimide Activated mcKLH Kit (cat #77666) from Thermo Scientific. Peptide identity was confirmed using a Bruker Ultraflextreme MALDI-TOF mass spectrometer, using α-cyano-4-hydroxyc innamic acid as the matrix.

To prepare immunizations, lyophilized OVA-Q11, ESAT-Q11 or pESAT peptides were dissolved in sterile water at 8 mM and stored overnight at 4 °C. Then, sterile and endotoxin-free 1× PBS (HyClone SH30028.02) was added to bring the peptide to a working concentration of 2 mM, and the solution was incubated 3–5 h at room temperature before immunization. This step, mixing with PBS, induced assembly into nanofibers, as we have previously reported [15,17,18]. For adjuvanted peptide/carrier groups, 0.1 mg Cys-pESAT conjugated to KLH was mixed 1:1 v/v with Imject Alum (Pierce) and vortexed for 30–60 min to allow adsorption. Endotoxin measurements were conducted on the same peptide solutions that were used for immunizations, using the Limulus Amebocyte Lysate chromogenic endpoint assay (Lonza). Endotoxin levels of all peptide solutions used for immunization were <0.1 EU/mL (<0.01 EU per 100 μL dose).

For heat treatments, peptides were either heated as lyophilized powders (before the initial water dissolution step), or after they had been formed into final nanofibers following the water and PBS dilution steps described above (2 mM in PBS final peptide concentration). Peptides were heated in one or the other condition to between 45 and 80 °C for time periods ranging from 1 day to