PROTEIN-TYROSINE PHOSPHATASE ACTIVITY OF *COXIELLA BURNETII* THAT INHIBITS HUMAN NEUTROPHILS

Y.P. LI, G. CURLEY, M. LOPEZ, M. CHAVEZ, R. GLEW, A. ARAGON, H. KUMAR, O.G. BACA*

Biology Department, University of New Mexico, 167 Castetter Hall, Albuquerque, NM 87131-1091, USA

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Summary – Supernatants prepared from disrupted *Coxiella burnetii* possess acid phosphatase (ACP) activity that apparently accounts for the inhibition of the metabolic burst of formyl-Met-Leu-Phe(fMLP)-stimulated human neutrophils. Results are presented regarding purification and biochemical-biological characterization of the ACP. The highly purified enzyme, which exhibited an apparent M_r of 91 K and optimal activity at pH 5.0, also inhibited neutrophils. The enzyme retained full activity at pH 4.5, 5.5, and 7.4, when incubated overnight at 0°C and room temperature; at pH 5.5, it retained full activity after overnight incubation at 37°C. Apparently, the enzyme contains asparagine-linked but not serine- or threonine-linked glycan residues since its treatment with N-glycosidase F (PNGase F) decreased its M_r to 87 K and no changes were detected with O-glycosidase. The enzyme's capacity to hydrolyze phosphate from a number of phosphate-containing compounds was examined; five phosphocompounds were significantly hydrolyzed: 5'-CMP > fructose 1,6-diphosphate > tyrosine phosphate > 3'-AMP > 5'-AMP. The ACP also dephosphorylated ^{32}P-Raytide, a phosphotyrosine-containing peptide. Dephosphorylation of Raytide was inhibited by the following phosphatase inhibitors: sodium molybdate, potassium fluoride, sodium ortho-vanadate and D_2, a heteropolymolybdate compound. These results indicate that *C. burnetii* ACP may play a role in disrupting tyrosine phosphorylation/dephosphorylation reactions associated with the signal transduction pathway culminating in the metabolic burst. Interestingly, Western blot analysis of ACP-inhibited neutrophils showed a marked increase in tyrosine phosphorylation of a 44 K protein as compared to uninhibited cells.

Key words: *Coxiella burnetii*; tyrosine phosphatase; human neutrophils

Introduction

The Q fever agent, *C. burnetii,* inhibits the metabolic burst and the accompanying generation of toxic oxygen metabolites in human neutrophils during and after entry into the phagocytes (Ferenčík *et al.,* 1984; Akporiaye *et al.,* 1990). Infected macrophage cells (J774 cell line) also exhibit a diminished oxidative metabolism and reduced capacity to generate superoxide anion (Baca *et al.,* 1984). Supernatants (100,000 x g) prepared from disrupted *C. burnetii* contain significant levels of ACP activity and also inhibit fMLP-stimulated neutrophils (Baca *et al.,* 1993). Heteropolymolybdate compounds – inhibitors of ACPs – obviate this inhibition, thus implicating the *C. burnetii* ACP as the inhibitory factor in the supernatants.

In this communication, we report that highly purified ACP from *C. burnetii* inhibits neutrophils. In addition, we present extensive characterization of the enzyme, including substrate specificity data that indicate that the enzyme is a protein-tyrosine phosphatase (PTPase); such an enzyme

*Corresponding author.

Abbreviations: ACP = acid phosphatase; AEBSF = 4-(2-aminoethyl)-benzenesulphonyl fluoride; BCA = bicinchonic acid; fMLP = formyl-Met-Leu-Phe; ECL = enhanced chemiluminiscence; EDTA = ethylenediamine tetraacetate; HPLC = high performance liquid chromatography; IP_3 = inositol 1,4,5-triphosphate; MEM = Eagle's Minimum Essential Medium; MUP = methylumbelliferyl phosphate; NP-40 = Nonidet P-40; PNGase = N-glycosidase F; PTPase = protein-tyrosine phosphatase; SDS-PAGE = polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate

from *Yersinia* has been implicated as a neutrophil inhibitor (Bliska *et al.*, 1991).

Materials and Methods

Propagation and purification of C. burnetii. Plaque-purified phase I *C. burnetii* (Nine Mile isolate, RSA493, originally obtained from M. Peacock of the Rocky Mountain Laboratory, Hamilton, MT) was purified from persistently infected L929 fibroblasts, continuously infected and maintained in culture for more than ten years (Baca *et al.*, 1993; Roman *et al.*, 1986). The pelleted organisms were overlaid with 10% (v/v) glycerol in 10 mmol/l HEPES buffer pH 7.4 and stored at -70°C.

Purification of ACP from C. burnetii. After thawing, the glycerol-HEPES freezing medium was decanted and the *C. burnetii* pellet was washed with 10 mmol/l HEPES pH 7.4 – 0.8% NaCl by centrifugation at 10,000 x g. This and the following procedures were carried out at 4°C. The washed organisms were resuspended in the HEPES-NaCl medium containing 0.2% (v/v) Triton X-100 and 0.4 mmol/l AEBSF, and gently stirred for 80 mins at 4°C followed by centrifugation at 100,000 x g for 30 mins. The supernatant was transferred to a hydroxylapatite column (8 x 65 mm) and eluted with 10 mmol/l potassium phosphate buffer pH 6.4 containing 0.1% Triton X-100. The fractions containing ACP activity were pooled and concentrated with the aid of a Centricon-50 centrifugal microconcentrator (Amicon, Beverly, MA). For some experiments, the concentrated ACP was further purified by high performance liquid chromatography (HPLC) using a Superose-12 column (10 x 300 mm) eluted with 10 mmol/l potassium phosphate pH 6.4 and 0.1% Triton X-100.

ACP activity was determined fluorometrically with 4-methylumbelliferylphosphate (MUP) as the substrate. The standard assay was carried out at 37°C for 15 mins in 0.1 ml. The mixture contained 0.2 mol/l sodium acetate buffer pH 5.5 and 5 mmol/l MUP (Baca *et al.*, 1993). One unit of enzyme activity was defined as the amount of enzyme required to convert 1 nmole of substrate to product in 1 hr.

The release of inorganic phosphate (P_i) from potential low-molecular phospho-monoester substrates was estimated using the method of Lanzetta *et al.* (1979).

Proteins were assayed by the micro bicinchonic acid (BCA) protein assay procedure (Pierce Chemical Co., Rockford, IL) with bovine serum albumin as the standard. The manufacturer's protocol was followed.

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) was carried out according to Laemmli (1970). Gels were silver-stained (Sambrook *et al.*, 1989).

Neutrophils were purified from freshly obtained peripheral blood of healthy laboratory personnel by Ficoll-Hypaque density gradient centrifugation, as previously described by English *et al.* (1981). Residual erithrocytes were lysed in 0.85% ammonium chloride containing 0.1% potassium bicarbonate pH 7.4. The cell pellet was resuspended at a concentration of 5 x 10^7 cells/ml in Eagle's Minimum Essential Medium (MEM) with 0.1% glycerol.

Superoxide anion assay. The generation of superoxide anions by fMLP-stimulated neutrophils was measured as superoxide dismutase-inhibitable cytochrome c reduction using the continuous assay of Saha *et al.* (1985).

Kinetic analysis. Determination of the Michaelis-Menten parameters K_m and V_{max} for the ACP was performed in the presence and absence of various inhibitors using six to eight substrate (MUP) concentrations in the range of 0.4 –7.0 x K_m. The initial reaction rates and their corresponding substrate concentrations were subjected to computer analysis using the mathematical procedure described by Cleland (1979) which fits the data to a rectangular hyperbola. Lineweaver-Burk plots were generated. A replot of the reciprocal of V_{max} versus the inhibitor concentration was constructed, and the abscissa intercept was calculated by linear regression analysis. The inhibition constant for each inhibitor (K_i) was calculated from the abscissa intercepts of the replots.

Deglycosylation by PNGase F. 20 µl of ACP (1 mg/ml) was mixed with 10 µl of 1% SDS, boiled for 10 mins and slowly cooled to room temperature. After the addition of 90 µl of the incubation buffer containing 20 mmol/l sodium phosphate pH 7.0, 10 mmol/l sodium azide, 50 mmol/l EDTA, and 0.5% Triton X-100, the sample was boiled again for 2 mins and cooled to room temperature. The resulting mixture was incubated with 0.4 U of PNGase F at 37°C for 20 hrs. The deglycosylated protein was analyzed by SDS-PAGE, and visualized by silver-staining.

Radiolabelling of Raytide. The tyrosine residue present in the peptide Raytide was phosphorylated (^{32}P) with $p60^{c\text{-}src}$ kinase as described by Shibata *et al.* (1994). One hundred µg of Raytide was incubated with 20 U of $p60^{c\text{-}src}$ at 30°C for 24 hrs in a total volume of 300 µl in the following reaction buffer: 50 mmol/l HEPES pH 7.5, 10 mmol/l $MgCL_2$, 0.067% ß-mercaptoethanol, 0.05 mmol/l ATP (including 800 µCi of [r-^{32}P]ATP). The reaction was stopped by the addition of 1.2 ml of 10% phosphoric acid. A total of 150 µl of the reaction mixture was spotted onto P81 phosphocellulose paper sheets (5 x 5 cm) and extensively washed with 0.5% phosphoric acid. Phosphorylated Raytide (^{32}P-Tyr-Raytide) was eluted with 20 ml of 500 mmol/l ammonium carbonate, lyophilized, and suspended in 2 ml of water. Based on the known specific activity of [r-^{32}P]ATP and the amount of P_i incorporated into Raytide, the concentration of ^{32}P-Tyr-Raytide was calculated to be 22 nmol/l.

Dephosphorylation of ^{32}P-Tyr-Raytide by ACP. A reaction mixture containing 10 µl of 100 mmol/l sodium acetate pH 5.0, 10 µl of ACP (290 U of ACP in 10 mmol/l potassium phosphate buffer pH 6.4), and 25 µl of distilled water was incubated with 5 µl of 22 nmol/l ^{32}P-Tyr-Raytide (approx. 40,000 cpm) at 37°C for indicated time periods. The reaction was stopped with 50 µl of 15% (w/v) trichloroacetic acid in 0.1 mmol/l potassium dihydrogen phosphate, and 100 µl of the molybdate reagent prepared according to Martin and Doty (1949). The ^{32}P-phosphate was extracted with 500 µl of isobutanol-benzene (1:1, v/v) and after centrifugation its radioactivity was determined.

Tyrosine phosphorylation assay was performed using the Western blot procedure described by Kusunoki *et al.* (1992) with some modifications. Freshly separated human neutrophils (5 x 10^6) in 100 µl of MEM with 0.1% glycerol were incubated with ACP or fMLP for indicated times at 37°C in a total of 1 ml of Krebs-Ringer phosphate buffer pH 7.4. After incubation, cells were

pelleted in a microcentrifuge, lysed with 100 µl of ice-cold lysis buffer containing 1% NP-40, 20 mmol/l Tris pH 8.0, 150 mmol/l NaCl, 1 mol/l AEBSF, 5 µg/ml aprotinin, 1 µg/ml pepstatin A, and 0.2 mmol/l sodium ortho-vanadate, and kept on ice for additional 15 mins. Insoluble material was removed by centrifugation at 15,000 x g for 10 mins at 4°C. Eighty µl of the supernatant was then mixed with 35 µl of 4xSDS loading sample buffer, boiled for 5 mins and electrophoresed on a 10% polyacrylamide gel. The banded proteins were then blotted to a nitrocellulose (Hybond-ECL, Amersham) membrane, and the phosphotyrosine-containing proteins were detected with anti-phosphotyrosine monoclonal antibody (MoAb) by an enhanced chemiluminescence (ECL) technique decsribed in the manufacturers' protocol.

Reagents. Pepstatin A, AEBSF, aprotinin, leupeptin, and monopoly resolving medium (Ficoll-Hypaque, 1.114 g/ml) were purchased from ICN Biomedicals, Inc., Aurora, OH. MEM was from Gibco BRL Life Technologies, Inc., Grand Island, NY. Hydroxylapatite was from Bio-Rad Laboratories, Hercules, CA. HEPES, fMLP, cytochrome C, NP-40, antiphosphoserine MoAb and anti-phosphothreonine MoAb were from Sigma Chemical Co., St. Louis, MO. Antiphosphotyrosine (clone 4G-10) was obtained from Upstate Biotechnology Inc., Lake Placid, NY. The heteropolymolybdate complexes was synthesized by M.T. Pope of Georgetown University, Washington, D.C. PNGase F and O-glycosidase were acquired from Boehringer Mannheim Co., Indianopolis, IN. [r-^{32}P]ATP (6,000 Ci/mmol/l was from New England Nuclear, Boston, MA. Raytide and p60$^{c\text{-}src}$ tyrosine kinase were purchased from Oncogene Science, Inc., Uniondale, NY. Ion-exchange chromatography paper (P-81) was the product of Whatman, Maidstone, UK.

Results

Purification and M_r of C. burnetii ACP

The 100,000 x g *C. burnetii* supernatant, which contained approximately 90% of the total ACP activity present in the intact organisms, was chromatographed on a hydroxylapatite column. About 50-60% of the ACP activity was eluted with the elution buffer. The fractions which had the highest levels of ACP activity were pooled and concentrated with the aid of Centricon-50 (Amicon) filters. The concentrated ACP was then chromatographed on HPLC Superose-12 columns (Fig. 1). Overall, the procedure resulted in a 66-100-fold purification (Table 1).

To determine M_r of the *C. burnetii* ACP, 20 U of the enzyme from different fractions containing the highest levels of ACP activity eluted from HPLC Superose-12 column were independently analyzed by SDS-PAGE. As shown in Fig. 2, most of the fractions examined contained only one major protein band of 91 K. It provided a convincing evidence that this band seen on SDS-PAGE represents the *C. burnetii* ACP.

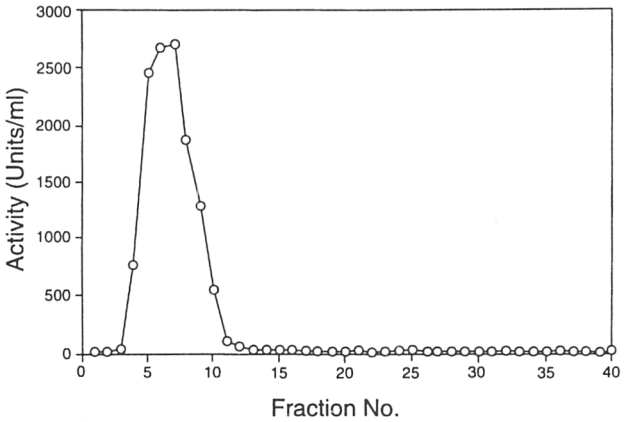

Fig. 1
HPLC of *C. burnetii ACP*
Fractions obtained from hydroxylapatite columms containing high levels of ACP activity were pooled, concetrated and applied to the HPLC column.

Table 1. Purification of ACP from *C. burnetii*

Purification step	ACP activity (U/ml)	Protein conc. (µg/ml)	Specific activity (U/mg protein)	Purification (-fold)
1.Crude Triton X-100 extract	867	301	2,881	–
2.100,000 x g supernatant	777	181	4,302	1-3
3.Hydroxylapatite	332	6	58,326	20-40
4.HPLC	2,353	12	189,792	66-100

*One unit = 1 nmole of methylumbelliferyl phosphate hydrolyzed per hr at 37°C.

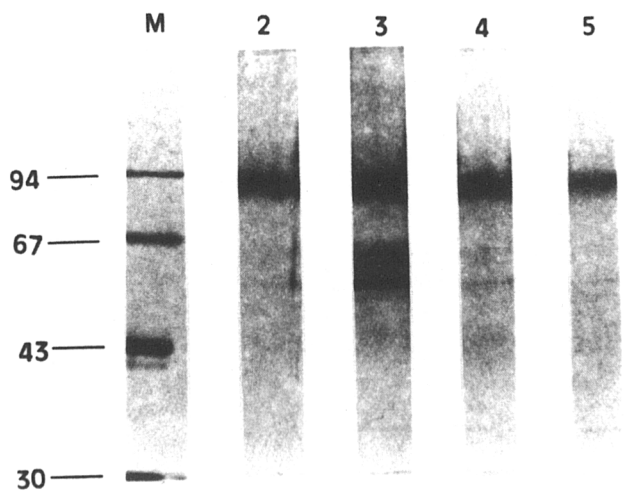

Fig. 2
SDS-PAGE of *C. burnetii* ACP
Twenty U of the enzyme obtained from different fractions of the peak region in HPLC (lanes 2-5) were electrophoresed. Size markers (lane M).

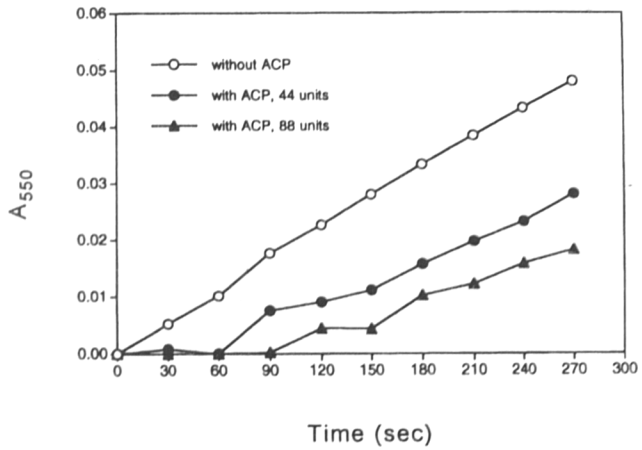

Fig. 3
Inhibition of superoxide anion production in human neutrophils by highly purified *C. burnetii* ACP
Hydroxylapatite-purified and concentrated *C. burnetii* ACP was employed. Experimental neutrophils (10^6) were preincubated with 44 U (10 µl) or 88 U (20 µl) of ACP in Krebs Ringer phosphate buffer pH 7.4 (final volume of 1.0) at 37°C for 2 mins; control, untreated neutrophils (10^6) received 1.0 ml of the buffer alone. Measurement of superoxide anion production began with the addition of 10^{-7} mol/l fMLP. Absorbance readings were made at 30 sec intervals during the 5-min-incubation period.

Inhibition of human neutrophils

The effect of highly purified ACP an human neutrophils was examined. The ACP inhibited superoxide anion production by human neutrophils (Fig. 3). Approximately 65 U of the enzyme reduced superoxide anion production by 50%.

Optimum pH for ACP activity

Optimum ACP activity was observed at pH 5.0 in both the 100,000 x g supernatants derived from Triton X-100-treated Nine Mile organisms and the highly purified hydroxylaptite fractions (Fig. 4). Assays were performed in pH range 3.0 – 8.0 with MUP as the substrate.

Stability of ACP at different temperatures and pH

Fifty U of the purified enzyme were incubated in 36 µl aliquots at different pHs [pH 4.5 P-25 buffer of Hackstadt and Williams (1981); pH 5.5 0.2 mol/l sodium acetate buffer; pH 7.4 0.01 mol/l HEPES with 0.8% NaCl] and temperatures (0°C, room temperature, 37°C) for 30 mins to 24 hrs. After 24 hrs of incubation at pH 4.5, 5.5, and 7.4, the enzyme retained full enzymatic activity at room temperature and at 0°C. After overnight incubation at 37°C, the enzyme retained 100% of its original activity at pH 5.5 and lost approximately two-thirds of its activity at pH 4.5 and 7.4.

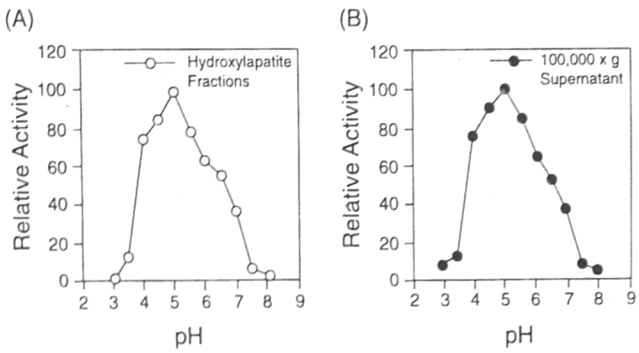

Fig. 4
Effect of pH on *C. burnetii* ACP activity
The enzyme activities at various pHs were determined for the ACP prepared with the aid of hydroxylapatite chromatography (A) and that present in the supernatant (S100) (B). The buffers were 0.2 mol/l sodium acetate (pH 3 to 5.5), 0.2 mol/l cacodylate (pH 6), and 0.2 mol/l Tris-HCl (pH 6.5 and 8).

Comparison of C. burnetii and L929 host cell ACPs on hydroxylapatite columns

ACP preparations from purified *C. burnetii* and uninfected L929 cells were subjected to hydroxylapatite chromatography. The ACP activity of *C. burnetii* was found in the breakthrough fractions when the column was eluted with 10 mmol/l potassium phosphate buffer pH 6.4, while the L929 host cell ACP was obtained only when a sodium chloride gradient (0 to 0.6 mol/l) was applied (Fig. 5). The different elution profiles clearly distinguished *C. burnetii* ACP from that of the host cell.

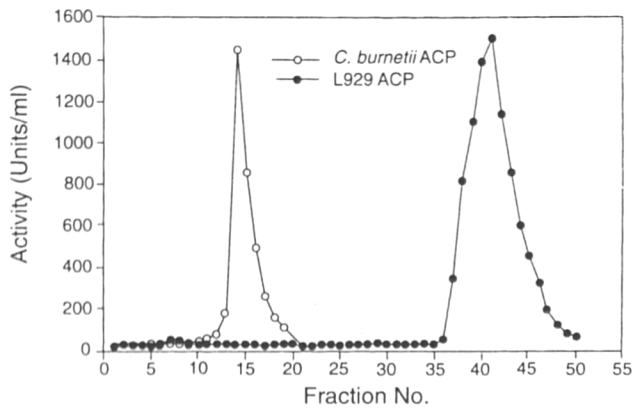

Fig. 5
Chromatography of *C. burnetii* and L929 ACPs on hydroxylapatite
The supernatants (S100) from *C. burnetii* and L929 cells were independently chromatographed on a hydroxylapatite column. The *Coxiella* ACP was eluted with 10 mmol/l phosphate buffer pH 6.4 containing 0.1% Triton X-100; a gradient ranging from 0 to 0.6 mol/l NaCl, which was initiated at fraction No. 15 and terminated at fraction No. 50, was used to elute the L929 ACP. Fractions (0.5 ml) were collected and assayed for ACP activity.

Table 2. List of heteropolymolybdate complexes

Designation	Formula
B'	$[C(NH_2)_3]_2[(CH_3)_2AsMoO_{15}H]$
B_2	$(Bu_4N)_2(C_6H_5)_2AsMo_4O_{15}H)$
B_3	$(Bu_4N)_2(CH_3)_2AsMo_4O_{15}H)$
L	$(Bu_4N)_4Mo_8O_{28}$
N	$(Bu_4N)_2Mo_6O_{19}$
S	$(NH_4)_3FeMo_6O_{24}H_6.ca6H_2O$
D_2	$(NH_4)_4GeMo_{12}0_{40}.xH_2O$
M	$Na_3PMo_{12}O_{40}.xH_2O$
P	$(NH_4)_8ThMo_{12}O_{42}.7H_2O$
Q	$(NH_4)_8CeMo_{12}0_{42}.8H_20$
R	$(NH_4)_4(CH_3As)_4Mo_{12}O_{46}.xH_2O$
E_2	$(NH_4)_6As_2Mo_{18}O_{62}.xH_2O$

Bu = $N\text{-}C_4H_9$

Effects of heteromolybdate compounds and sodium orthovanadate on C. burnetii and L929 host cell ACPs

Heteromolybdate compounds inhibit ACP activity (Saha *et al.*, 1991), including that present in *C. burnetii* (Baca *et al.*, 1993). In addition, sodium orthovanadate is a known inhibitor of PTPase (Cui *et al.*, 1994). Using MUP as substrate, the potential inhibitory effects of 12 heteromolybdate complexes (Table 2) and sodium orthovanadate on the purified ACP from *C. burnetii* were investigated and compared with those on ACP activity present in uninfected L929 mouse fibroblasts. As shown in Fig. 6, most of the heteropolyanions except E2 were generally weak inhibitors of L929 host cell ACP; compared to the untreated controls, the percent inhibition ranged from 1% to 28%. In contrast, most of the heteromolybdates tested – except P and Q – were strong inhibitors of *C. burnetii* ACP; inhibitions ranged from 55% to 89%. Of these compounds, complexes B', D2 and M were the most selective. Sodium ortho-vanadate inhibited both host and *C. burnetii* ACP by 55% and 32%, respectively. The differential effects of the molybdate compounds in general indicate that the ACPs of the parasite and the host are distinct.

Deglycosylation of C. burnetii ACP with PNGase F

The extensively purified *C. burnetii* ACP was treated with PNGase F which hydrolyzes all classes of asparagine-linked glycans. The results were analyzed by SDS-PAGE (Fig. 7). M_r of the ACP after digestion decreased from 91 K to 87 K, suggesting the presence of asparagine-linked glycans in the enzyme. A treatment with O-glycosidase did not produce a detectable shift in the position of the ACP in gels, indicating the absence of serine- or threonine-linked carbohydrate residues in the ACP.

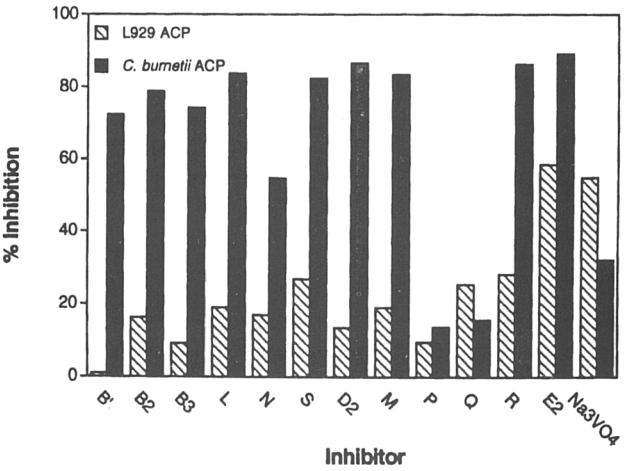

Fig. 6
Effects of heteropolymolybdate complexes and sodium ortho-vanadate on the *C. burnetii* and L929 ACPs
Hydroxylapatite-purified and concentrated *C. burnetii* and L929 ACPs were employed. Two U of *C. burnetii* or L929 ACP were incubated at 37°C for 1 hr in 0.1 ml of reaction mixture containing 0.2 mmol/l sodium acetate buffer pH 5.5, 5 mmol/l MUP, and 5 µmol/l of the various heteropolymolybdates (see Table 2).

Substrate specificity

A number of potential substrates for the ACP were investigated using the malachite green assay which measures the release of inorganic phosphate (Table 3). MUP was the

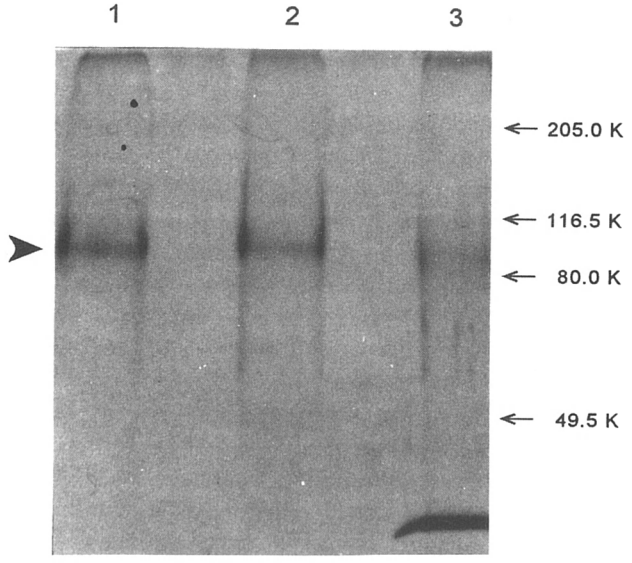

Fig. 7
Deglycosylation of the C. burnetii ACP with PNGase F
Ten µg of ACP were subjected to SDS-PAGE (7% acrylamide). Lane 1, ACP without any treatment; lane 2, ACP treated with all components except PNGase F; lane 3, ACP treated with PNGase in complete reaction mixture.

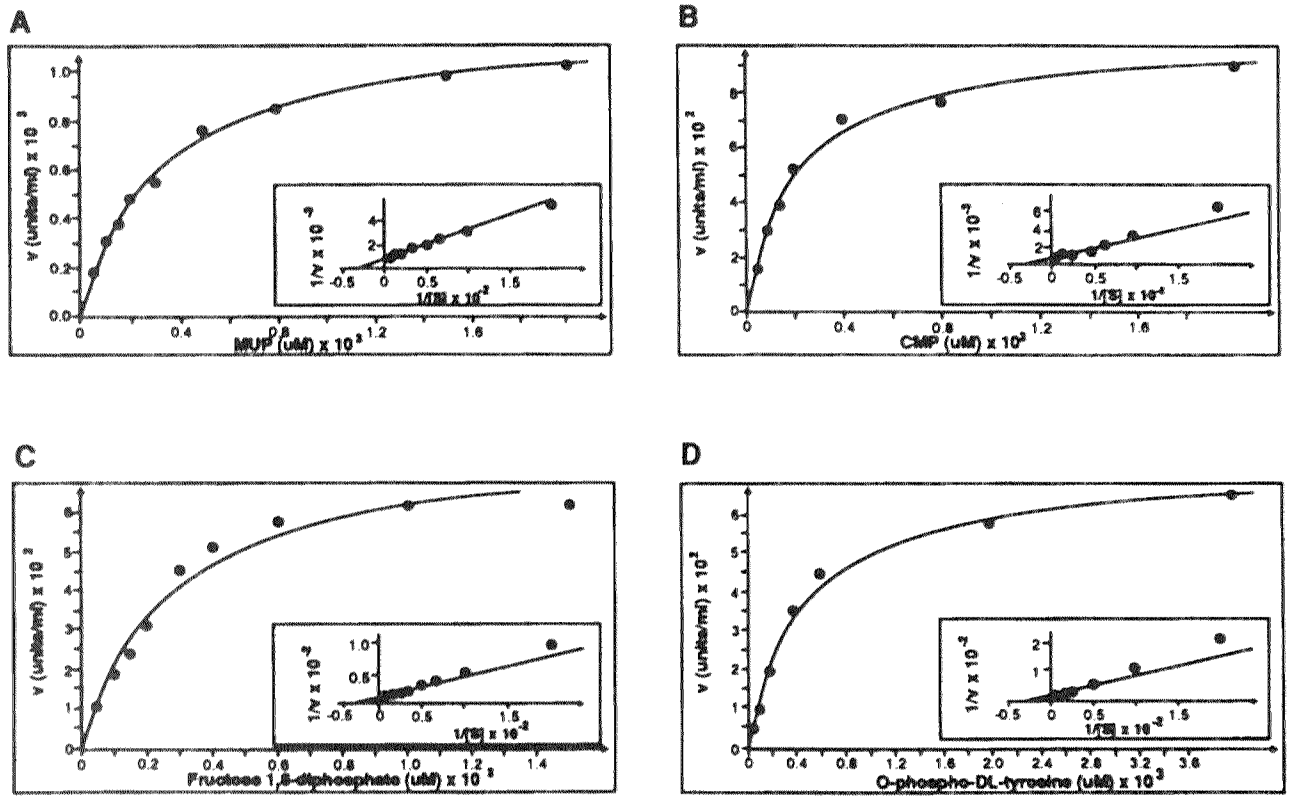

Fig. 8

Determination of Michaelis-Menten constants (K_m) with purified *C. burnetii* ACP and MUP (A), CMP (B), fructose 1,6-diphosphate (C) and O-phospho-DL-tyrosine (D) as substrates

Fourteen U of ACP were included in each assay. The reactions were conducted at 37°C for 2 hrs in a final volume of 100 µl of 10 mmol/l sodium acetate pH 5.5 and 0.1% Triton X-100. The amount of P_i released was measured by the malachite green assay. The K_m values were estimated to be: (A) MUP = 289 µmol/l; (B) CMP = 222 µmol/l; (C) fructose 1,6-diphosphate = 273 µmol/l; (D) O-phospho-DL-tyrosine = 463 µmol/l.

best substrate for the enzyme. Of the physiologically relevant substrates tested, fructose 1,6-diphosphate, 5'-CMP and O-phospho-DL-tyrosine were most effectively dephosphorylated. In addition, 3'-AMP and 5'-AMP were relatively good substrates for the enzyme. O-phospho-DL-threonine and O-phospho-L-serine were very poor substrates.

K_m values

K_m values for ACP were determined using four effective substrates in the malachite green assay (Fig. 8). The K_m values with MUP, 5'-CMP and fructose 1,6-diphosphate were 289 µmol/l, 222 µmol/l, and 273 µmol/l, respectively. The K_m value with O-phospho-DL-tyrosine was estimated to be 463 µmol/l.

Kinetic analysis of the inhibition of C. burnetii ACP by complexes D2 and M

Initial reaction rates were determined in the presence of various concentrations of complexes D2 and M while varying the substrate (MUP) concentration over 20-fold and 7-fold, respectively. Reciprocal reaction rates (1/v) were plotted as a function of the reciprocal substrate concentration (1/[S]) at various concentrations of D2 (Fig. 9) or M (Fig. 10). By plotting the slopes of the lines versus the concentrations of D2 or M, the inhibition constants for complexes D2 and M were estimated to be 2.4 µmol/l and 3.9 µmol/l, respectively. Both inhibited the enzyme non-competitively; complex D2 was a stronger inhibitor as compared to complex M.

PTPase-like activity of C. burnetii ACP

Several bacteria, including *Yersinia*, utilize PTPase to shut down the metabolic burst of neutrophils (see Discussion). The substrate specificity data indicated that tyrosine-phosphorylated proteins might also be the targets of the *C. burnetii* ACP. Because Raytide is often used as a peptide substrate for characterizing PTPases, we examined whether ACP dephosphorylated ^{32}P-Tyr-Raytide. In the presence of

Table 3. Substrate specificity of C. burnetii ACP

Substrate	Concentration (mmol/l)	P_i released (% of MUP)
4-MUP	1	100.0
O-phospho-DL-threonine	1	<1
O-phospho-L-serine	1	<1
O-phospho-DL-tyrosine	1	74.6
5'-ATP	1	21.5
5'-ADP	1	7.2
5'-AMP	1	46.4
3'-AMP	1	58.6
5'-CTP	1	15.4
5'-CDP	1	9.1
5'-CMP	1	93.5
Sodium pyrophosphate	1	8.8
O-phosphorylethanol amine	1	<1
Pyridoxal phosphate	1	25.7
p-nitrophenyl phosphate	1	27.0
Glucose 6-phosphate	1	25.0
Glucose 1-phosphate	1	5.0
Fructose 6-phosphate	1	14.2
Fructose 1-phosphate	1	37.4
Fructose 1,6-diphosphate	1	91.6
Mannan	10 mg/ml	<1
Phosphorylcholine	1	<1
Mannose 1-phosphate	1	7.5
Mannose 6-phosphate	1	3.6
Phosphatidylinositol 4-monophosphate	0.02	<1
Phosphathidylinositol 4,5-bisphosphate	0.02	<1
Inositol 1,4,5-triphosphate	0.02	1.7

Fifteen U of ACP concentrated by HPLC-gel filtration (Superose-12) were used in each assay. Enzyme activity was estimated by measuring the release of P_i by the malachite green assay.

complex D2 (10 μmol/l), the rate of dephosphorylation decreased significantly, indicating that the ACP possessed PTPase-like activity (Fig. 11).

The effect of pH on dephosphorylation of ^{32}P-Tyr-Raytide by ACP was examined. The oprimum pH was about 5.0 (Fig. 12). This result correlated with the pH optimum for MUP which was also about 5.0 as shown above.

To further characterize the PTPase activity of ACP, the effect of different chemicals on the activity was investigated (Fig. 13). Sodium molybdate, sodium ortho-vanadate, and potassium fluoride, known inhibitors of PTPases, markedly inhibited the ACP. In addition, complex B' also significantly inhibited the enzyme.

Neutrophils treated with C. burnetii ACP exhibited enhanced tyrosine phosphorylation of a 44 K protein

To assess possible protein-tyrosine phosphorylation/dephosphorylation in human neutrophils before and after treatment with ACP, cells were pretreated with ACP, stimulated

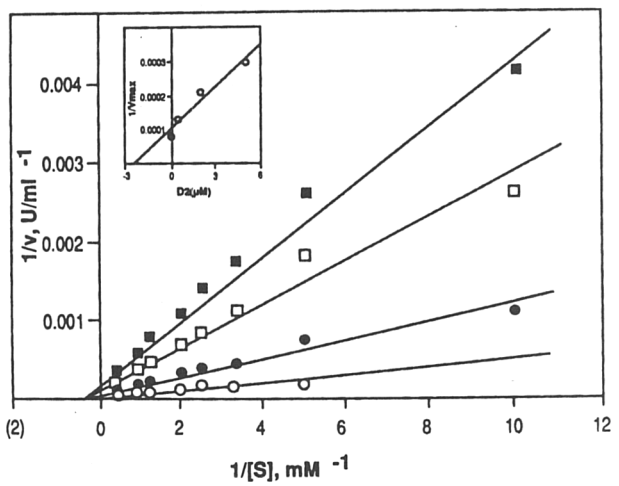

Fig. 9

Lineweaver-Burk plots of the *C. burnetii* ACP activity measured in the presence of varying concentrations of complex D_2

ACP activity was measured using the standard ACP assay over a 20-fold range of MUP concentration (0.1 – 2.0 mmol/l) in the absence (o) or presence of 0.5 μmol/l (●), 2 μmol/l (□), and 5 μmol/l (■) complex D_2. The inser is a replot of the Lineweaver-Burk slope as a function of the inhibitor concentration and was used to calculate K_i of the inhibitor. The correlation coefficient was 0.98.

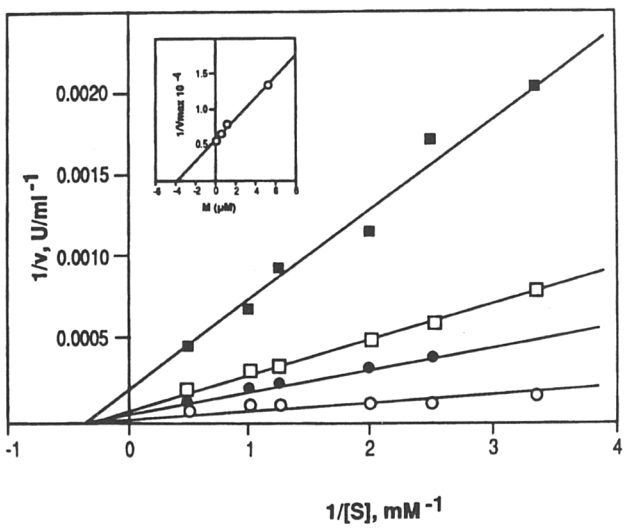

Fig. 10

Lineweaver-Burk plots of the *C. burnetii* ACP activity measured in the presence of different concentrations of complex M

Activity was measured over a 7-fold range of MUP concentration (0.3 – 2.0 mmol/l) in the absence (o) or presence of 0.5 μmol/l (●), 1 μmol/l (□), and 5 μmol/l (■) complex M. The inser is a replot of the Lineweaver-Burk slope and was used to calculate K_i of the inhibitor. The correlation coefficient was 0.99.

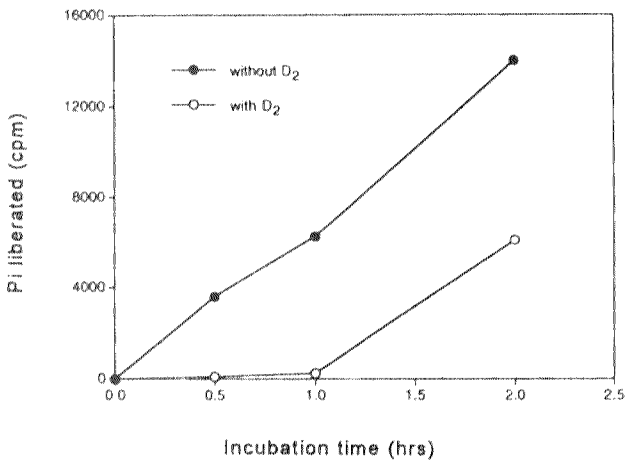

Fig. 11
Dephosphorylation of ^{32}P-Tyr-Raytide by the *C. burnetii* ACP
^{32}P-Tyr-Raytide was incubated at 37°C with 870 U of purified ACP with or without 10 µmol/l heteromolybdate complex D_2 for different time periods. The P_i liberated was measured.

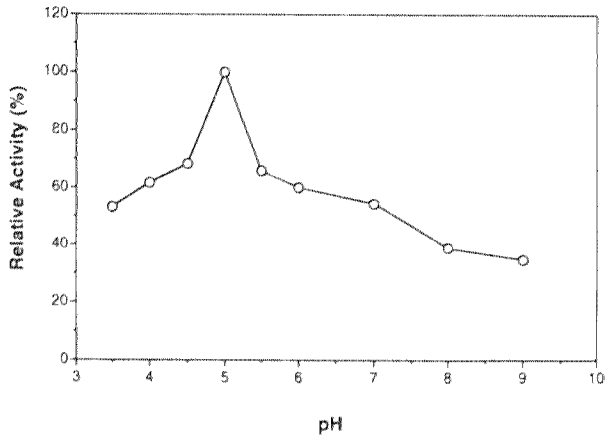

Fig. 12
pH profile of desphosphorylation of 32p-Tyr-Raytide by the *C. burnetii* ACP
Dephosphorylation was measured by using 0.1 mol/l Tris-acetate buffer (pH 4.5–9.0) and 0.1 mol/l sodium acetate buffer (pH 3.5–4.0). The incubation was at 37°C for 2 hrs.

with fMLP and lysed with 1% NP-40 in the presence of various protease inhibitors. The Western blots were then probed with anti-phosphotyrosine MoAb and visualized by ECL. Interestingly, ACP-treated (metabolically inhibited) neutrophils exhibited a significantly enhanced phosphorylation of a 44 K protein band (Fig. 14). This result was in contrast to what was anticipated: dephosphorylation of phosphotyrosine-containing protein(s). Neither the identity, function nor location (external or internal) of this 44 K protein is known at present.

The possibility that the ACP preparation also contained tyrosine kinase activity which might account for the enhanced phosphorylation was explored with the aid of a tyrosine kinase assay kit (ELISA based, Pierce). Neither the *C. burnetii* 100,000 x g supernatant nor the extensively purified *C. burnetii* ACP had detectable tyrosine kinase activity (data not shown).

Anti-phosphoserine and anti-phosphothreonine MoAbs were also used in the immunoblot analysis: no difference in serine or threonine phosphorylation were detected.

Discussion

Successful parasitization of phagocytes by the Q fever agent may be due, in part, to the parasite's suppression of the phagocytes' capacity to generate adequate concentrations of microbicidal oxygen metabolites during and after parasite entry. Several years ago it was noted that macrophage cells (J774 cell line) infected with *C. burnetii* exhibited diminished levels of oxidative metabolism and reduced capacity to generate superoxide anion (Baca *et al.*, 1984). Moreover, while oxidative metabolism occured in J774 cells during the phagocytosis of antibody-treated *C. burnetii*, it could not be detected in cells phagocytosing unopsonized *C. burnetii*. It was also noted that the phagocytosis of either opsonized or unopsonized organisms failed to trigger a significant production of superoxide anion in human neutrophils (Akporiaye *et al.*, 1990; Ferenčík *et al.*, 1984).

Recently we demonstrated that supernatants (S100) prepared from *C. burnetii* contained a factor that blocked superoxide anion production by human neutrophils stimulated with fMLP (Baca *et al.*, 1993). Because the addition of heteromolybdate compounds (demonstrated inhibitors of ACPs) to the S100 prevented the shut-down of anion production, it was concluded that the inhibitory factor was an ACP. The ACP was localized in the parasite's periplasm.

The data presented in this communication clearly show that the extensively purified *C. burnetii* ACP inhibits superoxide anion production in neutrophils and that the inhibitory substance is an ACP. That the *C. burnetii* ACP is different from the host cell ACP is indicated by their distinct chromatographic patterns and differential inhibitory effects of heteromolybdate compounds and sodium ortho-vanadate on them. Its M_r is approximately 91 K and its pH is abouit 5.0. The enzyme appears to be glycosylated because its treatment with N-glycohydrolase reduced its M_r by approximately 4 K.

The substrate specificity data exclude inositol phosphates as potential substrates of *C. burnetii* ACP. Such compounds have been reported (Das *et al.*, 1986; Saha *et al.*, 1988) to

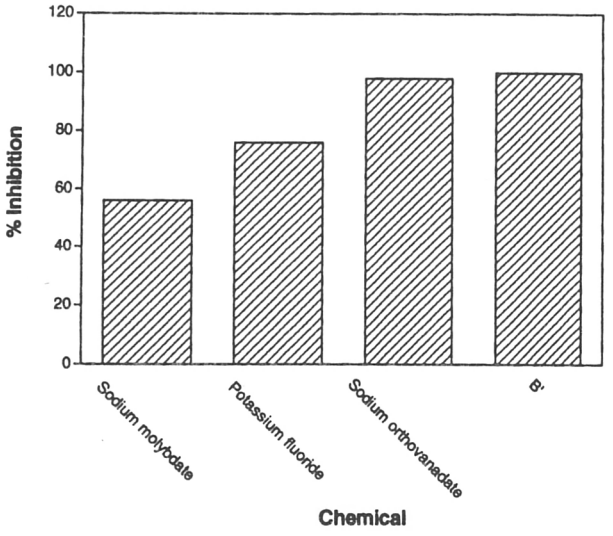

Fig. 13
Effect of various chemicals on dephosphorylation of ^{32}P-Tyr-Raytide by the *C. burnetii* ACP
^{32}P-Tyr-Raytide was incubated with the ACP in the absence (control) or presence of various chemicals (10 µmol/l) at 37°C for 2 hrs. The percentage of inhibition was calculated using the control as 0%.

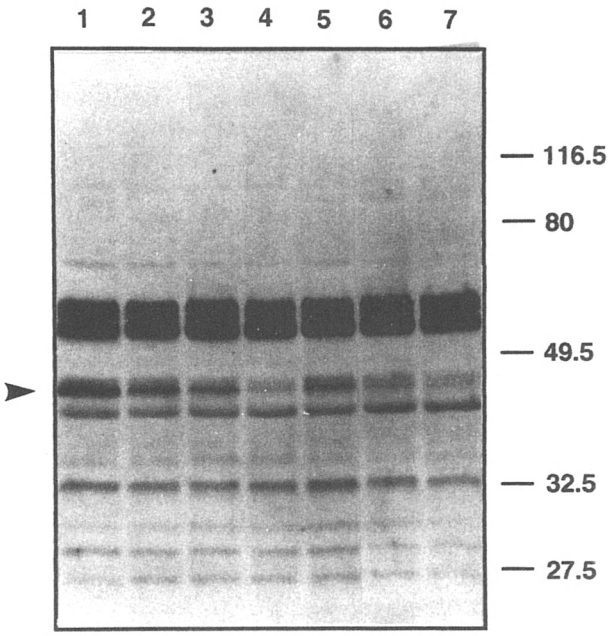

Fig. 14
Effect of the *C. burnetii* ACP on tyrosine phosphorylation of fMLP-stimulated human neutrophils
Human neutrophils (5 x 10^6) in Krebs-Ringer phosphate buffer were incubated with or without 100 U of the ACP and stimulated with 10^{-6} mol/l fMLP at 37°C for 2 mins. Tyrosine phosphorylation was determined by Western blot analysis. Lane 1, ACP, 15 mins, then fMLP 2 mins; lane 2, ACP and fMLP together, 2 mins; lane 3, ACP alone, 15 mins; lane 4, ACP alone, 2 mins; lane 5, fMLP alone, 2 mins; lane 7 and 8, control unstimulated cells were incubated for 15 mins and 2 mins, respectively. Arrowhead points to the 44 K phosphotyrosine protein band which was more heavily phosphorylated in neutrophils pretreated with the ACP followed by fMLP (lane 1). The latter cells also generated significantly less superoxide anion than control cells that were not pretreated with the ACP.

be substrates of ACPs isolated from the facultative intracellular agents *Legionella micdadei* and *Leishmania promastigotes*. Both parasites also grow in phagocytes and possess ACPs that inhibit the respiratory (oxidative) burst of neutrophils, apparently by reducing the amount of the second messengers, inositol 1,4,5-triphosphate (IP_3) and *sn*-1,2-diacylglycerol, compounds generated following receptor-mediated stimulation.

That *C. burnetii* ACP dephosphorylates Raytide, the phosphotyrosine-containing peptide commonly used in determing substrate specificity of such phosphatases, suggests that the enzyme targets host cell phosphotyrosine-containing proteins. The latter have been implicated in the signal transduction process associated with phagocytosis and the metabolic burst (Cui *et al.*, 1994). Endogenous neutrophilic plasma membrane PTPase apparently modulates fMLP-induced superoxide release by reversing the effects of tyrosine kinases activated in the initial phases of cell stimulation (Cui *et al.*, 1994). The treatment of human neutrophils with the *C. burnetii* ACP and the resulting enhanced phosphorylation of a host protein observed by us may at first glance be an apparent contradiction; however, it may be the result of activation/enhancement of internal kinase activity triggered by surface dephosphorylation. The ACP preparation did not contain kinase activity.

PTPase activity has been reported in *Yersinia*, and it has been shown that this secreted, plasmid-encoded enzyme (YopH) is essential for pathogenesis (Bliska *et al.*, 1991). The phosphatase inhibits the Fc-receptor-mediated signal transduction process in phagocytes which culminates in the oxidative burst. Recently, Bliska and Black (1995) demonstrated that *Yersinia's* YopH also inhibited Fc receptor-mediated oxidative burst in macrophages. Host cell surface-bound *Yersinia* translocates YopH by a polarized mechanism into the target cell (Persson *et al.*, 1995). Conceivably, *C. burnetii* ACP may also be transferred directly into host cells upon contact via a similar mechanism, and may not be secreted directly into the surrounding medium. Ideed, we were unable to detect the *C. burnetii* ACP in culture medium (pH 4.5, P-25 buffer) after incubating purified organisms at various temperatures (0°C – 37°C) for various time periods (minutes to overnight; data not shown).

The location of the ACP gene – plasmid or chromosome – is unknown. Because this enzyme appears to be a significant virulence factor of *C. burnetii*, additional

studies should be performed on its biological-clinical significance as well as its mechanism of action.

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