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# Elimination of trace endotoxin protein from rough chemotype LPS

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*SUMMARY.* **The phenol-chloroform-petroleum ether (PCP) extraction is the method of choice for isolating LPS chemotypes from rough mutant bacteria. However, we have observed that a high percentage of PCP-purified LPS preparations contain trace levels of protein contaminants, and these protein contaminants may exhibit endotoxin-like activity. To obtain protein-free rough LPS, a modified phenol-water extraction procedure was developed for use as a final step to follow PCP extraction. Using this procedure, *Salmonella minnesota* Ra, Rc, Rd and Re LPS were recovered in yields of 82–100% from the original PCP-extracted preparations. Yields were determined by recovery of KDO and supported by analysis of silver stained SDS-PAGE gels. Although the original PCP-purified chemotypes were contaminated by major 41, 38.5, 29.5, 23, 17 and 14 kDa proteins (detected by Western blotting and gold staining), the repurified LPS contained no detectable protein. The repurified LPS retained all of its original bioactivity as defined by the ability to stimulate normal C3H/OuJ macrophages to secrete TNF. The importance of utilizing repurified LPS in biologic studies was illustrated by the loss of > 99% of activity when repurified LPS was used to stimulate TNF secretion by LPS 'hyporesponsive' C3H/HeJ macrophages.**

The outer membrane and periplasmic space of Gram-negative bacteria are richly populated with a variety of bacterial proteins.[1] Because certain of these polypeptides bind specifically to the lipid A moiety of lipopolysaccharides (LPS), and because other proteins, such as lipoprotein are present in great abundance, the isolation of protein-free LPS is difficult.[2–7] Although the levels of protein in purified LPS preparations are frequently small (< 1%), the protein contaminants, known historically as endotoxin protein[8] or lipid A-associated protein,[2] are known to be bioactive, i.e. the protein contaminants activate macrophage TNF secretion and tumoricidal activity and are mitogenic for B lymphocytes[2,8–15] At least one characterized endotoxin protein, lipoprotein, may be as potent as LPS as a macrophage stimulus.[15] Since concentrations of LPS commonly cited in the literature may differ by as much as 6-logs, a 0.1% protein contaminant can, in theory, be problematic to assigning a bioactivity to the LPS or to the trace protein contaminant.

Due to their attenuated polysaccharides, LPS chemotypes of rough mutant bacteria are poorly soluble in aqueous solution. Consequently, attempts to purify rough chemotypes using a Westphal-type[16] phenol-water extraction results in low recoveries of LPS in the aqueous phase.[17] To achieve high yields of rough chemotypes, Galanos et al[17] developed the phenol-chloroform-petroleum ether (PCP) extraction method, and the PCP method has since been the method of choice for isolating LPS from rough mutant bacteria. Nonetheless, in addition to causing the release of LPS from rough mutant bacteria, PCP also extracts at least one LPS binding protein,[5] and the subsequent precipitation of LPS from these extracts may be associated with trace

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son, Lincoln Park, NJ, USA). Cells were cultured at 37°C and 6% $CO_2$. Non-adherent cells were removed by washing 2–4 h after plating. Adherent C3H/OuJ macrophages were then stimulated directly with stimuli as described below, but adherent C3H/HeJ macrophages were first cultured an additional 4 h with media containing 20 U/ml murine recombinant IFNγ. Media were then removed and adherent cells in triplicate wells were cultured with the indicated dilution of LPS. Culture supernatants were harvested after 4–5 h. Supernatants harvested from triplicate wells were pooled and submitted to TNF bioassay. Thioglycollate broth, media and serum contained 3, < 0.03, and < 0.03 EU/ml, respectively, by Limulus ameobocyte lysate assay.

### TNF bioassay

Culture supernatants were assayed for TNF bioactivity in a standard cytotoxicity assay using actinomycin D-treated L929 cells as described previously.[20]

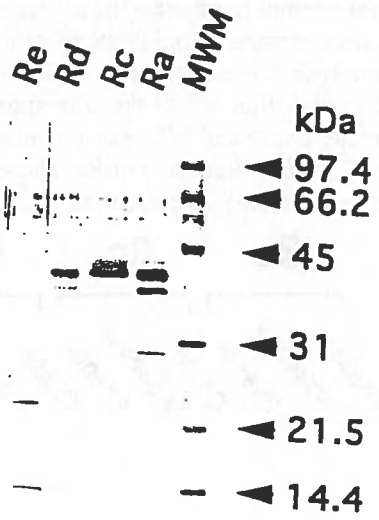


**Fig. 1** — Protein species present in PCP-extracted *S. minnesota* Ra, Rc, Rd and ReLPS. PCP-extracted rough LPS chemotypes (7.5 µg/lane) and molecular weight markers (MWM) were submitted to SDS-PAGE and Western blotting, and proteins were detected using colloidal gold as described in Materials and Methods. Minor 55 and 65 kDa proteins found in each lane are from protein (probably keratin) contamination of 4x loading buffer.

## RESULTS

### PCP-extracted rough LPS may contain endotoxin protein

We have observed that PCP-extracted LPS frequently contains trace levels of endotoxin proteins. To visualize proteins present in commercially available PCP-extracted Ra, Rc, Rd and Re LPS, 7.5 µg of each preparation was submitted to SDS-PAGE and blotted onto nitrocellulose. Proteins on the blots were stained using colloidal gold (Fig. 1). Ra, Rc and Rd LPS contained major proteins with molecular weights of 41, 38.5 and 29.5 kDa. Rd LPS contained an additional 17 kDa protein, and Re LPS was contaminated with major proteins exhibiting molecular weights of approximately 23 and 14 kDa. Colloidal gold, rather than silver staining, was selected for protein detection due to the superior protein sensitivity of colloidal gold and because it does not stain LPS.

### PCP-extracted rough chemotypes can be separated from endotoxin protein and recovered quantitatively using a modified phenol-water extraction procedure

To obtain rough LPS free of protein, we sought a rapid procedure to repurify PCP-extracted LPS under gentle conditions. Initially, we subjected Ra LPS to an additional extraction using the PCP method, but were unable to increase significantly the purity of the Ra LPS. Specifically, the 41, 38.5 and 29.5 kDa proteins were largely retained in the repurified Ra LPS (data not shown). In other experiments, essentially protein-free Ra LPS could be obtained by extracting the proteins into the phenol phase of a Westphal[16] phenol-water procedure. However, roughly 20% of the Ra LPS remained precipitated even after several rounds of aqueous extraction. More importantly, Rc, Rd and Re LPS contain progressively shorter core oligosaccharides and exhibit progressively lower water solubilities. Consistent with previous reports,[17] attempts to repurify Re LPS by phenol-water extraction resulted in < 5% recovery of Re LPS in the aqueous phase, severely limiting the utility of the standard Westphal extraction for the general repurification of PCP-extracted chemotypes.

Three modifications were made in the Westphal method that allowed rapid, quantitative isolation of protein-free Ra, Rc, Rd and Re LPS under mild conditions: the inclusion of 0.5% DOC in the aqueous phase, the use of short extraction times, and the use of room temperature conditions. DOC caused the dissociation of LPS from endotoxin protein and increased the aqueous solubility of the rough chemotypes. Results of a typical repurification of Ra, Rc, Rd and Re LPS are illustrated in Figure 2. 5 mg of each PCP-extracted chemotype was repurified as described in Materials and Methods, and equal volumes of the starting materials (Crude), the resuspended aqueous extract (Aqueous), and the phenol phase (Phenol) were submitted to SDS-PAGE. LPS, detected by silver stain, was recovered exclusively in the aqueous phase of each re-extraction (Fig. 2A). Note that the levels of protein contamination are below levels readily detected by silver stain. Therefore, a second series of samples was resolved by SDS-PAGE and blotted onto nitrocellulose, and the protein content of each sample was evaluated by colloidal gold staining of blots (Fig. 2B). In contrast to LPS, protein was recovered exclusively in the phenol phase of each re-extraction. Some dissoci-

measure protein levels was due to the low protein content of samples relative to assay detection limits (~1 μg/ml) and to false positives resulting from > 100 μg/ml LPS. Consequently, colloidal gold staining of Western blots was used to estimate the relative protein content of the chemotypes before and after repurification. Protein bands were minimally detectable in 75 ng Ra LPS, 150 ng Rc LPS and 250 ng Rd LPS or Re LPS before repurification. In contrast, following repurification, protein was undetectable in 15 μg Ra, Rc, Rd and Re LPS, and, as a control, 15 μg of repurified LPS within the same lane did not prohibit the detection of protein in threshold quantities of PCP-extracted LPS. At a minimum, the results indicated that 99.5–98.4% of contaminating proteins had been removed by phenol extraction. The limit of protein detectability by colloidal gold staining was determined to be between 2 and 0.3 ng for a variety of protein standards. Thus, at worst, the level of protein contamination in the repurified chemotypes was < 0.014%.

### LPS-induced TNF secretion by C3H/OuJ *Lps*$^n$ macrophages: repurified chemotypes are as potent or more potent than the original PCP-extracted chemotypes

A bioassay was selected as a final approach to verify that the aqueous phase recovery of rough chemotypes was essentially quantitative. C3H/OuJ *Lps*$^n$ macrophages were stimulated with 4 pg – 10 ng/ml PCP-extracted LPS or volumes of repurified LPS containing 4 pg – 10 ng/ml LPS assuming 100% recovery of LPS in the aqueous phase. 4 h culture supernatants were then assayed for TNF (Fig. 3). Repurified Ra, Rc and Rd LPS were equipotent with PCP-extracted Ra, Rc and Rd LPS, respectively. Unexpectedly, repurified Re LPS was nearly 10-fold *more* potent than the original PCP-extracted Re LPS.

### LPS-induced TNF secretion by C3H/HeJ *Lps*$^d$ macrophages: potency of PCP-extracted chemotypes are markedly reduced upon repurification

C3H/HeJ mice and their macrophages are markedly hyporesponsive to LPS by virtue of an undefined mutation in the gene (*Lps*) that controls LPS responsiveness (reviewed in [21]). However, LPS preparations that contain endotoxin protein are known to stimulate IFNγ-primed macrophages from C3H/HeJ mice to secrete TNF.[20] To

**Table.** Repurification of PCP-extracted LPS by a modified Westphal extraction*

| | KDO (μg) | | |
|---|---|---|---|
| | Crude | Repurified | % recovery |
| Ra LPS | 268 | 248 | 92.5 |
| Rc LPS | 502 | 517 | 103 |
| Rd LPS | 510 | 464 | 92.8 |
| Re LPS | 673 | 557 | 82.7 |

*5 mg of PCP-extracted Ra, Rc, Rd and Re LPS were each repurified and KDO content of the original PCP-extracted preparations (Crude)

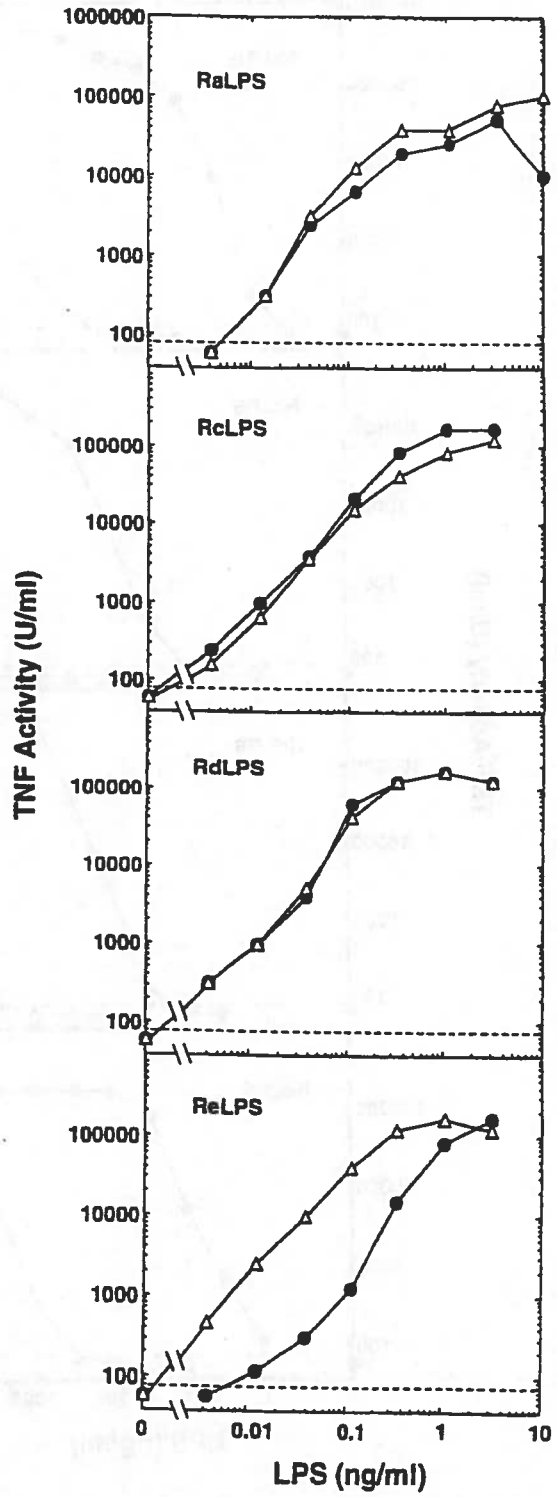


Fig. 3 — TNF secretion by C3H/OuJ *Lps*$^n$ macrophages: induction by PCP-extracted chemotypes before and after repurification. C3H/OuJ *Lps*$^n$ macrophages were stimulated with 0.004–10 ng/ml PCP-extracted Ra, Rc, Rd and ReLPS or volumes of repurified LPS containing 0.004–10 ng/ml LPS (assuming 100% recovery of LPS in the aqueous phase). 4 h culture supernatants were then assayed for TNF as described in Materials and Methods. Closed circles and open triangles represent results obtained with PCP-extracted LPS before and after repurification, respectively. Dashed lines represent the limit

LPS remained in the phenol phase in association with protein. Because DOC had been shown previously to convert LPS from a micellar state to a principally monomeric state,[22,23] we reasoned that inclusion of DOC in the extraction mixture might also release protein-bound LPS and increase Ra LPS recovery within the aqueous phase. Not only was DOC found to increase the recovery of Ra LPS, but it also facilitated the aqueous recovery of Rc, Rd and Re LPS chemotypes, previously considered too hydrophobic for phenol-water extraction. Thus, DOC appeared to have increased the aqueous solubility (or decreased the phenol solubility) of attenuated rough LPS chemotypes. Recovery of Ra, Rc, Rd and Re LPS in the resuspended aqueous phase was determined to be essentially quantitative as assessed by recovery of KDO, SDS-PAGE and silver staining, and by potency to stimulate TNF secretion by $Lps^n$ C3H/OuJ macrophages. Thus, we have reported herein an adaptation to the Westphal method that provides an ideal final step for removing trace protein contaminants from PCP-extracted rough mutant LPS. The procedure is rapid, mild, and results in quantitative recovery.

The value of utilizing protein-free LPS in examining LPS-macrophage interactions was illustrated by the effects of repurification on rough chemotype-induced TNF secretion by C3H/HeJ ($Lps^d$) and C3H/OuJ ($Lps^n$) macrophages. Although PCP-extracted rough LPS stimulated $Lps^d$ macrophages to secrete TNF, stimulation of $Lps^d$ macrophages required 100- to 7,200-fold greater concentrations of rough chemotypes than required to stimulate $Lps^n$ macrophages. The large differential sensitivity suggested the existence of a bioactive contaminant within the LPS preparations, and, indeed, removal of ≥ 99% of endotoxin protein resulted in the loss of ≥ 99% of stimulatory activity. In marked contrast, removal of a phenol extractable component of Re LPS, ***increased*** 10-fold the ability of Re LPS to stimulate $Lps^n$ C3H/OuJ macrophages. These latter results suggest that one or more endotoxin proteins may actually downregulate the actions of LPS on normal macrophages. Precedence for bacterial proteins that negatively regulate macrophage responses to LPS has been provided by a highly purified 39 kDa protein from the cell walls of *Proteus mirabilis* that inhibits LPS-induced IL-1 secretion, although this same protein appears to enhance LPS-induced TNF secretion.[24] Also possible is that trace levels of DOC retained within the repurified LPS preparations may reduce the micellar size of Re LPS and result in greater availability of the lipid A moiety. In any event, the differential effects of phenol-extractable components on cellular responses to LPS underscores the importance of utilizing well defined LPS preparations in biological studies.

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