kept in culture, thus indicating that an interval is required for intracellular JV to appear as infectious virus; c) unpublished data from our laboratory indicate that JV antigens can be faintly detected by immunofluorescence on acetone-fixed or living PBMC, but only after 5 days in culture.

The increased frequency of IC detection when 7 or more days have elapsed since the clinical onset of AHF (Table 2), can be interpreted as indicative of the extent of haemopoietic tissues involvement in JV infection. The undetectable α-IFN activity in the supernatants of patients' PBMC cultures is consistent with the presence of very low numbers of JV carrying cells. Concomitantly, the finding of high titres of α-IFN in the sera of patients rendering negative IC assays is another evidence suggestive of a larger population of JV-infected lymphoid cells, which cannot be estimated from the peripheral blood cells alone.

The few IC which occasionally appear among non-adherent cells could represent monocyte contaminations, although previous findings would allow to regard them as an underestimation of infected lymphocytes. Studies on peripheral blood cells of JV infected guinea pigs described some morphological alteration in lymphocytes (Carballal *et al.*, 1981*a*), as well as diminished numbers of T cells (Carballal *et al.*, 1981*b*). In patients with AHF, a decrease in T and B lymphocyte populations (Arana *et al.*, 1977) and a transient inversion of T_4/T_8 ratio have been described during the acute period of the disease (Vallejos *et al.*, 1989).

Infectious cells are apparently cleared from the blood of the patients by some mechanism(s) not yet elucidated. On day 4 after the treatment with immune plasma, JV cannot be isolated from the patients' PBMC (Ambrosio *et al.*, 1986), and concomitantly, titres of endogenous α-IFN drop to normal levels (Levis *et al.*, 1984). The low numbers of infectious cells make difficult to rule out the possibility of cytocidal JV infection of PBMC.

The results reported herein demonstrate that in patients with AHF peripheral blood monocytes (macrophages) carry intra-cellular JV, which can replicate in these cells. Circulating monocytes contribute to the spreading of JV different tissues. Viral infection of macrophages can interfere with the role of these cells in the immune response of the patients, thus partially explaining the immunodepression that characterizes the acute period of AHF. Furthermore, if JV multiplication in macrophages is ended by any cytocidal mechanism, subsequent release of reactive products would contribute to a chain of alterations (Peters, 1984). The results of the present study in patients with AHF are consistent with the central role assigned to macrophages in the pathogenesis of arenavirus infections.

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COXIELLA BURNETII FAILS TO STIMULATE HUMAN NEUTROPHIL SUPEROXIDE ANION PRODUCTION

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Summary. — This study investigated the release of superoxide anion (O_2^-) as an indicator of the oxidative metabolism of human neutrophils during the phagocytosis of Phase I *Coxiella burnetii*. Human neutrophils were incubated for 1 hr at 37 °C with opsonized or unopsonized viable Phase I *Coxiella burnetii* (MOI was 100 : 1) and superoxide anion formation was measured by the reduction of ferricytochrome C. The data revealed that during its phagocytosis by human neutrophils, *C. burnetii* (opsonized or unopsonized) fails to stimulate superoxide anion production. In contrast, the uptake of *Staphylococcus aureus* or zymosan was accompanied by the release of measurable O_2^-. This release of O_2^- was abrogated by the addition of 100 μg/ml of superoxide dismutase (SOD). These results suggest that the establishment of *C. burnetii* within neutrophils, as occurs during persistent infection, may be due to the failure to stimulate the metabolic burst during phagocytosis.

Key words: *Coxiella*; *phagocytosis*; *human neutrophils*; *superoxide anion*

Introduction

The role of phagocytes in the control of infectious agents is well document (Klebanoff 1975; Roos 1980). By eliciting a burst of metabolic activity du ing the ingestion of microorganisms, polymorphonuclear leukocytes, macr phages and monocytes generate an array of reactive oxygen species (All 1977; Salin and McCord 1975; Sasada and Johnston 1980; Wilson *et a* 1980) which are considered microbicidal (McRipley and Sbarra 1967; Murr and Cohn 1979; Pearson *et al.*, 1982).

Coxiella burnetii, the etiological agent of Q fever, is known to infect pr fessional phagocytes *in vitro* where it proliferates within the phagolysosom to establish a persistent infection (Akporiaye *et al.*, 1983, Moulder 198 Although a number of studies have examined the uptake and fate of *burnetii* within its phagocyte host, little is known about the mechanism that may account for parasite survival after ingestion.

The study described examined the oxidative metabolism of human neutrophils phagocytizing phase I *C. burnetii*. The release of superoxide anion was used as an indicator of oxidative metabolism.

We present evidence that opsonized *C. burnetii*, whether viable or killed, failed to elicit superoxide anion production in human neutrophils during phagocytosis. Opsonization of *C. burnetii* with high titre antibody (1 : 2000) at a very high bacteria/neutrophil ratio (1000 : 1) caused only a marginal production of superoxide anion.

Materials and Methods

Coxiella burnetii propagation and purification. Cloned phase I *C. burnetii*, Nine Mile Strain, was obtained from R. A. Ormsbee (Rocky Mountain Laboratory, U.S. Public Health Service, Hamilton, Montana). The organisms were propagated in mouse fibroblast (L-929) cells and purified by differential centrifugation as previously described (Baca *et al.* 1981). Purified rickettsiae were suspended in Dulbecco's balanced salt solution, pH 7.4. Rickettsial concentrations were determined by the method of Silberman and Fiset (1968) and stored at −70 °C until needed.

Neutrophil isolation. Thirty ml of normal human heparinized blood (20 units heparin/ml) were collected from healthy donors. The blood was carefully layered over 15 ml of Ficoll-Hypaque (Pharmacia, Piscataway, NJ) and centrifuged for 30 min at 400 xg at 18−20 °C. The layer above the packed red blood cells (RBCs) was decanted and an equal volume of Hank's Balanced Salt Solution (HBSS), pH 7.2, was added. The cell suspension was added in an equivalent volume to Plasma Gel (Cellular Products Inc., Buffalo N.Y.) and mixed gently. Fifteen ml of cell suspension were transferred into 20 ml syringes (Becton Dickinson, Rutherford, NJ) and incubated upright for 45 min at 37 °C. At the end of the incubation period, the supernatant fluid containing neutrophils was aspirated into a 50 ml polypropylene Falcon tube (Becton Dickinson, Oxnard, CA) and centrifuged for 10 min (400 xg, 4 °C). The resulting supernatant fluid was discarded, leaving a pellet of neutrophils. The cell pellet was resuspended in 2 ml HBSS and cell count and viability were performed (Akporiaye *et al.* 1985). Contaminating RBCs were lysed for 30 sec in distilled water. The neutrophils were adjusted to a final concentration of 1.1×10^6/ml prior to use.

Opsonization of organisms. Normal pooled human serum and immune human serum (complement fixation titer = 1 : 2000) were diluted to 10% in phosphate buffered saline (PBS), pH 7.2. Viable or formalin-killed phase I *C. burnetii*, suspended in PBS, were added to normal or immune human serum in 1 ml volume to give a final concentration of 1×10^{10} organisms/ml. Viable or formalin killed *Staphylococcus aureus* were similarly treated. Suspensions of rickettsia or *S. aureus* in normal or immune human sera were incubated for 60 min at 37 °C on a rotor at 60 rev/min. At the end of the incubation period, the opsonized organisms were washed twice (1000 xg. 10 min, 4 °C) in PBS and adjusted to a concentration of 1×10^{10} or 1×10^9 bacterial/ml.

Opsonization of zymosan. Zymosan (Catalog No. Z-4250; Sigma, St. Louis, MO) was boiled for 30 min in a small volume of distilled water. The zymosan particles were next centrifuged at 16 318 xg for 10 min at 4 °C. The resulting pellet was washed once with distilled water (16,318 xg, 10 min, 4 °C). The zymosan pellet was resuspended in normal pooled human serum (10 mg zymosan per ml of serum) and incubated for 1 hr at 37 °C on a rotor rack. Zymosan particles were again centrifuged at 16 318 xg for 15 min at 4 °C. The serum supernatant was decanted and the zymosan was washed twice in PBS (16 318 xg, 10 min, 4 °C). Zymosan was resuspended to 10 mg/ml in PBS. The samples were aliquoted and frozen at −70 °C until needed.

Superoxide anion assay. Superoxide anion production was determined by modification of a previously described method (Weening *et al.*, 1975). Approximately 1×10^8 neutrophils were added to tubes containing 80 μmol/l ferricytochrome C (Horseheart type III; Catalog No. C-2506; Sigma, St. Louis, MO) and 1×10^8 or 1×10^9 *C. burnetii* in a final reaction volume of 2.1 ml. Controls included tubes containing cytochrome C alone in buffer or neutrophils alone in buffer. Neutrophils incubated with zymosan (1 mg) or *Staphylococcus aureus* ($1 \times 10_8$ were used as positive controls. In some experiments, 100 μg/ml superoxide dismutase (SOD) were added to reaction tubes in order to assess the superoxide-mediated reduction of ferricytochrome C.

Superoxide anion production was inhibited 90—95 percent by the addition of SOD. Tubes were incubated for 60 min at 37 °C on a shaker water bath (100 rev/min). At the end of the incubation period, the tubes were centrifuged at 1000 xg for 10 min at room temperature. The supernatants were transferred to glass tubes. The absorbance of the supernatant fractions was measured at 550 nm against a control tube containing cytochrome C in HBSS buffer. Change in absorbance was determined by subtracting values of the control tubes from the experimental tubes. The amount of cytochtome C reduced was used as an indicator of superoxide formation and was calculated using the extinction coefficient of $2.1 \times 10^4\ M^{-1}\ cm^{-1}$ at 550 nm.

Statistical analysis. Statistical significance of results was determined using one-way analysis of variance and Least Significant Difference (LSD) tests (Snedecor and Cochran 1987). Any data whose analysis of variance showed that one of more means differed significantly from the others were then analyzed by the LSD test to identify which mean(s) differed significantly from the others. Probability values (P) of <0.05 were considered to indicate significant differences between data sets.

Results

Role of immune serum pre-treatment of C. burnetii on superoxide anion release

Coxiella burnetii, phase I was pretreated with human normal or hyperimmune anti-rickettsial serum prior to incubation with neutrophils in the

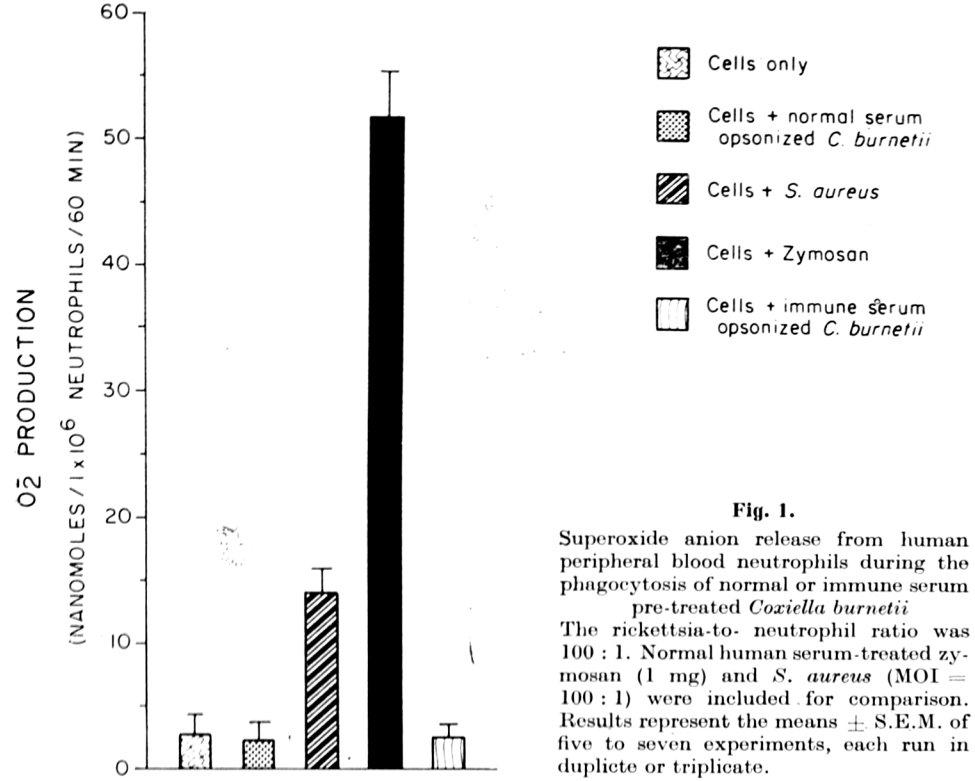

Fig. 1.
Superoxide anion release from human peripheral blood neutrophils during the phagocytosis of normal or immune serum pre-treated *Coxiella burnetii*
The rickettsia-to- neutrophil ratio was 100 : 1. Normal human serum-treated zymosan (1 mg) and *S. aureus* (MOI = 100 : 1) were included for comparison. Results represent the means ± S.E.M. of five to seven experiments, each run in duplicte or triplicate.

superoxide anion (O_2^-) assay. *Coxiella burnetii* in either form did not eltici O_2^- release by human neutrophils (Fig. 1). In contrast opsonized zymosan and *S. aureus*, used as controls, stimulated significant production of O_2^-.

Effect of rickettsial concentration on superoxide anion production

Immune serum opsonized *C. burnetii* were incubated with neutrophils at a multiplicity of infection (MOI) of 100 : 1 or 1000 : 1 in order to determine the effect of rickettsial concentration on O_2^- production. *Coxiella burnetii* at MOI of 100 : 1 did not stimulate O_2^- release (Fig. 2). However when the bacteria-to-cell ratio was increased to 1000 : 1, *C. burnetii* caused only a modest stimulation of O_2^- production. This amount of O_2^- production. This amount of O_2^- constituted only 10 percent of that obtained by zymosan stimulation.

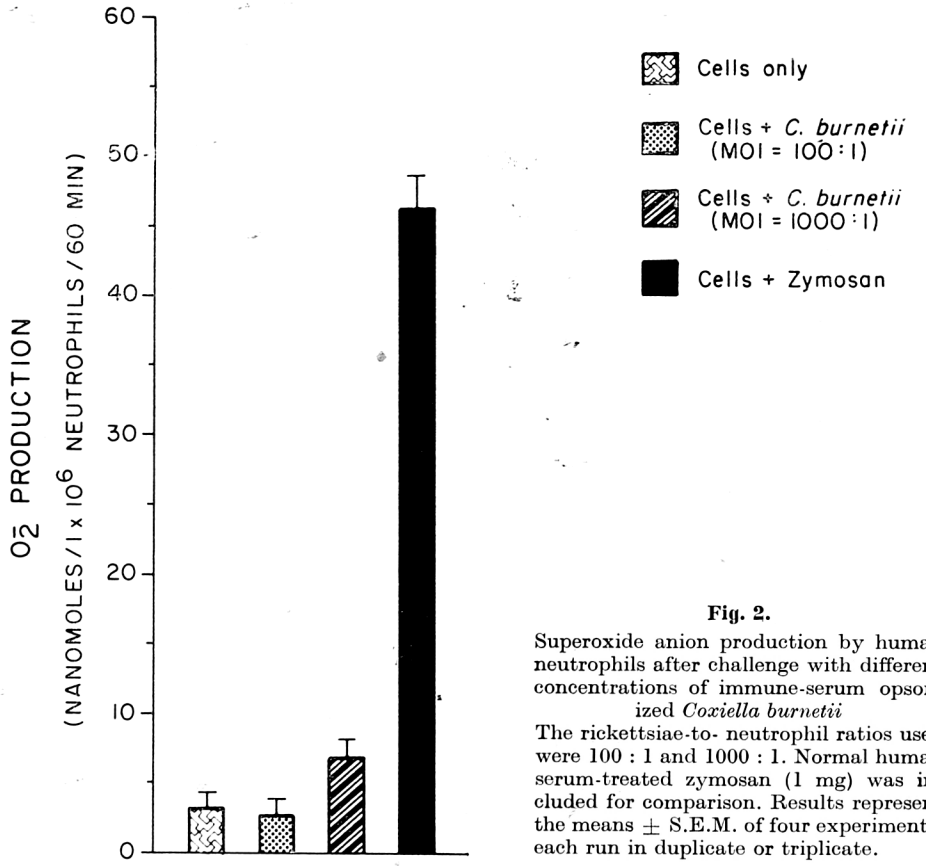

Fig. 2.

Superoxide anion production by human neutrophils after challenge with different concentrations of immune-serum opsonized *Coxiella burnetii*

The rickettsiae-to- neutrophil ratios used were 100 : 1 and 1000 : 1. Normal human serum-treated zymosan (1 mg) was included for comparison. Results represent the means ± S.E.M. of four experiments, each run in duplicate or triplicate.

Effect of rickettsial viability on superoxide anion release

Superoxide anion release by neutrophils incubated with viable or formalin-killed *C. burnetii* was determined. As shown in Fig. 3, neither viable nor killed *C. burnetii* stimulated O_2^- formation by human neutrophils. In contrast, zymosan and *S. aureus* (viable or formalin-killed) caused the release of significant amounts of O_2^-.

Discussion

The importance of the oxidative metabolic burst in the destruction of intracellular parasites by phagocytes has been demonstrated by several investigators (Allen *et al.* 1972; Babior *et al.* 1973; Buchmuller and Mauel 1981; Murray and Cohn 1979; Sasada and Johnston 1980). Our studies shown that phagocytosis of normal or immune human serum-treated rickettsiae failed to trigger a significant production of superoxide anion by human neutrophils. The observation that viable or formalin-killed normal serum-opsonized rickettsiae failed to elicit the respiratory burst is in agree-

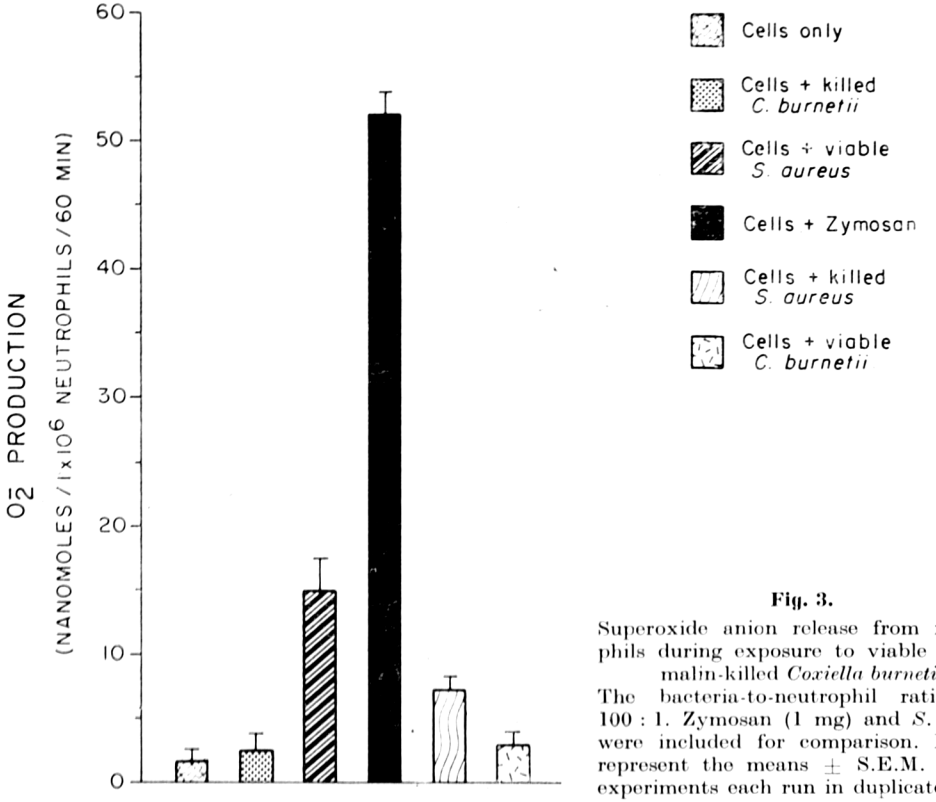

Fig. 3.

Superoxide anion release from neutrophils during exposure to viable or formalin-killed *Coxiella burnetii*

The bacteria-to-neutrophil ratio was 100 : 1. Zymosan (1 mg) and *S. aureus* were included for comparison. Results represent the means ± S.E.M. of two experiments each run in duplicate.

ment with the findings of Ferencik *et al.* (1984, 1985) using killed *C. burnetii*. However while they demonstrated a significant production of O_2^- (85% of zymosan controls) using rabbit immune-serum treated organisms, we report only a modest increase in O_2^- production (10% of zymosan controls) using viable *C. burnetii* opsonized with human immune serum containing antibodies to phase I and phase II rickettsiae. The difference may be accounted for, in part, by the higher bacteria-to neurophil ratio (1900 : 1) used in their study (Ferenčik *et al.* 1984).

The absence of measurable O_2^- production by neutrophils during the phagocytosis of viable or killed *C. burnetii* suggests that what happens during rickettsial ingestion is a failure to elicit O_2^- release rather than the dismutation of O_2^- by parasite superoxide dismutase (SOD). Viable *C. burnetii* was recently reported by us to contain SOD (Akporiaye and Baca 1983).

By failing to elicit an adequate metabolic burst during its uptake by human neutrophils, *C. burnetii* bears a resemblance to intracellular pathogens such as *Mycobacterium leprae* (Holzer *et al.*, 1986), *Toxoplasma gondii* (Wilson *et al.*, 1980) and *Leishmania donovani* (Haidaris and Bonventre 1982) which undergo unlimited replication within phagocytic cells.

Successful parasitization of phagocytes by *C. burnetii* may be due in part to the inability of the phagocyte to generate an adequate flux of reactive oxygen species during rickettsial uptake. The propagation and eventul consolidation of the parasite is further facilitated by its ability to resist the degradative lysosomal enzymes and survive within the phagolysosomes (Akporiaye *et al.*, 1983).

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