Oxygen-derived radicals from *Trypanosoma cruzi*-stimulated human neutrophils

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This study provides biochemical and electron spin resonance spectroscopic evidence that contract of human polymorphonuclear leukocytes with antibody-coated *Trypanosoma cruzi* triggers the respiratory burst. Oxygen consumption, superoxide anion and hydrogen peroxide release were stimulated under conditions of polymorphonuclear leukocyte-mediated killing. This stimulation did not occur under non-killing conditions when antibody was omitted. A common mechanism of cytotoxicity of human polymorphonuclear leukocytes against different *T. cruzi* forms is suggested by the triggering of the respiratory burst by antibody-coated epimastigotes and trypomastigotes.

DMPO *Neutrophil* *Hydrogen peroxide* *Free radical* *Superoxide* Trypanosoma cruzi

1. INTRODUCTION

Human polymorphonuclear leukocytes (PMN) are an important host defense mechanism against bacterial, fungal, viral and possible parasite invasion [1–4]. The mechanisms utilized by the PMN to destroy parasites are not well understood, but increasing attention has been focused on the ability of the PMN to generate potentially cytotoxic metabolites [5–7]. Following specific membrane perturbation, the PMN exhibit a burst in oxygen consumption associated with the generation of $O_2^{\cdot-}$, H_2O_2 and possible $OH^{\cdot}$ and singlet oxygen ($^1O_2$) (reviewed in [2]). Although evidence is accumulating on the cytotoxicity of these agents to parasites when generated by either cell-free model systems [7–11] or parasiticidal compounds [12–16], little is known about the destructive potential of cell-derived oxygen metabolites. Intact human PMN exposed to antibody-coated *Trypanosoma cruzi* epimastigotes or trypomastigotes are capable of destroying this target [3–5,17,18]. Although the cytotoxic reaction was postulated to be dependent on the products of the partial reduction of oxygen, the final mediators of the destruction were not identified [5].

This study provides biochemical and ESR spectroscopic evidence that oxygen consumption and production of $O_2^{\cdot-}$ and H_2O_2 are markedly stimulated in PMN that are in contact with antibody-coated *T. cruzi* epimastigotes and trypomastigotes.

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2. MATERIALS AND METHODS

PMN were prepared from fresh heparinized human blood by Ficoll–Hypaque separation [19] after dextran sedimentation [4]. The contaminating erythrocytes were lysed by hypotonic shock. Leukocytes in the PMN suspension were 90–95% neutrophils. Cell viability, assayed by the trypan blue exclusion test, was $\geqslant 95\%$ after purification and assays. *Trypanosoma cruzi* epimastigote [14] and trypomastigote [15] forms were prepared as cited. Sera from rabbits immunized with viable *T. cruzi* epimastigotes in complete Freund's adjuvant, agglutinating titre $> 1/4096$ for trypsin-treated antigen or 1/200 for viable cells, and human anti-*T. cruzi* sera obtained from patients with chronic infections were used [4,6,20]. The sera were inactivated at 56°C for 30 min and centrifuged for 1 h at 60000 × *g*.

Oxygen consumption by PMN was assayed in a Gilson K-IC oxygraph with a Clark-type electrode. Hydrogen peroxide was measured by determining O_2 release after the addition of catalase [21]. Production of $O_2^{\cdot-}$ was determined with the adrenochrome assay [22] by measuring the absorption at 480–575 nm using an absorption coefficient of 2.96 $mM^{-1}.cm^{-1}$ [12]. An Aminco-Chance spectrophotometer (American Instrument Company, Silver Spring MD) was utilized. Unless stated otherwise, all measurements were made at 37°C. The reactions were recorded under resting conditions and after the addition of unsensitized *T. cruzi* or antibody-coated *T. cruzi* at a 1:1 PMN:parasite ratio. Antibody-coated *T. cruzi* were obtained by incubating the parasites with a 1/200 dilution of anti-*T. cruzi* serum for 15 min at 30°C.

Spin-trapping experiments were carried out using 10^7 PMN/ml in RPMI medium containing 200 mM DMPO (5,5′-dimethyl-1-pyrroline-*N*-oxide) and 0.1 mM DETAPAC (diethylenetriamine penta-acetic acid). Neither DETAPAC (1 mM) nor DMPO (100 mM) affected the resting or stimulated rates of oxygen consumption by neutrophils [23,24]. After the addition of the stimulus (10^7 parasites/ml) the suspension was incubated at 37°C in the presence or absence of superoxide dismutase or catalase as indicated. At various intervals after the addition of the stimulus a portion (0.5 ml) of the cell suspension was transferred to the chamber of a flat cell using a syringe. The DMPO was purified according to [25].

[DMPO] was determined spectrophotometrically ($\epsilon_{234} = 7.7$ $mM^{-1}.cm^{-1}$) in ethanol. ESR spectra were recorded using a Varian E-9 spectrometer with an E_{238} TM_{110} cavity operating at 100 kHz field modulation in the first derivative mode.

3. RESULTS

To determine if the respiratory burst and hydrogen peroxide production by PMN could be triggered by contact with antibody-coated *Trypanosoma cruzi* epimastigotes or trypo-

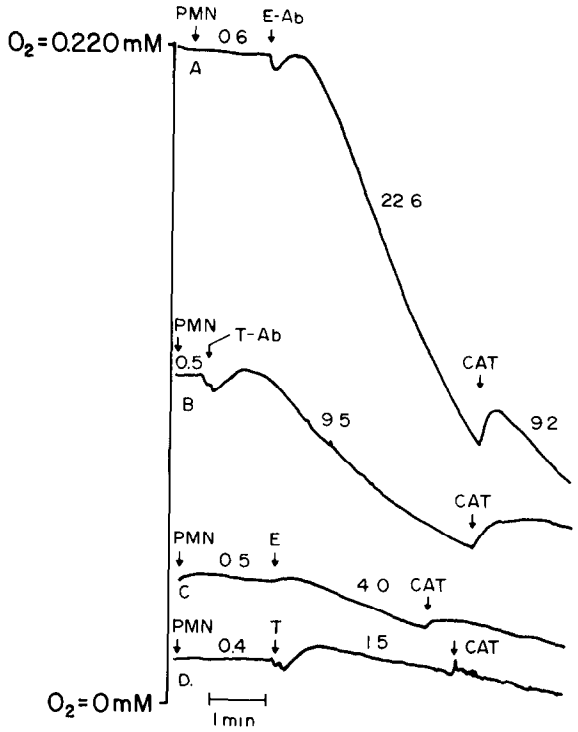

Fig.1. Oxygen consumption by PMN in the presence of *T. cruzi*. The arrows indicate the addition of PMN (2×10^7 cells/ml), antibody-coated epimastigotes (E-Ab), antibody-coated trypomastigotes (T-Ab), epimastigotes (E) and trypomastigotes (T) at 2×10^7 cells/ml, and catalase (CAT, 250 μg/ml). The numbers indicate the rate of oxygen consumption in $nmol.min^{-1}.10^7$ PMN^{-1}. The incubations were at 37°C in a modified Krebs–Ringer phosphate medium containing 0.12 M NaCl, 5 mM KCl, 10 mM phosphate buffer (Na_2HPO_4–NaH_2PO_4, pH 7.4), 1 mM Mg_2SO_4, 10 mM glucose, and 0.9 mM $CaCl_2$.

Table 1

Oxygen consumption and hydrogen peroxide release during ingestion of different *Trypanosoma cruzi* forms[a]

Form	Oxygen consumption (nmol.3 min^{-1}.2 × 10^7 PMN^{-1})[b]	Hydrogen peroxide release (nmol.3 min^{-1}.2 × 10^7 PMN^{-1})[b]
Epimastigote	117.0 ± 4.2	26.4 ± 3.2
Trypomastigote	41.1 ± 5.3	11.1 ± 2.1

[a] Parasite-to-PMN ratios were 1:1; both forms were antibody-coated as in fig.1 and section 2
[b] Results are expressed as the mean ± SD (3 independent determinations)

mastigotes, oxygen consumption and H_2O_2 release were measured under conditions where PMN-mediated cytotoxicity occurs [5,6].

Fig.1 shows the recorded tracings of the polarographic assays of oxygen uptake and H_2O_2 generation by PMN from human blood in the presence of antibody-coated *T. cruzi* epimastigotes (fig.1A) or trypomastigotes (fig.1B). Unstimulated cells consumed oxygen at 0.5 ± 0.2 nmol/10^7 cells.min, a value which agrees with that reported in [24]. Oxygen concentration fell rapidly after a latency of ~30 s in the presence of different antibody-coated *T. cruzi* forms. The effect was higher in the presence of antibody-coated *T. cruzi* epimastigotes than in the presence of antibody-coated *T. cruzi* trypomastigotes. Since the rate of oxygen consumption depended on the PMN preparation, the same PMN preparations were used in comparative experiments. Unsensitized *T. cruzi* epimastigotes (fig.1C) or trypomastigotes (fig.1D) caused significantly less activation of PMN. Antibody added separately caused no significant activation (not shown). Oxygen consumption by the concentration of *T. cruzi* used was negligible. The accumulation of H_2O_2 in the medium is indicated by the increase in oxygen concentration after catalase addition. The release of H_2O_2 into the medium accounts for 22–27% of the oxygen consumed (table 1).

The rate of formation of superoxide anion by PMN as detected by adrenochrome formation is illustrated in fig.2. Addition of PMN did not stimulate adrenochrome formation. Further addition of antibody-coated *T. cruzi* epimastigotes caused a marked stimulation of adrenochrome formation, which was inhibited only 50% by superoxide dismutase independently of the time of its addition, indicating that not only $O_2^{\cdot-}$ is involved in adrenochrome formation by this system. There was a lag of ~30 s before adrenochrome formation started. No significant adrenochrome formation was observed in incubations containing unsensitized parasites.

For a more direct demonstration of $O_2^{\cdot-}$ and/or $OH^{\cdot}$ production by PMN in the presence of *T. cruzi*, spin-trapping experiments were performed with DMPO. With this technique, short-lived radical species, such as $O_2^{\cdot-}$ and $OH^{\cdot}$, react with 'spin traps' to yield relatively long-lived nitroxide adducts, which may be characterized by electron spin resonance spectroscopy.

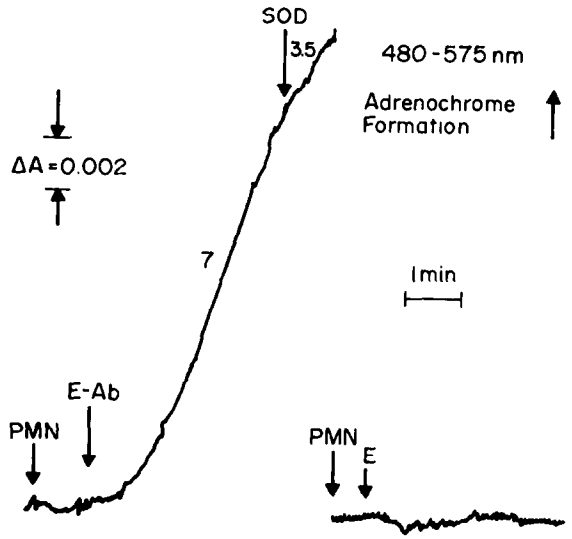

Fig.2. Adrenochrome formation by PMN on addition of *T. cruzi* epimastigotes. The incubation medium contained 1 mM epinephrine and 2 × 10^7 PMN/ml. Where indicated, antibody-coated epimastigotes (E-Ab) or unsensitized epimastigotes (E) at 2 × 10^7 cells/ml and 10 μg/ml superoxide dismutase (SOD) were added. The numbers indicate the rate of adrenochrome formation in nmol.min^{-1}.10^7 PMN^{-1}. Other experimental conditions as in fig.1 and section 2.

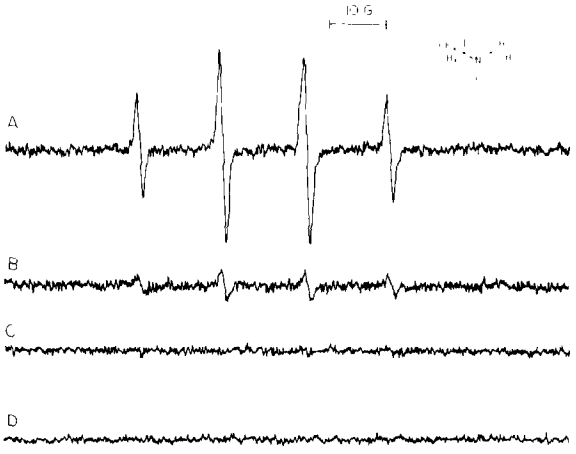

Fig.3. ESR spectra obtained after incubation for 4 min at 37°C of: (a) human PMN (10^7 cells/ml), DMPO (200 mM), and DETAPAC (0.1 mM) with antibody-coated *T. cruzi* epimastigotes (10^7 cells/ml; (B) same as (A) but unsensitized *T. cruzi* epimastigotes; (C) same as (A) but in the presence of SOD (10 μg/ml); (D) same as (A) but in the absence of parasites. The nominal power was 20 mW and the modulation amplitude was 1 G. Other experimental conditions as in section 2.

Incubation of PMN with antibody-coated *T. cruzi* epimastigotes (fig.3A) resulted in an ESR signal with splitting constants of $a^N = a^H = 15.02 \pm 0.16$ G and the 1:2:2:1 intensity distribution characteristic of the DMPO-hydroxyl radical adduct [27–29]. This signal was not observed when either PMN (not shown) or *T. cruzi* (fig.3D) were omitted and was lower when unsensitized *T. cruzi* were used (fig.3B). Superoxide dismutase at a concentration of 10 μg/ml strongly inhibited DMPO-hydroxyl radical adduct formation (fig.3C). In contrast, catalase at 250 μg/ml had no significant inhibitory effect. Comparable results were obtained with *T. cruzi* trypomastigotes.

4. DISCUSSION

These data demonstrate that contact of PMN with antibody-coated *T. cruzi* triggers the respiratory burst. Oxygen consumption, $O_2^{\cdot-}$ and H_2O_2 release were stimulated under conditions of PMN-mediated killing [5,6]. This stimulation did not occur under non-killing conditions when antibody was omitted. A common mechanism of cytotoxicity of human PMN against different *T. cruzi* forms is suggested by the triggering of the respiratory burst by antibody-coated epimastigotes and trypomastigotes.

The trypomastigote forms chosen for these experiments were obtained from the blood of infected animals and may be coated by antibodies developed by the host. However, it has been postulated [30] that capping on the surface membrane of trypomastigotes may represent the mechanism by which the parasites escape destruction by host antibodies. Accordingly, they did not trigger the respiratory burst if they were not preincubated with antibodies (T-Ab). Such a resistant mechanism would thus explain why trypomastigotes escape destruction by host PMN in vivo and the persistent parasitemia found in hosts chronically infected with *T. cruzi*.

The latency of ~30 s observed for the increase in oxygen consumption may indicate that a fixed period of time exists between contact of *T. cruzi* with critical portions of the PMN cell membrane and the stimulation of the formation of oxygen radicals [31].

Evidence of extracellular H_2O_2 was provided by demonstration of an immediate O_2 release when catalase was added to phagocytizing cells, implying an accumulation of H_2O_2 in the medium. Hydrogen peroxide release into the medium was observed to parallel the respiratory burst. Taking into account the rate of O_2 consumption of *T. cruzi*-stimulated neutrophils, 22–27% of the H_2O_2 formed was released into the medium. However, this calculation does not take into consideration that endogenous catalase or peroxidases may destroy H_2O_2, thus it can be considered only as an approximation.

The irreversible oxidation of epinephrine to adrenochrome provides a sensitive assay for $O_2^{\cdot-}$ at $\sim 10^{-7}$ M [22,32]. However, it has been stated that the specificity of the reaction appears to be rather poor [32]. In our system adrenochrome formation was only partially inhibited by superoxide dismutase. A more direct assay for superoxide is the technique of spin trapping [27–29]. Using this technique, two groups have detected the DMPO-hydroxyl radical adduct with stimulated neutrophils and reported that this was proof of hydroxyl radical formation by the neutrophils [23,26,33].

Detection of hydroxyl radical by spin trapping

with DMPO has been questioned in systems in which the level of hydroxyl radical production is < 3% of the rate of superoxide generation [34]. The introduction of nitrone spin traps into these systems can lead to the de novo production of the hydroxyl radical from the decomposition of the DMPO–superoxide radical adduct [34]:

$$\text{DMPO–OOH} \longrightarrow \text{DMPO (diamagnetic product)} + {}^{\cdot}\text{OH}$$

In addition, the DMPO–hydroxyl spin adduct is formed by the decomposition of the DMPO–superoxide spin adduct [35]:

$$\text{DMPO–OOH} \longrightarrow \text{DMPO–OH}$$

Thus, the generation of the DMPO–hydroxyl radical adduct by *T. cruzi*-stimulated neutrophils can be interpreted as due to the generation of $O_2^{\cdot -}$. In support of this assignment, superoxide dismutase completely inhibited the formation of the spin adduct, and catalase had no effect on the observed spectra. These results show that this hydroxyl spin adduct does not arise from hydrogen peroxide via either a Fenton, a Haber–Weiss, or some other H_2O_2-dependent reaction [35].

The mechanism for detoxifying oxygen species in *T. cruzi* is not clear. Protective enzymes such as glutathione peroxidase and catalase, which act in mammalian tissues to destroy relatively low and high concentrations of H_2O_2, respectively [32], appear to be of very little or no metabolic significance in *T. cruzi* [36,37]. The organism is particularly sensitive to $O_2^{\cdot -}$- and H_2O_2-generating agents such as naphthoquinones and nitrofurans [12–15]. This sensitivity may contribute to the susceptibility of antibody-coated *T. cruzi* to the cytotoxic effects of PMN leukocytes.

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