Published in final edited form as:

Vaccine. 2013 January 11; 31(4): 653–660. doi:10.1016/j.vaccine.2012.11.044.

Disparate adjuvant properties among three formulations of “alum”

Derek W. Cain^a, Sergio E. Sanders^a, Michael M. Cunningham^a, and Garnett Kelsoe^a

^aDepartment of Immunology, Duke University, Durham, North Carolina 27710, USA

Abstract

Aluminum adjuvants, commonly referred to as “alum,” are the most widespread immunostimulants in human vaccines. Although the mechanisms that promote humoral responses to alum-adsorbed antigens are still enigmatic, alum is thought to form antigen depots and induce inflammatory signals that, in turn, promote antibody production. It was recently noted that Imject® alum, a commercial aluminum-containing adjuvant commonly used in animal studies, is not the physicochemical equivalent of aluminum adjuvant present in human vaccines. This difference raises concerns about the use of Imject® alum in animal research as a model for approved aluminum adjuvants. Here, we compared the capacity of Imject® alum, Alhydrogel®, and a traditional alum-antigen precipitate to induce humoral responses in mice to the hapten-carrier antigen, NP-CGG [(4-hydroxy-3-nitrophenyl)acetyl-chicken γ -globulin]. The magnitude of humoral responses elicited by Alhydrogel® and precipitated alum was significantly greater than that induced by Imject® alum. The strength of the humoral responses elicited by different alum formulations was correlated with the quantity of pro-inflammatory cytokines induced and the numbers of inflammatory cells at the site of immunization. Moreover, Imject® exhibited a severely reduced capacity to adsorb protein antigens compared to Alhydrogel® and precipitated alum. These findings reveal substantial differences in the immunostimulatory properties of distinct alum preparations, an important point of consideration for the evaluation of novel adjuvants, the assessment of new alum-based vaccines, and in mechanistic studies of adjuvanticity.

Keywords

alum; adjuvant; antibody; inflammation

© 2012 Elsevier Ltd. All rights reserved.

Corresponding Author: Garnett Kelsoe DUMC Box 3010 Durham, NC 27710 Telephone: 1-919-613-7815 Fax: 1-919-613-7878 ghkelsoe@duke.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Author Contributions

D.W.C.: conceived and designed the study; acquired, analyzed, and interpreted data; wrote and approved the manuscript

S.E.S.: acquired and analyzed data; wrote and approved the manuscript

M.M.C.: acquired and analyzed data; wrote and approved the manuscript

G.K.: conceived and designed the study; interpreted data; wrote and approved the manuscript

Disclosure Statement

The authors declare that they have no financial conflicts of interest.

1. Introduction

Aluminum adjuvants (collectively, “alum”) are the primary immunostimulants of human vaccines. Alum enhances antibody production against associated antigens, but elicits Th2 biased responses, rendering alum ineffective for protective responses controlled by Th1-mediated immunity [1]. This limitation has prompted investigation into alternative adjuvants and, because of its use in approved vaccines, alum commonly serves as a benchmark to evaluate novel immunostimulants.

Several alum formulations are available for research. Alhydrogel® is a commercial aluminum hydroxide gel approved by the FDA for human use, and structural studies have revealed the aluminum phase as crystalline aluminum oxyhydroxide [2-4]. Imject® alum, in contrast, is not licensed for human immunization, but is commonly used in animals as a comparator for new adjuvants [5-7], in tests of immunogenicity [8-10], and in mechanistic investigations of adjuvanticity [11-14]. Although the manufacturer describes Imject® as containing aluminum hydroxide, a recent report revealed the aluminum phase of Imject® alum as amorphous aluminum hydroxycarbonate, rather than the crystalline aluminum oxyhydroxide or amorphous aluminum hydroxyphosphate compounded in human vaccines [15]. The different aluminum composition between Imject® and approved alum formulations has raised concerns that Imject® may not reflect the adjuvant properties of aluminum in licensed vaccines [15-17].

Here, we compared the capacity of Imject® and Alhydrogel® alums and traditional precipitated alum to induce humoral responses in mice to a hapten-carrier antigen, NP-CGG [18]. Whereas all three aluminum formulations promoted high affinity, class-switched, antigen-specific antibodies following *i.p.* immunization, the magnitude of the humoral response was lowest when NP-CGG was adjuvanted with Imject® alum. Imject® was also the least potent inflammogen and exhibited little capacity to adsorb antigens compared to Alhydrogel® and precipitated alum. Our observations indicate that Imject® alum cannot be used as an adjuvant standard, in studies of antigen immunogenicity, and in determinations of alum's adjuvant properties.

2. Materials and methods

2.1. Mice

Female C57BL/6 mice (8-12 weeks) were obtained from The Jackson Laboratory and housed in a specific pathogen-free facility with sterilized chow and water *ad libitum*. All procedures were approved by the Duke University Institutional Animal Care and Use Committee and carried out in accordance with Directive 2010/63/EU.

2.2. Preparation of antigen/alum complexes

The succinic anhydride ester of (4-hydroxy-3-nitrophenyl)-acetyl (NP) was reacted with chicken γ -globulin (CGG; Sigma Aldrich), ovalbumin (Ova; Sigma Aldrich), or recombinant Anthrax Protective Antigen (rPA, BEI Resources) to generate antigens at average hapten:carrier ratios of 11:1, 6:1, and 5:1, respectively.

For Imject® and Alhydrogel® adjuvants, antigen/adjuvant preparations followed the methods recommended by the manufacturer; precipitated alum/antigen preparations were generated as described [19]. Briefly, antigen was adsorbed to Imject® alum (Thermo Scientific; 40 mg/ml aluminum hydroxide, 40 mg/ml magnesium hydroxide) in a solution (1 mL volume) of sterile PBS containing 200 μ g/ml antigen. Imject® alum (1 ml) was added drop-wise with constant mixing and the mixture placed on a rotator for 30 minutes, yielding

an antigen concentration of 100 µg/ml and an aluminum hydroxide concentration of 20 mg/ml. This preparation was used immediately for immunization.

Antigen was adsorbed to Alhydrogel® (“Alhydrogel 2%” CAS no. 21645-51-2, Brenntag Biosector; 10 mg/ml aluminum hydroxide) by the same method, yielding an antigen concentration of 100 µg/ml and an aluminum hydroxide concentration of 5 mg/ml.

In some experiments, Imject® and Alhydrogel® alum immunogen preparations were centrifuged (1000 × G; 10 min.) to remove any unadsorbed antigen. Supernatants containing soluble antigen were recovered and replaced with an equal volume of PBS; after extensive mixing the washed alum preparations were used in immunizations.

Antigen/aluminum precipitates were generated as follows [19]: 5 g of Al₂(SO₄)₃·(14-18) H₂O (EM Science) was dissolved in 50 ml water to make a 10% Al₂(SO₄)₃ solution; 1 ml 200 µg/ml antigen solution was mixed with 1 ml 10% Al₂(SO₄)₃ solution. Subsequently, KOH (1 M) was added drop-wise with constant stirring until pH 6.5. This precipitate was washed 3x in PBS with final resuspension to 1.8 ml and an antigen concentration of 100 µg/ml.

2.3. Immunizations

C57BL/6 mice were injected *i.p.* with 100 µl of alum/antigen mixture.

2.4. ELISA for serum antibody analysis

Serum IgM and IgG were quantified by ELISA on (4-hydroxy-5-iodo-3-nitrophenyl) acetyl (NIP)₂₅-BSA, NIP₅-BSA, and BSA coated plates as described [20].

2.5. ELISpot analysis for antigen-specific antibody-forming cells (AFC)

NP-specific AFC in spleens and bone marrow (BM) of mice were enumerated by ELISpot [21]. The H33Lγ1 (IgG1) and B1-8µ (IgM) cell lines served as positive controls [22].

2.6. Histology

Spleens were prepared for histology [20] and stained with APC-conjugated B220, PE-conjugated TCRβ, and FITC-conjugated GL-7. Sections were imaged using a Zeiss LSM 510 inverted confocal microscope.

2.7. Cytokine measurements

Six hours after immunization, serum cytokines levels were quantified using a Bio-Plex Pro Group I 23-Plex kit (Bio-Rad) at the Duke Human Vaccine Institute Immune Reconstitution & Biomarker Shared Resource Facility.

2.8. Flow cytometry

The peritoneal cavity was lavaged with 5 ml PBS. Cells were pelleted by centrifugation and the acellular fraction saved. Cells were prepared for flow cytometry as described [23] and labeled with antibodies from BD Biosciences, eBioscience, or AbD Serotec. One antibody panel contained FITC Ly-6G (clone 1A8), PE Ly-6B (clone 7/4), PE-Cy5 TER119, APC TCRβ, APC-AlexaFluor780 CD11b, PE-Texas Red B220, and PE-Cy7 IgM; another contained FITC F4/80, PE Siglec F, PE-Cy5 TER119, APC-AlexaFluor780 CD11b, biotin I-A/I-E, and PE-Cy7 IgM. Biotinylated antibodies were revealed by PE-Texas Red streptavidin; propidium iodide labeling excluded dead cells. Flow cytometric data were acquired on a LSRII flow cytometer (BD Biosciences) and analyzed with FlowJo software (TreeStar).

2.9. Cytotoxicity Measurement

Acellular peritoneal lavage fluid was assayed for DNA using a Quant-iT dsDNA assay kit (Invitrogen) and lactose dehydrogenase (LDH) using a Cytotoxicity Detection kit (Roche); standard curves were generated using rabbit skeletal muscle LDH (Roche).

2.10. Quantification of unadsorbed antigen in alum preparations

Unadsorbed NP-CGG, NP-Ova, and NP-rPA were quantified by ELISA using carrier-specific antibody for capture and NP-specific antibody (H33Lγ1; [20]) for detection, and purified antigen standards. CGG concentrations were determined by Bradford assay.

2.11. Statistics

Significant differences across treatments were determined via Anova and square-root transformation of data with JMP Pro (SAS) or Spotfire S+ (TIBCO) software. Post-hoc comparisons were evaluated by Tukey-Kramer test.

3. Results

3.1 Imject®, Alhydrogel®, and precipitated alum do not promote equivalent antibody responses

To evaluate the adjuvanticity of Imject® alum, NP₁₁-CGG was adsorbed to Imject® by the manufacturer's protocol. As a comparator, NP₁₁-CGG was adsorbed in the same way to Alhydrogel®, an alum standard often used to evaluate new adjuvants[24]. Finally, NP₁₁-CGG was also precipitated with alum as a third comparator [19]. C57BL/6 mice were immunized *i.p.* (10 μg NP₁₁-CGG) and were bled on days 8, 16, 25, and 42 post immunization for serum antibody determinations by ELISA.

The NP-specific serum IgM responses were similar in all immunized mice; NP reactive IgM peaked at day 8 and dropped to background levels by day 42 (Fig. 1A). Precipitated alum elicited modestly higher levels of NP IgM than did Imject® and Alhydrogel® (day 8; $P = 0.05$ and $P > 0.05$, respectively), but otherwise IgM responses did not significantly differ (Fig. 1A).

The kinetics of NP-specific serum IgG were also similar, with NP IgG antibody peaking on day 16 and persisting through day 42 (Fig. 1B). Concentrations of NP IgG antibody, however, differed substantially between the immunogen cohorts. At peak, mice immunized with precipitated alum exhibited average serum NP IgG concentrations of $2.0(\pm 0.1)$ mg/ml (mean $\pm$ SEM); mice immunized with Alhydrogel® or Imject® alums had lower concentrations of serum NP IgG with $0.6(\pm 0.07)$ and $0.3(\pm 0.03)$ mg/ml, respectively ($P = 0.05$, precipitated alum *vs.* Alhydrogel® *vs.* Imject®) (Fig. 1B). This rank pattern was present both for total and high affinity IgG antibody and persisted for 42 days postimmunization (Fig. 1B). Analogous immunizations using NP₆-Ova or NP₅-rPA antigens gave similar responses, with Imject® eliciting the lowest serum IgG antibody responses of the three formulations (Fig. 1C).

Immune responses elicited by alum adjuvants are influenced by aluminum content [25]; consequently, we calculated the aluminum concentrations of our Imject®, Alhydrogel®, and precipitated alum immunogens. Mice given precipitated alum or Alhydrogel® received 0.43 or 0.17 mg aluminum, respectively, a difference that might explain the higher IgG antibody levels elicited by precipitated alum (Fig. 1B). However, mice injected with Imject®/NP-CGG received 0.69 mg of aluminum but mounted the weakest IgG antibody responses (Fig. 1B). Excess aluminum can inhibit adjuvanted humoral responses [25]; however, when we reduced the aluminum content of the Imject® immunogen to that of the Alhydrogel®

preparation (0.17 mg) serum IgG antibody responses were lowered (Fig. 1D). We conclude that Imject® is a significantly less potent aluminum adjuvant than Alhydrogel® or precipitated alum.

3.2 Induction of plasmacytes and germinal centers by different alum formulations

Serum antibody is produced first by lower affinity, short-lived antibody-forming cells (AFC) in secondary lymphoid tissues and subsequently by higher affinity AFC that reside in the bone marrow (BM). These BM AFC are products of germinal center (GC) B cells [21, 26, 27]. In ELISpot assays, mice immunized with NP₁₁-CGG in precipitated alum or Alhydrogel® exhibited similar frequencies of NP-specific IgG AFC at day 8 (~70 AFC/10⁵ splenocytes; P>0.05); NP-specific AFC frequencies were significantly lower in mice immunized with Imject® alum (~25 AFC/10⁵ splenocytes; P = 0.05, Imject® alum vs. precipitated alum/Alhydrogel®; Fig. 2A).

Enumeration of IgG AFC in the BM of immunized mice (day 16) revealed that both precipitated alum and Alhydrogel® elicited comparable AFC responses (~6 AFC/10⁵ BM cells; P>0.05) whereas NP-specific IgG AFC in the BM of mice given Imject® alum were significantly lower (~1 AFC/10⁵ BM cells; P = 0.01, Imject® alum vs. precipitated alum/Alhydrogel®) (Fig. 2B).

Optimal vaccines elicit high affinity serum antibody and memory B cells as products of the GC reaction. We compared each alum formulation for GC induction. In naïve mice, the frequency of GC B cells (B220⁺GL-7⁺Fas⁺) was ~0.5% of splenic B cells; after immunization with NP-CGG adjuvanted with precipitated alum or Alhydrogel®, this frequency increased to >3% on day 8 and then fell to 1.5% by day 16 (Fig. 2C, D). With Imject®, the frequency of GC B cells at day 8 was <1.5% (P = 0.05 vs. precipitated alum/Alhydrogel®) but was comparable to other alum formulations on day 16 postimmunization (Fig. 2C, D). Histological examination of spleens (day 8) confirmed that Imject® elicited fewer, albeit comparably sized, GCs than did precipitated alum or Alhydrogel® (Fig. 2C).

Although the magnitude of humoral responses elicited by these alum formulations was precipitated alum >> Alhydrogel® >> Imject®, all alum types supported comparable rates of antibody affinity maturation (Fig. 2E). These different alums elicit, therefore, humoral responses that differ in quantity but not quality.

3.3. Cytotoxic and inflammatory properties of Imject®, Alhydrogel®, and precipitated alums

Alum is cytotoxic and the necrotic release of genomic DNA has been proposed as the basis for alum's adjuvanticity [28]. To determine if the magnitude of humoral responses elicited by different alum formulations was correlated to different cytotoxicities, we analyzed acellular peritoneal lavage fluid for DNA and as an independent measure of cell death, lactose dehydrogenase (LDH), 24 hours after *i.p.* immunization.

In mice injected with PBS or NP₁₁-CGG alone, DNA concentrations in peritoneal lavage fluid were <0.1 µg/ml (Fig. 3A). Injection of any alum formulation, however, resulted in free DNA concentrations of ~2 µg/ml with no differences between alum preparations (P>0.05) (Fig. 3A). LDH concentrations in lavage fluid were modestly elevated in mice injected with alum compared to controls, again with no significant differences among groups (P>0.05) (Fig. 3B). All alum preparations were equivalently cytotoxic.

Adjuvanticity is also associated with the inflammatory activation of innate immune cells [29]. As a measure of local inflammation, we analyzed peritoneal exudate cells 24 h after *i.p.* injection of the different alums, PBS, or NP-CGG alone. Peritoneal lavage cells were

analyzed by flow cytometry to enumerate macrophages (F4/80^{hi}), B cells (IgM⁺), eosinophils (F4/80^{low}Siglec F⁺), inflammatory monocytes (Ly6G⁺Ly6B⁺), and neutrophils (Ly6G⁺Ly6B⁺) (Fig. 3C). In mice injected with PBS or NP₁₁-CGG alone, B cells and macrophages dominated the lavages and the numbers of eosinophils, inflammatory monocytes, and neutrophils were comparably low (Fig. 3D). In contrast, peritoneal cell populations were dramatically different in mice immunized with antigen/alum formulations (Fig. 3D). As expected [29], F4/80^{hi} macrophages were virtually undetectable in immunized mice, regardless of alum formulation (P 0.01, all vs. PBS/NP-CGG; Fig. 3C, D), and B-cell numbers were also significantly reduced (P 0.05, all vs. PBS; Fig. 3D). The numbers of eosinophils, inflammatory monocytes, and neutrophils significantly increased in all alum-immunized mice (Fig. 3C, D). Among alum formulations, Alhydrogel® and precipitated alum elicited many more neutrophils (P 0.01, precipitated alum/Alhydrogel® vs. Imject®) and modestly more inflammatory monocytes (P 0.05, Alhydrogel® vs. Imject®; P=0.19, precipitated alum vs. Imject®) than did Imject® alum (Fig. 3D).

3.4 Cytokine induction by different alum formulations

Intraperitoneal immunization elicits systemic humoral response, as evidenced by GC induction in the spleen (Fig. 2); consequently, we determined the capacity of Imject®, Alhydrogel®, and precipitated alum to evoke cytokines that influence systemic antibody production.

Based on previous observations of alum-induced cytokine production [23], we measured serum cytokine concentrations in mice six hours after *i.p.* immunization. Concentrations of 23 cytokines (Supplemental Fig. 1) were determined in a multiplex bead array. In mice receiving PBS or NP₁₁-CGG alone, serum concentrations of cytokines were identical (Fig. 4, Supplemental Fig. 1). In mice injected with NP₁₁-CGG/precipitated alum, there were significant increases in IL-5 (~2000 pg/ml), IL-6 (~50 pg/ml), G-CSF (~1700 pg/ml), KC (~50 pg/ml), and MCP-1 (~250 pg/ml), as well as a decrease in RANTES (P 0.05 vs. PBS/NP-CGG; Fig. 4). Animals receiving NP-CGG/Alhydrogel® exhibited less dramatic changes in cytokine concentrations, with significant elevations in IL-5 (~1000 pg/ml), IL-6 (~35 pg/ml), and G-CSF (~500 pg/ml), and modest, but not significant, increases in KC (~35 pg/ml) and MCP-1 (~70 pg/ml) (Fig. 4). In general, the effects of Imject® alum were milder than both precipitated alum and Alhydrogel®; we only observed a significant increase in G-CSF (~1000 pg/ml), a modest elevation in IL-5 (~200 pg/ml, P=0.06 Imject® vs. NP-CGG alone, P=0.19 vs. PBS), and decreased RANTES (P 0.05 vs. PBS; Fig. 4). Overall, serum concentrations of IL-5, IL-6, KC, and MCP-1 were highest in animals injected with precipitated alum, intermediate in Alhydrogel®-immunized animals, and lowest in those given Imject® (Fig. 4). In general, the quantity of proinflammatory cytokines (Fig. 4) and the numbers of neutrophils and inflammatory monocytes recruited to the peritoneal cavity (Fig. 3D) by each alum formulation was correlated with the magnitude of elicited humoral responses (Figs. 1-2). Imject® alum may be a less potent adjuvant than Alhydrogel® or precipitated alum because it is a less potent inflammogen.

Eosinophil numbers in the peritoneal cavity were similar among all alum immunized mice (P>0.05; Fig. 3D), a surprising observation given IL-5's role in eosinophilia [30] and the 10-fold difference in IL-5 levels induced by precipitated vs. Imject® alum (Fig. 4). Apparently, the smaller amount of IL-5 elicited by Imject® (Fig. 4) is sufficient for maximal eosinophil responses.

3.5 Alum formulations differ in antigen adsorption capacity

Antigen adsorption is an important property of an aluminum adjuvant [31, 32]. To determine if Imject®, Alhydrogel®, and precipitated alum differ in antigen-binding capacity, we

measured concentrations of unadsorbed antigen in each formulation; with no antigen adsorption, the concentration of unbound NP₁₁-CGG is maximally 100 µg/ml. Immunogens prepared with Alhydrogel® or precipitated alum contained no detectable unbound antigen, indicating complete adsorption (Fig. 5A). In contrast, the concentration of unadsorbed NP₁₁-CGG in Imject®-antigen preparations was ~60 µg/ml (Fig. 5A), indicating that only ~40% of antigen bound to the adjuvant. Deficient antigen adsorption by Imject® alum was not due to haptentation, as Imject® poorly bound native CGG as well (Fig. 5B). To ensure that these adsorption/precipitation reactions were not impaired by phosphate in the buffer [33], we also generated Imject®-antigen immunogens in saline; they exhibited similarly poor adsorption of CGG (Fig. 5B). The reduced capacity of Imject® to adsorb antigen was not limited to NP₁₁-CGG and CGG, as Imject®'s ability to bind NP₆-Ova or NP₅-rPA was also substantially less than Alhydrogel® or precipitated alum (Fig. 5C).

Since the majority of antigen in Imject®-adjuvanted vaccines was unadsorbed, we determined if soluble antigen contributes to the elicited antibody response. We prepared two types of NP₁₁-CGG/Imject® and NP₁₁-CGG/Alhydrogel® vaccines, one containing both adsorbed and unadsorbed antigen, and one washed of soluble antigen prior to immunization. Due to low adsorptive capacity (Fig. 5A-C), mice immunized with a “washed” Imject® vaccine received a total antigen dose only 40% of that in the “unwashed” vaccine. The two Imject® immunogens, however, elicited identical antibody responses (Fig. 5D), indicating that unadsorbed antigen in Imject®-containing vaccines contributes little to the humoral response. As expected, both Alhydrogel® preparations elicited robust NP-specific IgG (Fig. 5D). We conclude that Imject®'s low adsorptive property limits its efficacy.

4. Discussion

We compared the capacities of three aluminum adjuvants - Imject® alum, Alhydrogel®, and a classic aluminum precipitate - to potentiate humoral responses to a well characterized antigen, NP-CGG. All three adjuvants elicited high affinity IgG antibody with similar kinetics (Fig. 1) but the magnitude of IgG antibody responses induced by each alum preparation differed. In general, serum IgG, AFC, and GC responses were highest in mice immunized with antigen/aluminum precipitate, followed by those injected with antigen/Alhydrogel®, and were lowest in mice given antigens compounded with Imject® (Fig. 1, 2).

Affinity maturation (Fig. 2E) in mice immunized with Imject® alum was normal, despite lower numbers of GC B cells and fewer GC (Fig. 2C), and reduced BM AFC numbers (Fig. 2B). We conclude that Imject® immunogens induce GC that are fully functional and that the deficiency of Imject® as a humoral adjuvant is quantitative.

Remarkably, the mechanism(s) of alum's adjuvant properties are enigmatic [34], but it is now thought that alum-induced inflammation promotes adaptive immunity [11, 13, 14, 23, 30, 35, 36]. Several inflammatory properties of alum have been associated with adjuvant activity, including its capacity to activate the NLRP3 inflammasome [11], to trigger cell necrosis and the release of endogenous immunostimulants [28, 37], and to induce prostaglandin release [38]. Consistent with a linkage between alum's inflammatory and adjuvant properties, we observed correlations between the capacity of these alum adjuvants to induce proinflammatory cytokines in the serum (Fig. 4) and modulate peritoneal leukocyte populations (Fig. 3C, D) to the induction of robust humoral responses (Fig. 1, 2). Precipitated alum, the strongest inducer of antibody, was the most potent inducer of serum IL-5, IL-6, G-CSF, MCP-1, and KC (Fig. 4). Alhydrogel® elicited intermediate antibody responses (Fig. 1) and moderate but significant increases in IL-5, IL-6, and G-CSF (Fig. 4). Finally, Imject® alum, the weakest promoter of humoral responses (Fig. 1) induced the smallest elevations serum cytokines, with a significant increase only in serum G-CSF (Fig.

4). Immunization with Imject® alum also resulted in the immigration of significantly fewer neutrophils to the immunization site compared to Alhydrogel® or precipitated alum (Fig. 3D). These observations confirm Imject® as the least potent inflammogen of the alum formulations surveyed.

Of the cytokines induced by these alum types, IL-5 varied most (Fig. 4). While the level of induced IL-5 (Fig. 4) correlated with serum IgG antibody production (Fig. 1), IL-5^{-/-} mice are capable of mounting robust antibody responses to antigen/alum precipitates [30]. This observation precludes IL-5 as the primary determinant of the adjuvant properties of Imject®, Alhydrogel®, and precipitated alum.

Alum exerts a “depot effect” by retaining antigen in insoluble form [39]; consequently, a signal property of alum adjuvants is the capacity to bind antigen [32, 40-44]. Whereas antigen adsorption was virtually complete in vaccines containing precipitated alum or Alhydrogel®, only 20-40% of antigen was bound in Imject® preparations (Fig. 5), suggesting poor adsorption as a cause of its modest adjuvanticity.

Conclusions

Three alum adjuvant formulations commonly employed in research and medicine exhibit significant differences in their capacity to promote humoral responses, elicit inflammation, and adsorb antigen. Imject® was the poorest adjuvant in all of these categories, Alhydrogel® was intermediate, and precipitated alum was the best. Differences in immunostimulation by Alhydrogel® and precipitated alum may reflect the aluminum content of each vaccine, but the weak humoral response elicited by Imject® was unassociated with aluminum concentration, and instead linked to poor antigen adsorption and modest inflammatory effects. These differences may reflect the chemical form of aluminum in each formulation [15]. Alum adjuvants are often used interchangeably, but the very modest adjuvant properties of Imject® must be considered when used to benchmark new adjuvants, to study alum's immunopotential, and to evaluate new vaccines.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This study was supported by NIH grants to G. Kelsoe (AI 81579; AK 91693; and NIAID Contract, Multiscale Systems Immunology).

Abbreviations

AFC	antibody-forming cells
BM	bone marrow
CGG	chicken γ -globulin
GC	germinal center
LDH	lactose dehydrogenase
NP	(4-hydroxy-3-nitrophenyl)-acetyl
NIP	(4-hydroxy-5-iodo-3-nitrophenyl) acetyl
Ova	ovalbumin

rPA recombinant protective antigen of *Bacillus anthracis*

References

1. Leroux-Roels G. Unmet needs in modern vaccinology: adjuvants to improve the immune response. *Vaccine*. 2010; 28(Suppl 3):C25–36. [PubMed: 20713254]
2. Vogel FR, Powell MF. A compendium of vaccine adjuvants and excipients. *Pharm Biotechnol*. 1995; 6:141–228. [PubMed: 7551218]
3. Shirodkar S, et al. Aluminum compounds used as adjuvants in vaccines. *Pharm Res*. 1990; 7(12): 1282–8. [PubMed: 2095567]
4. Edelman R. Vaccine adjuvants. *Rev Infect Dis*. 1980; 2(3):370–83. [PubMed: 6997966]
5. Krishnan L, et al. Archaeosome vaccine adjuvants induce strong humoral, cell-mediated, and memory responses: comparison to conventional liposomes and alum. *Infect Immun*. 2000; 68(1): 54–63. [PubMed: 10603368]
6. Wilmar A, et al. The cationic lipid, diC14 amidine, extends the adjuvant properties of aluminum salts through a TLR-4- and caspase-1-independent mechanism. *Vaccine*. 2012; 30(2):414–24. [PubMed: 22075093]
7. Yip HC, et al. Adjuvant-guided type-1 and type-2 immunity: infectious/noninfectious dichotomy defines the class of response. *J Immunol*. 1999; 162(7):3942–9. [PubMed: 10201913]
8. Gottwein JM, et al. Protective anti-*Helicobacter* immunity is induced with aluminum hydroxide or complete Freund's adjuvant by systemic immunization. *J Infect Dis*. 2001; 184(3):308–14. [PubMed: 11443556]
9. Ogunniyi AD, et al. Immunization of mice with combinations of pneumococcal virulence proteins elicits enhanced protection against challenge with *Streptococcus pneumoniae*. *Infect Immun*. 2000; 68(5):3028–33. [PubMed: 10769009]
10. Rao KV, He YX, Kalyanasundaram R. Expression of a 28-kilodalton glutathione S-transferase antigen of *Schistosoma mansoni* on the surface of filamentous phages and evaluation of its vaccine potential. *Clin Diagn Lab Immunol*. 2003; 10(4):536–41. [PubMed: 12853382]
11. Eisenbarth SC, et al. Crucial role for the Nalp3 inflammasome in the immunostimulatory properties of aluminium adjuvants. *Nature*. 2008
12. Gavin AL, et al. Adjuvant-enhanced antibody responses in the absence of toll-like receptor signaling. *Science*. 2006; 314(5807):1936–8. [PubMed: 17185603]
13. Kool M, et al. Cutting Edge: alum adjuvant stimulates inflammatory dendritic cells through activation of the NALP3 inflammasome. *J Immunol*. 2008; 181(6):3755–9. [PubMed: 18768827]
14. Li H, Nookala S, Re F. Aluminum hydroxide adjuvants activate caspase-1 and induce IL-1 β and IL-18 release. *J Immunol*. 2007; 178(8):5271–6. [PubMed: 17404311]
15. Hem SL, Johnston CT, HogenEsch H. Imject Alum is not aluminum hydroxide adjuvant or aluminum phosphate adjuvant. *Vaccine*. 2007; 25(27):4985–6. [PubMed: 17543429]
16. Exley C, Siesjo P, Eriksson H. The immunobiology of aluminium adjuvants: how do they really work? *Trends Immunol*. 2010; 31(3):103–9. [PubMed: 20153253]
17. Exley C. When an aluminium adjuvant is not an aluminium adjuvant used in human vaccination programmes. *Vaccine*. 2012; 30(12):2042. [PubMed: 22041301]
18. Imanishi T, Makela O. Strain differences in the fine specificity of mouse anti-hapten antibodies. *Eur J Immunol*. 1973; 3(6):323–30. [PubMed: 4586168]
19. Tesch H, Takemori T, Rajewsky K. The immune response against anti-idiotope antibodies II. The induction of antibodies bearing the target idiotope (Ab3 β) depends on the frequency of the corresponding B cells. *Eur J Immunol*. 1983; 13(9):726–32. [PubMed: 6413225]
20. Kuraoka M, et al. Activation-induced cytidine deaminase expression and activity in the absence of germinal centers: insights into hyper-IgM syndrome. *J Immunol*. 2009; 183(5):3237–48. [PubMed: 19667096]

21. Takahashi Y, et al. In situ studies of the primary immune response to (4-hydroxy-3-nitrophenyl)acetyl. V. Affinity maturation develops in two stages of clonal selection. *Journal of Experimental Medicine*. 1998; 187(6):885–95. [PubMed: 9500791]
22. Takemori T, Rajewsky K. Lambda chain expression at different stages of ontogeny in C57BL/6, BALB/c and SJL mice. *Eur J Immunol*. 1981; 11(8):618–25. [PubMed: 6791945]
23. Cain DW, et al. Inflammation triggers emergency granulopoiesis through a density-dependent feedback mechanism. *PLoS One*. 2011; 6(5):e19957. [PubMed: 21655273]
24. Stewart-Tull, DES.; Greogoriadis, G.; Allison, AC.; Poste, G., editors. *Immunological Adjuvants and Vaccines*. Plenum; New York: 1989. Recommendations for the assessment of adjuvants (immunopotentiators).; p. 213-226.
25. Gupta, RK.; Rost, BE. Aluminum Compounds as Vaccine Adjuvants.. In: O'Hagan, DT., editor. *Methods in Molecular Medicine*. Vol. 42. Humana Press; Totowa, N.J.: 2000. p. 25
26. Jacob J, Kassir R, Kelsoe G. In situ studies of the primary immune response to (4-hydroxy-3-nitrophenyl)acetyl. I. The architecture and dynamics of responding cell populations. *J Exp Med*. 1991; 173(5):1165–75. [PubMed: 1902502]
27. Jacob J, Kelsoe G. In situ studies of the primary immune response to (4-hydroxy-3-nitrophenyl)acetyl. II. A common clonal origin for periarteriolar lymphoid sheath-associated foci and germinal centers. *J Exp Med*. 1992; 176(3):679–87. [PubMed: 1512536]
28. Marichal T, et al. DNA released from dying host cells mediates aluminum adjuvant activity. *Nat Med*. 2011; 17(8):996–1002. [PubMed: 21765404]
29. Kool M, et al. Alum adjuvant boosts adaptive immunity by inducing uric acid and activating inflammatory dendritic cells. *J Exp Med*. 2008
30. McKee AS, et al. Alum induces innate immune responses through macrophage and mast cell sensors, but these sensors are not required for alum to act as an adjuvant for specific immunity. *J Immunol*. 2009; 183(7):4403–14. [PubMed: 19734227]
31. Gupta RK, et al. Adjuvant properties of aluminum and calcium compounds. *Pharm Biotechnol*. 1995; 6:229–48. [PubMed: 7551219]
32. Hem SL, White JL. Structure and properties of aluminum-containing adjuvants. *Pharm Biotechnol*. 1995; 6:249–76. [PubMed: 7551220]
33. Gupta RK. Aluminum compounds as vaccine adjuvants. *Adv Drug Deliv Rev*. 1998; 32(3):155–172. [PubMed: 10837642]
34. Marrack P, McKee AS, Munks MW. Towards an understanding of the adjuvant action of aluminium. *Nature Reviews Immunology*. 2009; 9(4):287–293.
35. Hornung V, et al. Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization. *Nat Immunol*. 2008; 9(8):847–56. [PubMed: 18604214]
36. Ueda Y, et al. IL-1R type I-dependent hemopoietic stem cell proliferation is necessary for inflammatory granulopoiesis and reactive neutrophilia. *J Immunol*. 2009; 182(10):6477–84. [PubMed: 19414802]
37. Wang Y, Rahman D, Lehner T. A Comparative Study of Stress-mediated Immunological Functions with the Adjuvanticity of Alum. *J Biol Chem*. 2012; 287(21):17152–60. [PubMed: 22474329]
38. Kuroda E, et al. Silica crystals and aluminum salts regulate the production of prostaglandin in macrophages via NALP3 inflammasome-independent mechanisms. *Immunity*. 2011; 34(4):514–26. [PubMed: 21497116]
39. Glenny AT, Buttle GAH, Stevens MF. Rate of disappearance of diphtheria toxoid injected into rabbits and guinea-pigs: Toxoid precipitated with alum. *Journal of Pathology and Bacteriology*. 1931; 34(2):267–275.
40. Aprile MA, Wardlaw AC. Aluminum compounds as adjuvants for vaccine and toxoids in man: a review. *Can. J. Pub. Health*. 1966; 57:343–354. [PubMed: 5329166]
41. al-Shakhshir R, et al. Effect of protein adsorption on the surface charge characteristics of aluminium-containing adjuvants. *Vaccine*. 1994; 12(5):472–4. [PubMed: 8023556]
42. Hem SL, White JL. Characterization of aluminum hydroxide for use as an adjuvant in parenteral vaccines. *J Parenter Sci Technol*. 1984; 38(1):2–10. [PubMed: 6423795]

43. Masood H, White JL, Hem SL. Relationship between protein adsorptive capacity and the X-ray diffraction pattern of aluminium hydroxide adjuvants. *Vaccine*. 1994; 12(2):187–9. [PubMed: 8147102]
44. Seeber SJ, White JL, Hem SL. Predicting the adsorption of proteins by aluminium-containing adjuvants. *Vaccine*. 1991; 9(3):201–3. [PubMed: 2042392]

\$watermark-text

\$watermark-text

\$watermark-text

Highlights

- The adjuvant properties of three common formulations of alum were compared
- Humoral adjuvant properties ranked precipitated alum > Alhydrogel® > Imject® alum
- Imject® exhibited reduced inflammatory properties and antigen adsorption capacity

Figure 1.

Alhydrogel®, Imject® alum, and precipitated alum differ in their capacity to promote production of antigen-specific IgG. (A) Concentrations (mean+SEM) of NP-specific IgM in serum of C57BL/6 mice after *i.p.* immunization with 10 µg NP₁₁-CGG precipitated in alum (black square) or adsorbed to Alhydrogel® (gray triangle) or Imject® alum (open diamond) are shown. (B) Concentrations (mean+SEM) of total (NIP₂₅-BSA) and high affinity (NIP₅-BSA) NP-specific IgG in serum after immunization with 10 µg NP₁₁-CGG precipitated in alum (black square) or adsorbed to Alhydrogel® (gray triangle) or Imject® alum (open diamond) are shown. (n=4-6 mice for each alum formulation at days 0, 8, and 16; n=3 for days 25 and 42). (C) Concentrations (mean+SEM) of total (NIP₂₅-BSA) NP-specific IgG in serum after immunization with 10 µg NP₆-OVA (left panel) or 10 µg NP₅-rPA (right panel) precipitated in alum (black square) or adsorbed to Alhydrogel® (gray triangle) or Imject® alum (open diamond) are shown (n=3 mice per data point). (D) Concentrations (mean+SEM) of total (NIP₂₅-BSA) NP-specific IgG in serum after immunization with 10 µg NP₁₁-CGG/Alhydrogel® containing 0.17 mg Al (gray triangle), NP₁₁-CGG/Imject® containing 0.69 mg Al (open diamond), or NP₁₁-CGG/Imject® containing 0.17 mg Al (closed diamond). (n=3-6 mice per data point). * P 0.05, ** P 0.01.

Figure 2.

Induction of antibody-forming cells and germinal centers by different alum formulations. (A) Mice were immunized *i.p.* with 10 μ g NP₁₁-CGG associated with various alum formulations. Frequencies (mean+SEM) of antigen-specific IgG AFC in spleens of naïve mice and mice immunized with NP₁₁-CGG adjuvanted with precipitated alum (black squares), Alhydrogel® (gray triangles), or Imject® alum (open diamonds), as determined by ELISpot assay, are shown. (B) The frequencies (mean+SEM) of NP-specific IgG AFC in bone marrow are shown on days 0, 8, and 16 after immunization with NP₁₁-CGG adjuvanted with precipitated alum (black squares), Alhydrogel® (gray triangles), or Imject® alum (open diamonds). (n=5-6 mice per data point). (C) Representative FACS plots of

splenic germinal center B cells (B220⁺TCR β ⁻GL-7⁺Fas⁺ cells) from naïve mice and mice immunized with various alum adjuvants (day 8). Representative histological images of B-cell follicles and GCs from spleens of naïve mice and immunized mice are shown at right (green = GL-7, red = TCR β , blue = B220; scale bar = 100 μ m; 10x objective lens, NA: 0.3). (D) Frequencies (mean $\pm$ SEM) of GC B cells (GL-7⁺Fas⁺ cells) within the splenic B-cell population (B220⁺TCR β ⁻ cells) at days 0 (naïve), 8, and 16 after immunization with NP₁₁-CGG adjuvanted with precipitated alum (black squares), Alhydrogel® (gray triangles), or Imject® alum (open diamonds) are shown. (E) Affinity maturation, as measured by the ratio of high affinity NP-specific IgG over total NP-specific IgG (mean $\pm$ SEM) from Fig. 1, is shown at various days after immunization with 10 μ g NP₁₁-CGG precipitated in alum (black square) or adsorbed to Alhydrogel® (gray triangle) or Imject® alum (open diamond). (n=3-6 mice per data point). * P 0.05, ** P 0.01.

Figure 3.

Cytotoxic and inflammatory effects of Imject® alum, Alhydrogel®, and precipitated alum. Mice were injected *i.p.* with PBS, 10 µg NP₁₁-CGG without adjuvant (“NPCGG”), or 10 µg NP₁₁-CGG adjuvanted with Imject® alum (“Imject”), Alhydrogel® (“Alhydr”), or precipitated alum (“Prec. alum”). Twenty-four hours later, the peritoneal cavity was lavaged and the acellular fraction was analyzed for DNA (A) and LDH (B). The concentrations (mean+SEM) of DNA (A) and LDH (B) for each treatment are shown (n=3-4 mice per treatment). (C) The cellular fraction of peritoneal lavages was analyzed by flow cytometry to characterize the inflammatory exudate. Macrophages were defined as F4/80^{hi}, B cells as IgM⁺, neutrophils as Ly6G⁺Ly6B⁺, inflammatory monocytes as Ly6G⁺Ly6B⁺, and eosinophils as Siglec F⁺F4/80^{low}. (D) The numbers (mean+SEM) of total peritoneal lavage cells and each leukocyte type are shown (n=4-6 mice per treatment). * P 0.05, ** P 0.01.

Figure 4.

Induction of serum cytokines by Inject® alum, Alhydrogel®, and precipitated alum. Mice were injected *i.p.* with PBS, 10 μ g NP₁₁-CGG alone (“NPCGG”), or 10 μ g NP₁₁-CGG adjuvanted with Inject® alum (“Inject”), Alhydrogel® (“Alhydr”), or precipitated alum (“Prec. alum”). Six hours after injection, mice were bled and sera were analyzed by multiplex assay for concentrations of 23 cytokines. Concentrations (mean+SEM) of IL-5, IL-6, G-CSF, KC, MCP-1, and RANTES are shown. (n=4-6 mice per treatment). * P 0.05, ** P 0.01

Figure 5.

Imject® exhibits a poor capacity to adsorb antigen. (A) Following NP₁₁-CGG preparation with precipitated alum, Alhydrogel®, or Imject®, the liquid phase of each vaccine was assayed by ELISA for soluble antigen. Concentrations of soluble NP₁₁-CGG in each vaccine are shown, with each point representing a separate vaccine preparation (n=3). The hashed line represents the antigen concentration in the absence of antigen adsorption (100 μg/ml). (B) The concentrations of soluble CGG (unhaptenated) in vaccines prepared with Alhydrogel® and Imject® are shown (n=2). CGG adsorption to alum formulations took place in PBS (left panel) or physiologic saline (right panel). (C) Concentrations of unadsorbed NP₆-Ova and NP₅-rPA in the various alum-containing vaccines are shown (n=3 preparations per antigen). (D) Two NP₁₁-CGG/Imject® vaccines were prepared, one according to the manufacturer's specifications ("unwashed", closed diamond) and containing both soluble and adsorbed antigen; the other vaccine was washed of unadsorbed antigen prior to immunization ("washed", open diamond). Two vaccines of NP₁₁-CGG/Alhydrogel® were prepared similarly ("unwashed," closed triangle and "washed," open triangle). Total NP-specific IgG concentrations (mean+SEM) in serum at days 8 and 16 after immunization with washed and unwashed vaccines are shown.