Adjuvant Activity of Emulsan, a Secreted Lipopolysaccharide from *Acinetobacter calcoaceticus*

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Several promising adjuvant candidates have been studied over the past 75 years; however, only alum is currently approved for human use. The complex acylated polysaccharide emulsan, secreted from *Acinetobacter calcoaceticus*, represents a new candidate. Unique features of this family of polymers are their amenability to structural tailoring and their emulsification behavior. We demonstrate that emulsan activates macrophages in a dose-dependent manner. This activation is dependent on the presence of the fatty acid side chains that decorate the polysaccharide backbone, and, furthermore, the level of activation can be affected by changes in the chemical characteristics of emulsan structural variants. One emulsan variant was examined in a classical hapten carrier immunization protocol and demonstrated significant adjuvant activity as determined by hapten-specific antibody titers. This immune response was characterized by a high immunoglobulin G2a titer, consistent with a Th1 response. The significant immunopotentiation demonstrated by this complex polymer establishes emulsan as an exciting new candidate adjuvant. Furthermore, by manipulating the chemical structure of this compound, we can explore the physical basis of pattern recognition receptors and macrophage activation.

Taking its name from the Latin word adjuvare, which means to help, an adjuvant is any compound that increases the strength and/or duration of an immune response to a foreign antigen over that caused by the antigen alone (19). The important characteristics of an adjuvant are its ability to enhance the immune response to the target antigen, long-term safety in widespread application, and flexibility in its use for different antigen and disease systems.

Since its adjuvant activity was first described over 75 years ago (6), alum remains the only adjuvant approved for use in humans (5). Several potential adjuvants are in various stages of research and development including monophosphoryl lipid A (20), CpG oligonucleotides (25), saponins (26), and lipid vesicles (10). One of the most significant limitations to these preparations, besides their often severe side effects (24), is their inability to form stable oil-in-water emulsions. Emulsan's polysaccharide backbone suggests that it might also share properties with chitin and chitosan derivatives that have demonstrated the ability to activate macrophages (16–18, 22). This family of polymers has structural similarities to bacterial lipopolysaccharide (LPS), which suggested that it might have proinflammatory activity. The polymeric nature of emulsan's polysaccharide backbone suggests that it might also share properties with chitin and chitosan derivatives that have demonstrated the ability to activate macrophages (16–18, 22). This study explores the ability of these polymers to activate the innate immune response and to serve as adjuvants for humoral immunity in a model hapten carrier system.

**MATERIALS AND METHODS**

Experimental animals and cell lines. CD-1 and BALB/c mice were purchased from Charles River Laboratories. L929 murine fibroblasts were maintained in Dulbecco's modified Eagle medium (DMEM) with 10% horse serum (GIBCO, Carlsbad, Calif.). The murine macrophage cell line RAW 264.7 was maintained in DMEM containing 10% fetal calf serum (FCS). Resident peritoneal cells of female mice from a sterile lavage were plated at 2 × 10^6 cells/well in RPMI 1640 media with 5% FCS and 5 μg of polymixin B/ml (600 U per μg) in flat-bottom 96-well tissue culture plates. After 1 to 3 h of incubation at 37°C, medium was replaced with RPMI 1640 containing 2 μg of indomethacin/ml and incubated for
30 min. Medium containing indomethacin was replaced with fresh RPMI 1640 prior to stimulation. All media contained 50 μg of gentamicin/ml.

Generation and culture of A. calcoaceticus RAG-1 mutants. A. calcoaceticus RAG-1 (ATCC 31012) was obtained from the American Type Culture Collection (Manassas, Va.). A. calcoaceticus RAG-1 transposon mutants 13D, 52D, 62C, and VRBS1, were generated with the mini-Tn10PttKm transposon (11, 12, 14). The parent strain and the RAG-1 transposon mutants were grown on minimal medium (0.1 M K2HPO4, 0.05 M KH2PO4, 2 mM MgSO4·7H2O, 0.03 M [NH4]2SO4) or Luria-Bertani (LB) broth supplemented with ethanol and undecanoic, myristic, palmitic, or stearic acid (1% [wt/vol]) as carbon sources in 500-ml baffled flasks each containing 100 ml of medium and incubated at 30°C in an orbital shaker (250 rpm) for 6 days. Emulsan variants were purified as previously described (7–9, 13, 21). Apoemulsan (protein-free emulsan) was produced by hot-phenol extraction (28). Emulsan was deacylated by basic hydrolysis for some experiments as previously described (9). Emulsan variants were analyzed by gas chromatography, gel permeation chromatography, and mass spectrometry for fatty acid content and polysaccharide backbone length as previously described (2, 9, 12). Apoemulsan was used for all experiments unless otherwise noted.

Macrophage activation and cytokine assay. Macrophages (primary or cell lines) were stimulated with emulsan preparations at various concentrations for 12 to 16 h. RAW 264.7 cells were plated at 8 × 10^4 cells per well in 200 μl of medium and incubated at 37°C for 3 h. The medium was replaced with RPMI 1640 containing 2 μg of indomethacin/ml and cells were incubated for 30 min prior to stimulation. Cell supernatants were collected for cytokine assays. Tumor necrosis factor (TNF) was measured by either the L929 cytotoxicity assay or by enzyme-linked immunosorbent assay (ELISA; Genzyme, Cambridge, Mass., and R&D Systems, Min-

### TABLE 1. Immunization protocol

| Group<sup>a</sup> | Adjuvant | Antigen          |
|-------------------|----------|------------------|
| 1                 | None     | 100 μg of KLH-DNP|
| 2                 | 200 μg of crude emulsan | None          |
| 3                 | 200 μg of apoemulsan | None          |
| 4                 | 20 μg of crude emulsan | 100 μg of KLH-DNP|
| 5                 | 200 μg of crude emulsan | 100 μg of KLH-DNP|
| 6                 | 20 μg of apoemulsan | 100 μg of KLH-DNP|
| 7                 | 200 μg of apoemulsan | 100 μg of KLH-DNP|
| 8                 | 100 μl of CFA and IFA<sup>b</sup> | 100 μg of KLH-DNP|

<sup>a</sup> Five mice per group.<br>
<sup>b</sup> CFA was used as the adjuvant in the primary immunization, and IFA was used at boost.

30 min. Medium containing indomethacin was replaced with fresh RPMI 1640 prior to stimulation. All media contained 50 μg of gentamicin (Cell-Gro, Herndon, Va.)/ml.

FIG. 1. Primary macrophages (A) and RAW 264.7 cells (B) were stimulated with crude emulsan (EM) (solid bars) or apoemulsan (open bars) isolated from the A. calcoaceticus RAG-1 parent strain fed on minimal medium and ethanol. TNF release was determined by the L929 bioassay. Control, nonstimulated cells; 100 ng/ml LPS, bacterial lipopolysaccharides. Error bars indicate standard errors of the means.
neapolis, Minn.). The L929 cytotoxicity assay was conducted as previously described (1). All stimulations were carried out in the presence of 5 μg of polymyxin B/ml unless otherwise noted. Bacterial LPS (E. coli O55:35; Sigma) was used as a contaminating protein, as removing this protein by phenol extraction did not decrease activity (Fig. 1).

Adjuvant activity for humoral immune response. Forty 6- to 8-week-old female BALB/c mice were randomly placed in eight groups of five mice and immunized as described in Table 1 (keyhole limpet hemocyanin [KLH]-2,4-dinitrophenol [DNP] was purchased from Pierce). Blood was collected from the tail 3 days prior to primary immunization. Antigen and adjuvant were mixed by repeated aspiration through an 18-gauge needle attached to a 3-ml syringe. Each mouse was immunized intraperitoneally (i.p.) with 200 μg of bovine serum albumin-DNP/ml in 0.05 M carbonate buffer, pH 9.5, was g周末祭祀 every 3 days after boost until day 21 postboost and then again at 6, 9, and 12 weeks. The total DNP-specific antibody titer was determined by ELISA as described above with the substitution of horseradish peroxidase-conjugated goat anti-mouse antibody. The dilution at which the absorbance was twice baseline was determined. The relative antibody titers were plotted to a sigmoidal curve (Graphpad Prizm), and the antibody titer was determined as for the total antibody titers.

TABLE 2. Fatty acid compositions and polysaccharide molecular weights of emulsan variants produced by mutant A. calcoaceticus RAG-1 strains

| Emulsan variant | Polysaccharide MW | Total FA content | Individual FA content for side chain:
|-----------------|------------------|-----------------|-------------------------|
|                 |                  |                 | 12 | 12:0, 2-OH | 12:0, 3-OH | 13 | 13:1 | 14 | 14, OH | 15 | 16 | 16:1 | 16:2 | 17 | 17:1 | 18 | 19 | 22 |
| 13D-LB          | 255,083          | 47.5            | 6  | 8         | 14         | 13    | 6  | 0.5 |
| 13D-LB11        | 344,214          | 94.0            | 3  | 1.5       | 33         | 4     | 0.5 | 2  | 20     | 24 |
| 13D-LB14        | 272,960          | 157.3           | 12 | 3.5       | 4          | 9     | 13  | 76  |
| 13D-LB16        | 351,348          | 154.1           | 4  | 19        | 2          | 22    | 0.05 | 13  | 65    | 0.05 | 29 | 0.02 |
| 13D-LB18        | 138,631          | 20.0            | 2  | 2         | 2          | 9     | 7   |
| 52D-LB          | 11,345           | 14.0            | 7  | 1.5       | 2          | 22    | 0.05 | 13  | 65    | 0.05 | 29 | 0.02 |
| 52D-LB11        | 251,533          | 0.5             | 0.5| 1         | 2          | 4     | 3   |
| 52D-LB14        | 222,467          | 12.0            | 3  | 1.5       | 2          | 22    | 0.05 | 13  | 65    | 0.05 | 29 | 0.02 |
| 52D-LB16        | 215,341          | 57.0            | 3  | 1.5       | 2          | 22    | 0.05 | 13  | 65    | 0.05 | 29 | 0.02 |
| 52D-LB18        | 142,337          | 264.0           | 21 | 24        | 3          | 13  |
| 62C-LB          | 13,227           | 5.0             | 2  | 1         | 1          | 2    |
| 62C-LB11        | 510,562          | 239.3           | 1.5| 1.5       | 1.5       | 2    | 26  | 48  | 2.5 |
| 62C-LB14        | 697,064          | 18.0            | 0.01| 156     | 16         | 2    | 26  | 48  | 2.5 |
| 62C-LB16        | 681,448          | 154.1           | 12 | 13       | 205        | 184  |
| 62C-LB18        | 651,043          | 89.0            | 12 | 13       | 205        | 184  |
| R1-LB           | 21,224           | 15.0            | 2  | 1         | 3          | 4    | 5   |
| R1-LB11         | 765,410          | 269.0           | 24 | 33        | 4          | 6    | 6   | 37  | 88  | 69  |
| R1-LB14         | 562,554          | 604.0           | 105| 109      | 315        | 72   | 3   |
| R1-LB16         | 236,732          | 680.0           | 288| 205       | 184        | 3    |
| R1-LB18         | 190,375          | 124.0           | 41 | 13        | 34         | 7    | 26  | 3   |
| VRBS1-LB        | 45,267           | 0.3             | ND | 0.3       |
| VRBS1-LB11d     | 219,261          | 28.0            | ND | 0.3       |
| VRBS1-LB14      | 53.0             | 10.5 12 | 0.2 |
| VRBS1-LB16e     | ND               |
| VRBS1-LB18e     | ND               |

a Variant identification is the strain followed by the carbon source. LB11, LB14, LB16, and LB18, LB media plus 1% (wt/vol) undecanoic, myristic, palmitic, and stearic acids, respectively.
b Polysaccharide molecular weight (MW) was determined by gel permeation chromatography after removal of acyl chains.
c Fatty acid (FA) content is in units of nanomoles of FA per milligram of apoemulsan.
d No emulsan was isolated from this variant.
e Insufficient emulsan was isolated to complete the FA analysis.
f Unk, unknown.
g ND, not done.

RESULTS

Emulsan activates macrophages. To determine if emulsan possesses any immunomodulatory properties, one variant of emulsan was tested to determine if it could activate macrophages, as determined by measuring TNF release. Emulsan isolated from the A. calcoaceticus RAG-1 strain, fed on minimal medium and ethanol, stimulated macrophages in a dose-dependent manner. This activation was independent of contaminating protein, as removing this protein by phenol extraction did not decrease activity (Fig. 1).

Structural variation affects emulsan bioactivity. To characterize the critical structural features of the emulsan with regard to macrophage activation, the fatty acids were stripped off the polysaccharide backbone of the ethanol-fed A. calcoaceticus RAG-1 emulsan. Removal of the fatty acid side chains destroyed the ability of emulsan to induce TNF from RAW 264.7 cells (data not shown). To explore the relationship between the fatty acid content of emulsan and its ability to activate macro-

idase-conjugated isotype-specific antibodies (Accurate, Westbury, N.Y.) for the goat anti-mouse antibody. The dilution at which the absorbance was twice baseline was determined as for the total antibody titers.
phages, several *A. calcoaceticus* RAG-1 mutants and the parent *A. calcoaceticus* RAG-1 strain were fed on different carbon sources and the resulting emulsan variants were isolated for chemical analysis and macrophage activation assays. Gas chromatography and mass spectroscopy analysis of the emulsan variants derived from these conditions indicated a wide array of fatty acid substitutions along their polysaccharide backbones, as well as differences in the average lengths of the polysaccharide backbones (Table 2).

These emulsan variants were then used to stimulate RAW 264.7 cells to explore the putative role of the fatty acid composition in activating macrophages. The emulsan variants tested induced a wide range of TNF releases from the RAW 264.7 cells, which were dependent not only on the bacterial

FIG. 2. RAW 264.7 cells were stimulated with 200 (solid bars) or 20 (open bars) ng of several emulsan variants/ml in 96-well tissue culture plates, and TNF release was determined. Cultures were grown in LB bacterial media with or without fatty acids (1% [wt/vol]) of a specified carbon length (LB11, LB14, LB16, and LB18 are defined in Table 2). The bacterial strains are identified and the emulsan variants resulting from each culture condition are characterized in Table 2. The data are grouped to show the relationship of TNF release to culture conditions for a given bacterial strain (A to E) or to show the relationship of TNF release to bacterial strain under particular culture conditions (F to J). N.D., not done. Error bars indicate standard errors of the means.
culture conditions used to generate the emulsan (Fig. 2A to E) but also on the genetics of the mutant strains (Fig. 2F to J). The detailed chemical analysis of each of these variants made it possible to attempt to correlate certain chemical and physical properties of the emulsan variants with the level of macrophage activation elicited. We tested the correlation between TNF release from RAW 264.7 cells and total fatty acid (nanomoles of fatty acid per milligram of emulsan) (Fig. 3A), the total degree of substitution (the ratio of total fatty acid to the molecular weight of the polysaccharide backbone) (Fig. 3B), the total amount of each individually identified fatty acid side chain, and the degree of substitution of each fatty acid (Fig. 3C, representative example). Macrophage activation was increased with either increased fatty acid content (Fig. 3A) or increased total degree of substitution of fatty acids along the polysaccharide backbone (Fig. 3B). However there was a great deal of variation, indicating a more complex relationship between fatty acid content and macrophage activation.

Examination of the individual fatty acid side chains and their correlation to macrophage activation indicated again that this relationship is complex. The most common side chains identified were C12;C12, 2-OH; C14;C16; and C18; each was represented in some quantity in 10 or more of the 23 emulsan variants analyzed. The presence of the C12, 2-OH side chain appeared to have an inhibitory effect on macrophage activation, as increased total content or degree of substitution of this side chain resulted in decreased macrophage activation (Fig. 3C). The C17:1 side chain appeared to have a positive effect on macrophage activation, as its degree of substitution increased. Several other side chains exhibited slight positive or negative effects on macrophage activation, but none were clearly correlated (data not shown).

Emulsan acts as an adjuvant. To test emulsan as an adjuvant, the ethanol-fed *A. calcoaceticus* RAG-1 emulsan before and after protein removal was used in a hapten carrier immunization protocol. Animals immunized with antigen in the presence of emulsan exhibited significantly higher DNP-specific antibody titers over the course of 12 weeks than those animals immunized with antigen alone. This heightened humoral response was similar to that achieved with the complete Freund’s adjuvant (CFA)-incomplete Freund’s adjuvant (IFA) combination (Fig. 4A).

The relative contributions of IgG1 and IgG2a isotypes to the total serum response of these immunized animals were examined at the ninth day after boost. Emulsan- and Freund’s adjuvant-immunized animals demonstrated a significant increase in the IgG2a response compared to antigen-alone controls, with little or no increase in IgG1 titers (Fig. 4B).

Animals used in these immunizations demonstrated no evidence of toxicity from emulsan during the course of these

![FIG. 3.](image-url) TNF release data for stimulations with emulsan (EM) at 20 ng/ml (Fig. 2) were used to analyze the relationship of total fatty acid (FA) (A), total degree of substitution (B), and individual fatty acid content (C). Degree of substitution is defined as a ratio of fatty acid content in nanomoles to polysaccharide molecular weight (MW). A linear regression line is displayed for each data set.
experiments. Mice were injected twice with as much as 200 μg of crude emulsan or apoemulsan without any obvious changes in behavior or survival.

**DISCUSSION**

The goal of these studies was to define the immunomodulatory properties of emulsan, a complex polysaccharide, and to characterize its ability to activate the innate immune response and to serve as an adjuvant for specific humoral responses. Initial studies demonstrated that emulsan could induce TNF release from primary murine macrophages and RAW 264.7 cells in a dose-dependent manner. This activation was independent of any LPS contamination, as polymyxin B was present in all of the stimulations.

During the process of purifying emulsan, contaminating protein may copurify at a weight percent as high as 16.4% (12). To eliminate the possibility that the macrophage activation could be due to the contaminating protein rather than the emulsan itself, preparations of emulsan that had been extracted with hot phenol to remove the protein were used to stimulate macrophages. There was no significant difference in the levels of macrophage activation by the crude emulsan and apoemulsan preparations. This result leads to the conclusion that the macrophage activation induced by emulsan is independent of the contaminating protein.

Emulsan has two main components, the polysaccharide backbone and the fatty acid side chains. To determine the relative contribution of each of these components to macrophage activation, emulsan from ethanol-fed *A. calcoaceticus* RAG-1 was stripped of its fatty acid side chains and then assayed for its ability to induce TNF release. Deacylation re-

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**FIG. 4.** (A) DNP-specific antibody titers for female BALB/c mice immunized with DNP-KLH, with emulsan (EM) as the adjuvant, as described in Table 1. The relative antibody titers indicated represent the dilutions at which absorbance was twice baseline (i.e., 0 represents a 1/100 dilution, 1 represents a 1/50 dilution, 2 represents a 1/250, etc.). PBS, phosphate-buffered saline; ApoEM, apoemulsan. (B) DNP-specific IgG1 (solid bars) and IgG2a (open bars) antibody titers were determined from antiserum collected at 9 days postboost. Results were normalized to the titers of antigen (Ag)-alone controls and presented as a fold increases over those for control animals. Error bars indicate standard errors of the means.
sulted in a complete inhibition of macrophage activation. Because the fatty acid component is critical to macrophage activation, we explored the relationship between fatty acid composition and macrophage activation. We have generated several *A. calcoaceticus* RAG-1 mutants (12; W. Blank and D. Kaplan, submitted for publication), which produce variant forms of emulsan. The overall amount of fatty acid decorating the emulsan polysaccharide backbone and also the identities of the side chains in these mutants are affected. It was also observed that the fatty acid content and identity could be manipulated simply by changing the carbon source provided to the mutant and parent strains. The results of macrophage activation assays with these emulsan variants demonstrate that the changes in composition lead also to differences in the macrophage-activating capabilities of the emulsan variants. Analysis of these data suggests that individual fatty acids may be identified as either stimulatory or inhibitory with regard to macrophage activation. This was most striking with the 10 emulsan variants that contained the C_{12} 2-OH side chain, in which the higher content of this fatty acid was correlated with diminished macrophage activation (Fig. 3C). However, it is likely that more-subtle structural properties, such as the clustering of particular side chains and the order of individual side chains, may be very important in recognition of these acylated polysaccharides by the macrophage.

The combination of emulsan’s ability to stimulate macrophages and its natural emulsifying properties led to the intriguing possibility that it could act as an adjuvant. The data presented here demonstrate its effectiveness in enhancing humoral immunity. Over the course of more than 130 days, mice immunized against an antigen in the presence of emulsan had antigen-specific antibody titers more than 100-fold higher than those in mice immunized with antigen alone. This increase in antibody titer was equivalent to that seen with CFA. The high antigen-specific titer supports emulsan’s future use as an adjuvant.

The nature of the immune response potentiated by emulsan was examined for its relative T-helper-subtype contribution. This was assessed by determining the relative amounts of an adjuvant. The data presented here indicate that the quantity and identity of a given side chain may affect the ability of that particular emulsan variant to activate macrophages. In vivo studies with several variants may correlate differences in structure and adjuvant activity.

While several questions remain to be answered with regard to the mechanism of emulsan-induced immune activation, it is clear that the combination of amenability to structural tailoring and emulsification properties of the emulsan variants presents a powerful system to examine structure-function relationships with regard to immune activation and adjuvantivity.

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