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A Novel Adjuvant for Vaccine Development in the Aged

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Abstract

A conformationally-biased, response-selective agonist of human $C5a_{65-74}$ (EP67) activated antigen presenting cells (APC) from aged C57Bl/6 mice *in vitro* and the generation of antigen (Ag)-specific antibody (Ab) responses in aged mice *in vivo*. EP67, induced the release of the pro-inflammatory cytokines IL-6, TNFα, and INFγ from splenic APCs obtained from both aged and young mice. Both aged and young mice produced high Ag-specific IgG Ab titers when immunized with EP67-containing vaccines to ovalbumin (OVA-EP67) and to a protein (rPrp1) from the cell wall of *Coccidioides* (rPrp1-EP67). Immunization with EP67-containing vaccines resulted in higher IgG titers in both young and aged mice compared to mice immunized with OVA adsorbed to alum (OVA/alum) and Prp1 admixed with CpG (rPrp1 +CpG). Aged and young mice immunized with the EP67-containing vaccines generated higher titers of IgG1 and IgG2b relative to their aged-matched counterparts immunized with OVA/alum or Prp1 + CpG. These results indicate that EP67 induces humoral immunity in aged mice not obtainable with alum and CpG. These results support the use of EP67 as a potential vaccine adjuvant suited to the elderly.

Keywords

Adjuvant; vaccine; aging; immunization; cytokines; antibody response responses

1. Introduction

A prominent manifestation of aging is a decline in immune system responsiveness. This puts the elderly at a therapeutic disadvantage when it comes to vaccination, particularly with vaccines that contain antigens (Ag) to which they have had no history of exposure. Obtaining efficacious immune outcomes in an age-compromised immune system therefore presents a significant challenge in vaccine development.

Changes in T lymphocyte function correlate with the poor responsiveness of the elderly to vaccination. These changes include decreased proliferative responses, impaired cytolytic activity, a shift from naïve to memory phenotype, alterations in cytokine/chemokine secretion, and an increase in suppressive regulatory T-cells (Tregs) [1-11]. Loss of T

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lymphocyte function may be, in part, a downstream manifestation of impaired/diminished function of antigen presenting cells (APC) in the elderly. It is known that APCs from aged individuals are less effective in their ability to process and present Ags, to up-regulate co-stimulatory factors for engagement of $CD4^+$ and $CD8^+$ lymphocytes, and to produce and respond to certain cytokines and chemokines [9,12-17]. The inability to induce efficient cytokine production (IL-6, IL-12, TNF-α, and INF-γ) by aged APCs is likely to play a significant role in the inability of a vaccine to provide optimal immunity in the elderly.

We have developed a conformationally-biased, response-selective agonist of the biologically active C-terminal region of human complement-derived component $C5a_{65-75}$ (ISHKDMQ-LGR). This analogue, EP67 (YSFKDMP(MeL)aR), is the result of residue substitutions designed to restrict backbone flexibility in $C5a_{65-74}$, which have biased specific topochemical features that enable a conformational distinction between inflammatory activities versus immune stimulatory activities. EP67 is devoid of C5a-like neutrophil and neutropenic activities yet retains C5a-like activities in terms of its ability to engage and activate antigen presenting cells (APC) and induce Ag-specific humoral and cell-mediated immune responses (18-20). We have recently reported that EP67 significantly enhanced OVA-specific Ab responses in young C57Bl/6 mice [18]. In this paper, we extend those findings by examining the response of aged animals to EP67 vaccine constructs. In addition, we compared the EP67 vaccine response with two established adjuvants (alum and CpG) with two different antigens for the ability to enhance antibody responses in aged mice.

2. Materials and Methods

2.1 Reagents

The reagents employed in these studies were obtained from the following suppliers: RPMI 1640 (Mediatech Cellgro, Manassas, VA); fetal bovine serum and newborn calf serum (Gibco/Invitrogen, Carlsbad CA); biotin-donkey anti-mouse IgG (Biosource, Camarillo, CA); AP-goat anti-mouse IgG1, anti-mouse IgG2a and IgG2b (Bethyl Laboratories, Montgomery, TX), anti-mouse IgG2c (Beckman Coulter, Fullerton, CA); ExtrAvidin-AP, Sigma Fast p-nitrophenyl phosphate Tablet sets, and ovalbumin (Sigma, St. Louis, MO); unconjugated and biotin-conjugated anti-mouse IL-6, TNFα, and INFγ (BD Biosciences Pharmingen, San Diego CA); recombinant mouse IL-6, TNFα, and INFγ (eBioSciences, San Diego CA); and Fmoc-protected amino acids, Wang resins, and reagents for peptide synthesis (AAPPTEC, Louisville, KY). Recombinant Prp1 was provided by Dr. Garry Cole, Department of Biology and South Texas Center for Emerging Infectious Diseases, University of Texas-San Antonio, San Antonio, TX.

2.2 Peptides

EP67 [YSFKDMP(MeL)aR] and the inactive control peptide scrambled EP67 (sEP67) [(MeL)RMYKPaFDS] were synthesized by solid-phase methods as previously described [19]. EP67 with an N-terminal succinimidyl-4-benzoylhydrazino-nicotinamide (S4BHyNic) linker was synthesized as previously described [20]. Peptides were purified by analytical and preparative reverse-phase HPLC on C18-bonded silica columns with 0.1% TFA as the running buffer and 60% acetonitrile in 0.1% TFA as the eluant. Peptides were characterized by molecular mass (MH^+) with MALDI mass spectrometry.

2.3 Vaccine Conjugates

Conjugation of EP67 to OVA and rPrp1 was accomplished by a single-step reaction using a version of EP67 in which its N-terminus was modified with succinimidyl-4-benzoylhydrazino-nicotinamide (S4BHyNic-EP67) as previously described [20]. These conjugates are designated OVA-EP67 or Prp1-EP67. Vaccine constructs were depleted of

endotoxin as previously described [21]. OVA was precipitated with, Al(OH)3/Ag using standard methodology and these constructs designated OVA/, Al(OH)3/Ag. A 10% solution of a, Al(OH)3 was mixed with a 1% solution of OVA, dropwise while stirring. The pH was adjusted to 6.5 by dropwise addition, while stirring, of 1 N NaOH. THe reaction wa allowed to stand for 30 min. The suspension was centrifuged at 1000 × g at 5 C for 10 min. Both the supernant and pellet were assayed for the amount of OVA present. The suspended pellet was dialyzed, sterile filtered and stored at −70 C in aliquots prior to injection.

CpG was simply admixed with the antigen and these constructs designated Ag+CpG.

2.4 Animals

Female C57Bl/6 mice were obtained from Harlan Laboratories (Los Angeles, CA) and used at 8-12 weeks of age. Aged mice (18-26 months old) were obtained through the NIA- aged rodent colony. Animals were maintained on a 12/12 light-dark cycle and fed standard rodent chow (Harlan #8604). All experiments were approved in advance by the institutional animal care and use committee and performed in accordance with institutional guidelines.

2.5 Preparation of Splenic Cells

Spleens were removed aseptically in complete media (CM) consisting of RPMI 1640, 10% FBS, 2mM L-glutamine, pen-strep, and 5×10^{-5} 2 ME. Single cell preparations were prepared as previously described [18,21]. Cells were maintained on ice in CM prior to use.

2.6 Cell Culture Procedures

For the generation of cytokines, spleen cells were cultured in CM at a concentration of 2.5×10^6/ml in 12 well or 96 well plates (Costar Corning, Cambridge, MA) in a humidified atmosphere containing 5% CO_2 for 24-48 hrs. Supernatants were collected and frozen at −20° C prior to analysis by ELISA.

2.7 Methods of Immunization

OVA—Mice were immunized IP with either 100 μg (delivered in 0.1 ml) of the OVA-EP67 or OVA/alum. For a secondary immune response mice were immunized on day 0 and boosted on days, 14 and 28. Blood was collected on days 14 and 42.

rPrp1—Mice were immunized IP on day 0 and boosted on days 14, 21 and 28 with either 5 μg of the rPrp1-EP67, 5 μg rPrp1 + 10 μg CpG, or 5 μg rPrp1 alone. Blood was collected on days 14 and 42.

2.8 Measurement of Cytokines

A sandwich ELISA was performed as previously described [18]. Samples were run in triplicate and the data presented as pg/ml ± SD. Each experiment was performed at least three times.

2.9. Measurement of Anti-OVA and Anti-Prp-1 Ab Responses

A direct ELISA was utilized to measure OVA- and Prp1-specific Abs derived from mouse serum as previously described [18]. Each experiment was performed at least three times.

2.10 Statistical Analysis

The student t test was utilized to evaluate the results.

3. Results

3.1 EP67-Containing Vaccines Induce Robust Ag-Specific Antibody in Aged Mice

Young and aged mice were immunized three times at two week intervals with ovalbumin either directly conjugated to EP67 (OVA-EP67), or precipitated with alum (OVA/alum). Two weeks following the last injection, sera from these animals were assessed for the presence of OVA-specific IgG. The results presented in Figure 1A indicate that mice vaccinated with the OVA-EP67 produced statistically higher anti-OVA Ab responses than those vaccinated with OVA/alum ($p<.01$). This was true for both young and aged mice.

In a second series of experiments, we compared the adjuvant activity of EP67 to that of CpG, using the recombinant protein rPrp1 as the vaccine antigen. rPrp1 is derived from the cell wall of the fungal pathogen *Coccidioides immitis*. As shown in Figure 1B, the anti-rPrp1 Ab response is significantly increased in both young and aged mice vaccinated with rPrp1-EP67 compared to age-matched animals given CpG-containing vaccines.

3.2 Immunoglobulin Subclass Distribution of Ag-Specific Immunoglobulin

Different adjuvants drive or polarize immune responses to distinct endpoints, characterized by different patterns of immunoglobulin subclass distribution. To determine in what manner EP67 might polarize immunoglobulin responses, the serum of animals immunized as described in Figure 1 were further analyzed for immunoglobulin subclass distribution profile. The results presented in Figure 2 indicate that in aged mice EP67 adjuvanted proteins induce increased production of both IgG1 and IgG2b relative to either CpG or alum. It is important to note the serum dilutions used for ELISA analysis of IgG subclasses varied based on the responses observed for total IgG. Serum from mice immunized with OVA/alum and rPrp1 + CpG were assayed at a dilution of 1:100 whereas serum from animals immunized with the EP67 conjugates was diluted to 1:1000 for assay in order to display all the data on the same graph.

3.3 Effectiveness of EP67 in a Single Dose Regimen

One purpose of a vaccine adjuvant is to reduce the total Ag required to induce a productive immune response. To evaluate EP67 in this regard, young and aged mice were administered a single dose of OVA-EP67 or OVA/alum. Fourteen days later, the animals were bled and the sera immunoglobulin levels quantified at a dilution of 1:100. As shown in Figure 3A, the total IgG response of aged mice given a single injection of OVA-EP67 is as robust as that of young animals (Panel A). Alum-adjuvanted OVA not only induced lower IgG levels, but was also much less effective in the aged animals than in the young. Immunoglobulin subclass analysis, shown in Figure 3B (IgG1) and 3C (IgG2b), mirrors the results of the total IgG analysis. Equivalent levels of both IgG1 and IgG2b are induced in young and aged mice by EP67, and both IgG1 and IgG2b levels are higher in the mice immunized with OVA-EP67 as compared with the age-matched animals immunized with OVA/alum.

3.4 EP67 Induces the Release of Cytokines from Aged Splenic Adherent Cells

Induction of immune responses in the aged is often improved by the presence of pro-inflammatory cytokines [1-4,7,22,23]. Here we compared the relative ability of the three adjuvants to induce IL-6 cytokine production by splenic adherent cells from aged and young mice. As shown in Table 1, alum was unable to trigger the production of IL-6 by either young or aged adherent cells. CpGs, while stimulating IL-6 production by the aged adherent cells, were less effective than EP67 in this regard. These results show that splenic adherent cells derived from aged mice respond to EP67 by the release of innate immune cytokines in a fashion similar to cells derived from young mice, and that EP67 is the most potent of the three tested adjuvants in triggering IL-6 production. The ability to induce other pro-

inflammatory cytokines by aged adherent cells was further assessed. We have previously reported that EP67 is a potent inducer of pro-inflammmatory cytokines from adherent spleen cells derived from young (3 mo) C57BL/6 mice [18]. Our purpose here was to compare production of the three pro-inflammatory cytokines, IL-6, IFN-γ and TNF-α induced by EP67, sEP67 and the potent toll receptor ligand, LPS in young and aged mice. Adherent spleen cells were prepared from 18-24 mo C57BL/6 mice and stimulated with EP67, s-EP67, or LPS for 24 hrs. Supernatants were collected and assayed for the presence of IL-6, TNF-α, and INF-γ. As shown in Figure 4, all three cytokines were effectively induced in the aged spleen cell cultures when stimulated either by LPS or EP67, while the negative control peptide, sEP67 was not effective. The EP67-induced cytokine response from aged mice was comparable to that induced in the cultures containing splenic adherent cells from young (3 mo) mice exposed to EP67.

4. Discussion

A hallmark feature of advancing age is reduced immune function resulting in dramatically lowered vaccine efficacy [24,25]. For example, the effectiveness of vaccination for influenza ranges from 70-90% in the young, but it falls to 17-45 % in persons older than 65 [26]. Similar declines with age in the percentage of the vaccinated population that is effectively protected occur with other vaccines as well [25]. Improving vaccines for the elderly by addition of an appropriate adjuvant could overcome aged immune deficiencies and improve levels of disease protection Results dissecting immune function in aged rodent models indicate that this may be a fruitful approach. Numerous analyses demonstrate that vaccine efficacy can be markedly improved in aged rodents by the inclusion of a potent adjuvant. [22-24,27-30]. The results presented in this study compare EP67 with two well-characterized adjuvants (alum and CpG). EP67 was found to be equivalent or better than these adjuvants in inducing Ag-specific Ab responses to multiple Ags.

The efficacy of a vaccine may be related in part to the Ig class and subclass induced by it. The choice of an adjuvant directly affects the type of the humoral immune response that is generated to the selected Ag. , Al(OH)3 primarily induces an IgG1 response, while CpG, a more potent adjuvant, shifts the humoral response towards increasing IgG2b [31,32]. The production of the IgG2 subclasses is of particular importance in anti-viral responses. The IgG2 subclasses are the best activators of complement, which plays an important role in viral elimination [33-35] and the IgG2a isotype is the most effective at binding to Fc receptors on macrophages and NK cells [36,37], enhancing their ability to eliminate virus [38-41]. We have previously reported that EP67, when combined with Ag, induced antigen-specific Ab responses response comprised of IgG1 and IgG2b Abs [18]. In this paper, we demonstrate that EP67 is equally effective in inducing IgG1 and IgG2b in aged mice as in young.

The results reported here indicate that EP67 is a potent adjuvant in the aged. It is likely that its function is due, at least in part, to the induction of inflammatory cytokine synthesis that secondarily drives increased T cell function. This is in line with the adjuvant properties of Toll-like receptor ligands, especially CpG, which have been shown to activate inflammatory cytokine production and promote vigorous humoral and cell-mediated antigen specific responses in the aged [27-30]. Using a transgenic mouse model, the Haynes group delineated several age-associated immune function deficiencies that could be rectified by appropriate co-stimulation or inclusion of adjuvants [22,23]. Their conclusion was that either inflammatory cytokines or adjuvants inducing their synthesis, could reverse immune response aging defects. Here, we demonstrate that EP67 is equal or better than CpG for induction of inflammatory cytokine production (IL-6, TNF-α, and INFγ) by splenic adherent cells derived from aged mice. Furthermore, the quantity of cytokines produced by the aged

cells following EP67 stimulation was comparable to that produced by young splenic adherent cells.

Together, these results underscore several important attributes of EP67 that make it a useful adjuvant in vaccines particularly well suited to the elderly or immuno-compromised. First, immunization with EP67-conjugated proteins induces potent Ig production, including antigen-specific IgG1 and IgG2b in aged mice. Secondly, EP67 drives the activation of aged APCs *in vitro* to an analogous extent as that seen with young APCs, inducing significant inflammatory cytokine release. These results support the further development of EP67 as a new class of adjuvant that holds particular promise for improving vaccine efficacy in the elderly.

Acknowledgments

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immunoglobulin G antipolysaccharide responses and specific CD4+-T-cell priming to young adult levels. Infect Immun. Apr; 2006 74(4):2177–86. [PubMed: 16552048]

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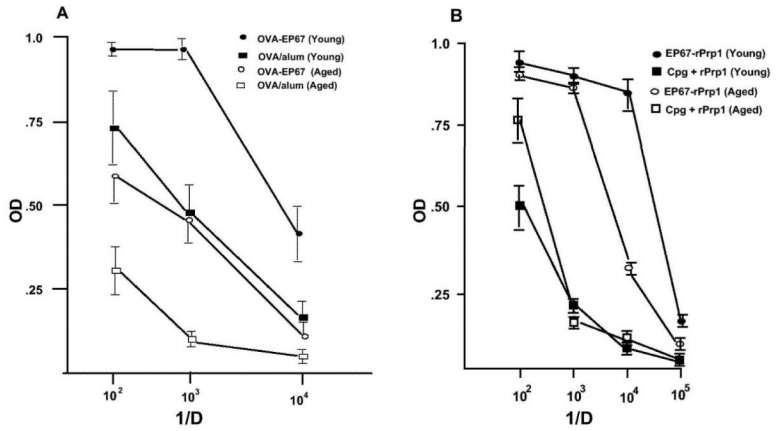

Figure 1.
Comparison of the Adjuvant Activity of Al(OH)3, CpGs and EP67 in Young and Aged Mice

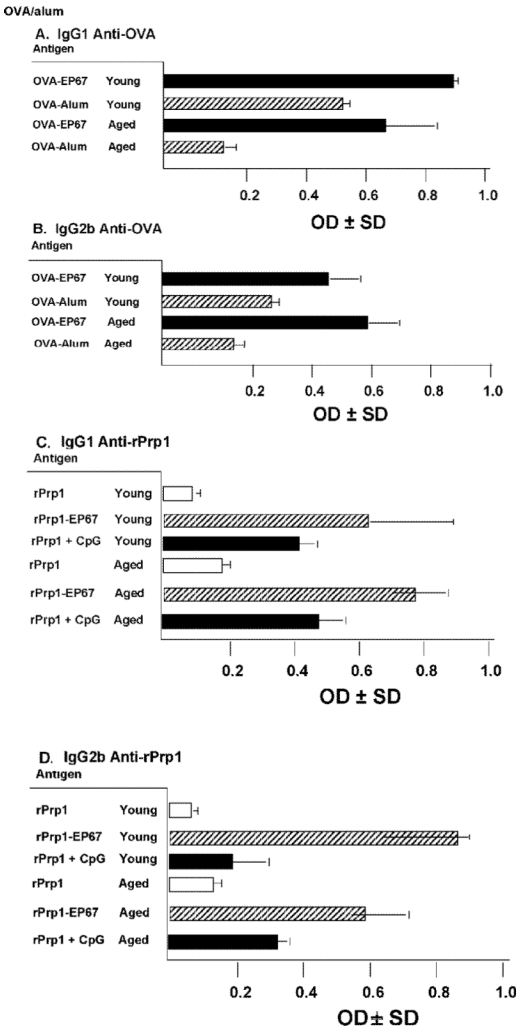

Figure 2.
Impact of Adjuvant on Immunoglobulin Subclass Distribution

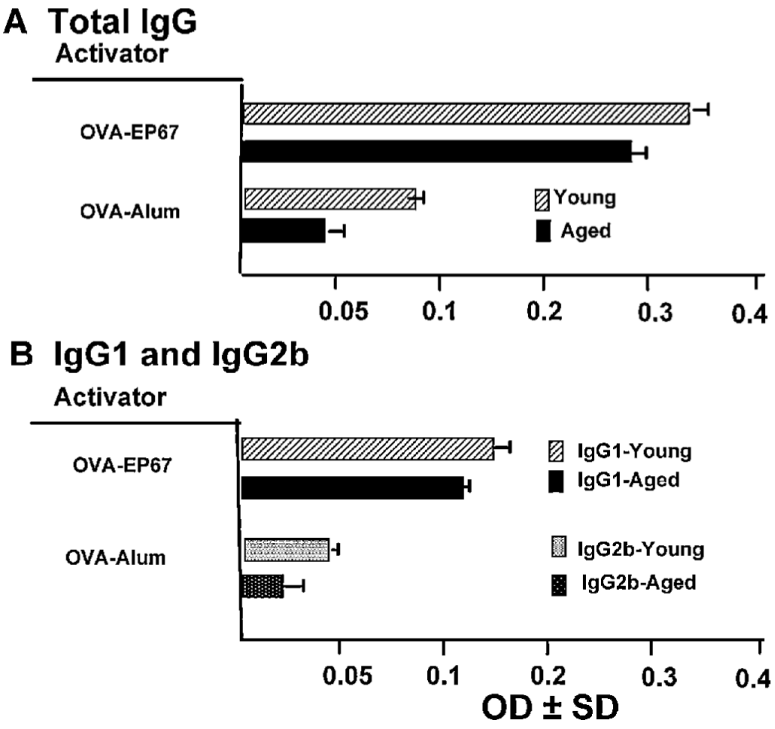

Figure 3.
Efficacy of EP67 Adjuvant Activity when Administered in a single dose.

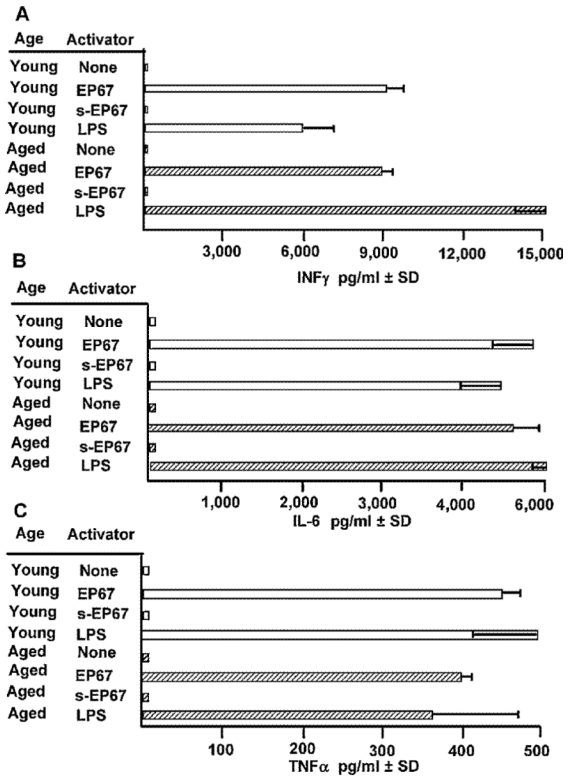

Figure 4.
EP67- Triggered Cytokine Release

Table 1

Induction of IL-6

Age	Activator (μg/ml)		IL-6 pg/ml ± SD
Young[1]	EP67	50	1440 ± 486
Young[1]	CpG	5	1060 ± 712
Young[1]	Al(OH)3	50	BKG
Aged[2]	EP67	50	787±400
Aged[2]	CpG	5	226 ± 30
Aged[2]	Al(OH)3	50	BKG

[1]**Young mice were 3 months of age**

[2]**Aged mice were 18-24 months of age**