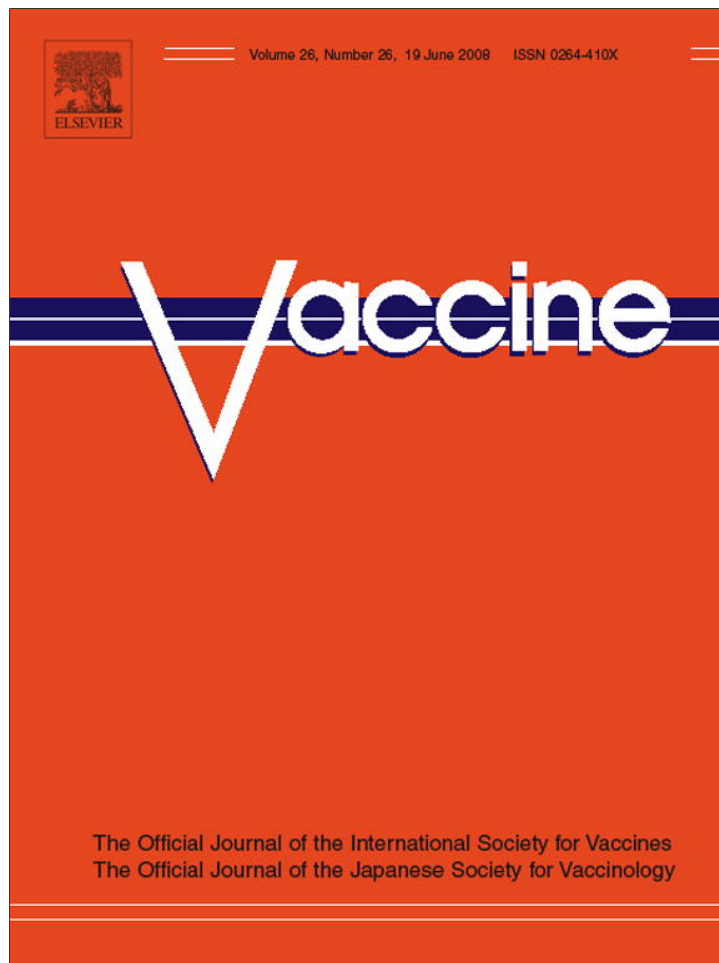


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Short communication

Adjuvant potential of aggregate-forming polyglutamine domains

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ABSTRACT

Aggregation may significantly affect the fate of a polypeptide, including its susceptibility to proteasome-dependent or autophagic degradation, its interaction with chaperones, etc. Since all these factors may affect the antigenicity of a polypeptide, we hypothesized that stimulating aggregation of an antigenic protein by its fusion to polyQ domain may enhance its antigenic potential. This hypothesis was tested with the weakly immunogenic model antigen GFP, which was fused to either long polyQ domain that triggers protein aggregation (103Q), or short polyQ domain that does not promote aggregation (25Q). Plasmids encoding control pGFP or soluble 25Q-GFP generated a very weak antibody response, while a significant increase in anti-GFP antibody titer was seen in groups immunized with DNA encoding aggregating 103Q-GFP. Similarly, fusion with 103Q strongly enhanced anti-GFP CTL activity, compared to fusion with 25Q. No apparent toxicity was observed after immunization with polyQ-GFP fusions. These data suggest that fusion of an antigen with expanded polyQ domains could have a significant adjuvant potential.

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1. Introduction

A need to develop novel vaccines against major infectious agents (HIV, SARS, influenza), and the low immunogenicity of recombinant vaccines warrant a search for adjuvants able to stimulate both cell-mediated and humoral immunity. Success in this area has been relatively limited since adjuvants that significantly augment the immune response (e.g., Freund's complete adjuvant), generally have unacceptable local or systemic toxicity that precludes their utilization in humans [1]. However, advances in dissecting the antigen presentation machinery offer new insights into mechanisms of adjuvant effects. For example, the recent finding that certain intracellular antigens are presented via the MHC-II pathway suggests that autophagic pathways may play a special role in the adaptive immune response. Indeed, it appears that autophagy could function as routes of delivery of cytosolic proteins to lysosome for generating antigenic peptides that bind to MHC class II molecules [2,3]. The role of autophagy in MHC class II-dependent presentation was demonstrated for

nuclear antigen 1 of Epstein-Barr virus [4], influenza matrix protein M1 [5,6], and other antigens. Based on these multiple data it appears that artificial targeting of an antigenic polypeptide to the autophagic pathway may lead to augmented immune responses.

Another important function of autophagy is the clearance from cells of protein aggregates or oligomers. This function is especially important for protection from toxic aggregates of misfolded proteins in various neurological disorders [7,8]. In several types of neurodegeneration (e.g., Huntington's disease) protein aggregation is caused by the expansion of polyglutamine (polyQ) domains. Indeed, when polyQ domain of huntingtin protein exceeds 36 glutamines, it starts to form insoluble aggregates eventually leading to the development of disease [9,10]. These aggregates can be cleared by autophagy. Fusion of such an expanded polyQ domain to a polypeptide could cause aggregation of the latter and targeting to the autophagic pathway. Accordingly, we hypothesized that attachment of the expanded polyQ domain to a cytosolic protein may augment its immunological presentation. To test this hypothesis, we fused the weakly immunogenic green fluorescent protein (GFP) with polyQ domains [11], and tested the immunogenicity of these modified GFP forms in a DNA vaccination model. Fusion to aggregate-forming polyQ domains resulted in a dramatic increase of GFP immunogenicity.

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2. Materials and methods

2.1. Cells and plasmids

All experiments were performed in 293 human embryo kidney (HEK) cells. PolyQ-GFP constructs were described in [12,13]. 103QP represents the entire exon 1 of huntingtin, including the 17 amino acids N-terminal region, polyQ domain with 103 glutamine residues, and the proline-rich region. 103Q represents exon 1 lacking the proline-rich domain. 25Q is a shorter version of 103Q, having stretch of 25 glutamines [13]. All polyQ-GFP fusion genes were sub-cloned into pCAGGS expression vector [14] and used for cell transfection and vaccination experiments.

PolyQ-GFP-expressing plasmids were transfected into 293 HEK cells at 60–80% confluence in 35 mm plates using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) for 4 h (1.5 μ g of total plasmid DNA per 3 μ l LF2000). Control cells were transfected with the same amount of GFP-containing pCAGGS. Green fluorescence of transfected cells was visualized at 24 h post-transfection. Fluorescent microscopy was performed at room temperature with Axiovert 200 (Carl Zeiss, Germany) microscope.

2.2. Immunization with polyQ-GFP-encoding plasmids and monitoring the immune response

20 μ g of polyQ-GFP or GFP-encoding plasmids were injected intramuscularly into each experimental mouse three times with 14-day interval (in 100 μ l of PBS, 8-wk Balb/c females, 3–4 animals per group). Blood was drawn at 10 days after the 3rd immunization or after 2nd and 3rd immunizations and used for ELISA against GFP (Millipore). At the time of the last bleed (10 days after the third DNA vaccination), mice were sacrificed, their splenocytes were purified and stimulated ($\sim 10^8$ total, plated at 5×10^6 /ml) *in vitro* by 5 μ g/ml of GFP (Millipore) for 5–7 days (incubated in DMEM containing 10% FCS and 2 μ M of 2-mercaptoethanol). Mouse mastocytoma p815 cells pulsed with GFP 4–24 h were used as a target and cytotoxic activity was measured by lactate dehydrogenase (LDH) release (CytoTox 96 Kit; Promega). Target p815 cells (0.3×10^5 /well) were mixed with 2-fold dilutions of stimulated effector cells starting with 3.0×10^6 cells/well and incubated in 100 μ l volume for 4 h at 37 $^{\circ}$ C. CTL activity as the percentage of cell lysis was calculated by the following formula: (experimental release – spontaneous release)/(maximum release – spontaneous release) $\times 100$. Target cells incubated in medium alone and with medium containing 1% detergent NP-40 were used to determine spontaneous and maximum LDH release. Results shown were means (\pm S.D.).

3. Results and discussion

To test the hypothesis that the attachment of a polyQ sequence could augment the antigenic properties of a polypeptide, we used three distinct polyQ domains derived from huntingtin [13]. As a model antigenic polypeptide we used GFP, which is known to be weakly immunogenic if unmodified and had been successfully used to demonstrate adjuvant potential of several molecular modifications [15,16]. Thus, GFP gene was fused with sequences containing either the entire exon 1 of huntingtin, including the 17 amino acids N-terminal region, the polyQ domain and the proline-rich segment (p103QP-GFP), or exon 1 of huntingtin lacking the proline-rich region (p103Q-GFP). 103 glutamines represent a polyQ domain of pathological length that is prone to aggregation. Two distinct aggregate-forming sequences (i.e., 103QP and 103Q) were used since the proline-rich region could affect the properties of the polypeptide [17,18]. As a control we used 25Q, a polyQ segment of normal length that is not prone to aggregation, resulting in the p25Q-GFP construct. Although the aggregation properties of these polypeptides have previously been investigated [12,13], in this study we used the highly expressing vector pCAGGS [14] for vaccination, making it essential to test aggregation of GFP fused to various length polyQ domains in this expression system.

293 cells were transiently transfected with these plasmids, and protein aggregation was monitored under the fluorescence microscope. As expected, fusion of expanded polyQ domains, 103Q or 103QP to GFP led to the formation of multiple fluorescent aggregates in more than 60% of cells (Fig. 1C and D), while the expression of GFP alone produced a diffuse cytoplasmic fluorescence (Fig. 1A). In the vast majority of cells, the expression of 25Q-GFP also produced the same type of cytoplasmic fluorescent pattern as GFP alone, with less than 5% of the cell population showing fluorescent aggregates (Fig. 1B). This was in agreement with previous data showing that polyQ domains are soluble if the length of polyQ sequence is less than 35, and have a tendency to aggregate if the length exceeds 36 glutamines [9,10]. Collectively, this system appears to be suitable for the study of the role of protein aggregation in antigen presentation.

To investigate the immunogenic properties of the polyQ-GFP fusions, we immunized several groups of Balb/c mice with pGFP, p103Q-GFP and p103QP-GFP plasmids and tested the humoral response against GFP. In addition, we assayed for the effects of various prime/boost combinations of GFP/polyQ-GFP on the anti-GFP immune response augmentation. The rationale for the latter experiment was that in many systems a heterologous prime/boost is known to be more potent than a repeated homologous immunization [19–23].

The immune response to vaccination with pGFP, p103Q-GFP and p103QP-GFP plasmids differed dramatically. In fact, while control

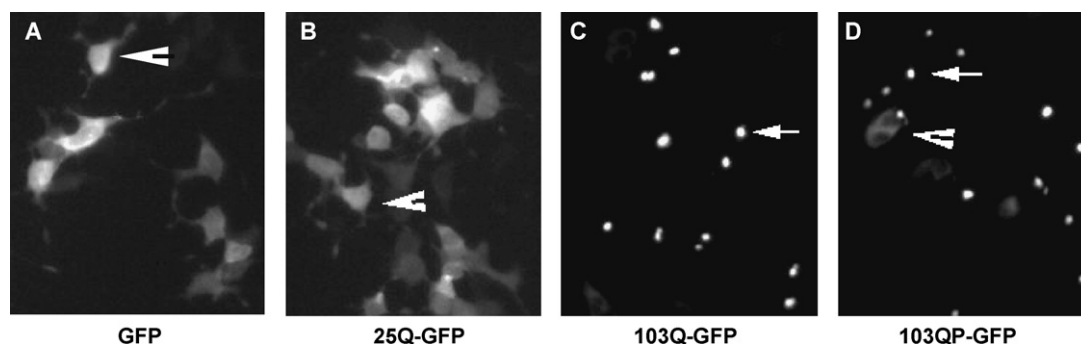


Fig. 1. Aggregation properties of polyQ-GFP constructs used in this study. HEK 293 cells were transfected by: (A) pGFP, (B) p25Q-GFP, (C) p103Q-GFP, (D) p103QP-GFP. GFP aggregates are indicated by arrows and the cells with diffused GFP fluorescence are indicated by arrowheads.

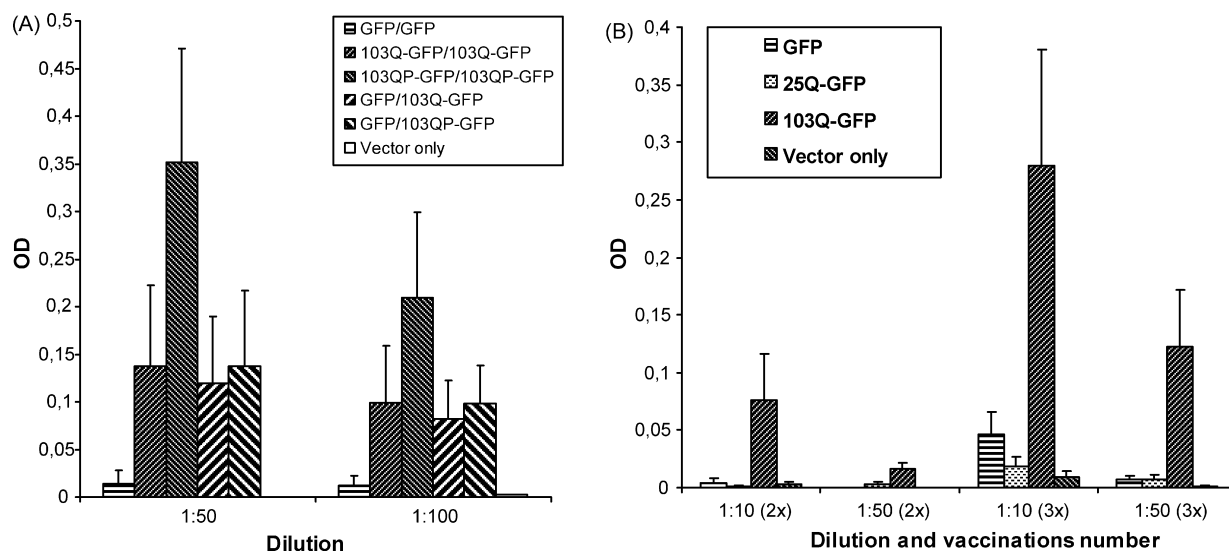


Fig. 2. Humoral immune response to polyQ-GFP fusion polypeptides. Mice were immunized as described in the text. Mean anti-GFP antibody titers are shown. (A) Immunization with indicated prime/boost combinations of GFP- or aggregate-forming polyQ-GFP-encoding plasmids. (B) Immunization with aggregate-forming and non-aggregate-forming polyQ-GFP plasmids.

GFP plasmid generated a very weak humoral immune response, plasmids encoding polyQ-GFP fusions generated 10–30 times higher anti-GFP antibody titers (Fig. 2A). Although both 103Q-GFP and 103QP-GFP markedly enhanced the antibody response, there was no significant difference in the magnitude of enhancement of the humoral responses generated by these two fusion proteins. Therefore, the presence of the proline-rich region does not seem to affect the adjuvant properties of polyQ. When pGFP prime was followed by two boosts by either p103Q- or p103QP-GFP (GFP/103Q-GFP and GFP/p103QP-GFP groups), a significant augmentation of humoral response to GFP was also observed (Fig. 2A), although this effect was not stronger than the effect of homologous immunization with polyQ-GFP constructs.

To address whether the adjuvant effects of polyQ domains are related to their aggregation properties, we compared the immunogenic capacity of aggregating 103Q-GFP and soluble 25Q-GFP (see Fig. 1). In these experiments, immunization with 25Q-GFP-encoding plasmid could not significantly augment the humoral immune response compared to GFP control, while 103Q-GFP dramatically enhanced the anti-GFP antibody response (Fig. 2B).

It is well known that DNA vaccination is an effective inducer of the antigen-specific CD8⁺ T-cell response. Therefore, in parallel experiments we evaluated the ability of polyQ domains to potentiate the CTL response against target antigen. Accordingly, anti-GFP CTL activity was assayed in spleens of animals from experimental groups immunized either with pGFP or polyQ-GFP (Fig. 3). While no cytotoxic activity was detected in splenocytes from pGFP-immunized mice, significant cytotoxic activity against GFP was exhibited by splenocyte cultures from animals immunized with p103QP-GFP or 103Q-GFP. Interestingly, 25Q-GFP also increased splenocyte cytotoxicity, although its effect was significantly lower than that of 103Q or 103QP (Fig. 3). These results indicate that polyQ-mediated aggregation strongly enhances both antibody and cytotoxic responses against a model antigen.

Our original rationale for testing the effects of polyQ fusion on the antigenic properties of a polypeptide was based on the hypothesis that polyQ-mediated aggregation could target the polypeptide to the autophagic pathway, which can lead to MHC class II presentation for CD4⁺ T-cell recognition [3,24] or even enhance CD8⁺ T-cell response via strengthening of immunological memory or other related pathways [25]. Antigen aggregation driven by the polyQ

domain may also promote formation of the so-called dendritic cell aggresome-like induced structures, DALIS, which are important for maturation of APC [26,27].

The augmentation of cytotoxic activity that we observed may also point to the possibility that polyQ-fused GFP efficiently triggers CD4⁺-mediated priming of Th-dependent CD8⁺ CTL responses. This priming is required for effective CTL response in a number of systems and has recently been shown to involve the acquisition of APC-presenting machinery by CD4⁺ cells, i.e., not only MHC-II, but also bystander MHC-I-peptide complexes [28,29]. It should also be noted that although the GFP-directed cytotoxicity that we observed following DNA vaccination is likely caused by CTL, complement-dependent cytotoxicity or antibody-dependent macrophage-mediated cytotoxicity could also contribute to this

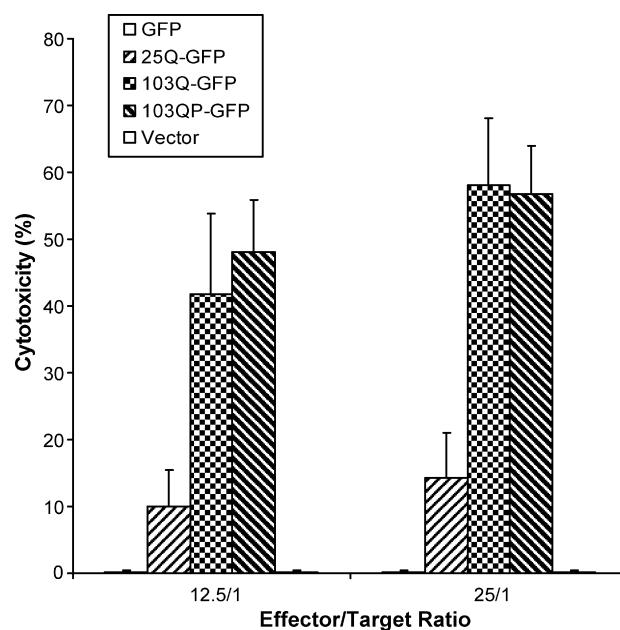


Fig. 3. Anti-GFP CTL induction by aggregating and soluble polyQ-GFP fusion polypeptides. Mean CTL activities in mice immunized with pGFP, p25Q-GFP, p103Q-GFP and p103QP-GFP are shown.

phenomenon. Additionally, it is possible that other mechanisms may also be involved in the augmentation of immunogenicity. For example, polyQ-GFP aggregates could be released from dying cells, and taken up by macrophages or other antigen-presenting cells, leading to their activation. Such mechanism of action would resemble the effect of aluminum-containing adjuvants, which are known to promote antigen internalization by APC by both macropinocytosis and phagocytosis [30] resulting in the augmentation of an antigen's immunogenicity via both Th1 and Th2 pathways [31]. Importantly, aluminum hydroxide adjuvant does not exist as primary solitary particles but forms aggregates [32], whose size and structure modulate immune response by trapping the antigen within the site of injection [33,34]. Here we have shown that independent of the mechanism, fusion of an antigen with expanded polyQ domains appears to have a significant adjuvant potential. Importantly, since this phenomenon was dependent on antigen aggregation, the immunostimulatory potential of other aggregate-promoting domains, sequences or proteins is also likely and should be evaluated in the future. Other aggregation-promoting domains may involve a variety of sequences derived from either naturally aggregating polypeptides (e.g., α -synuclein or prions), or from mutant polypeptides that aggregate due to mutation-promoted misfolding.

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