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# REDUCED CAPACITY OF PERITONEAL EXUDATE CELLS FOR OXIDATIVE KILLING OF *SCHISTOSOMA MANSONI* SCHISTOSOMULA

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*Peritoneal exudate cells from mice infected with* Schistosoma mansoni *(S-PEC) can kill schistosomula* in vitro *in the presence of immune serum. S-PEC produce a low level of respiratory burst, and schistosomula mortality in their presence is not reduced when exogenous antioxidants are added, suggesting that with S-PEC, oxidative killing is not important. Hydrogen peroxide ($H_2O_2$) and superoxide production by S-PEC, and cells from BCG and thioglycollate (THGL) injected non-infected mice, non-specifically stimulated with opsonized zymosan, were measured. Levels of $H_2O_2$ produced by S-PEC were significantly lower than BCG or THGL PEC, and were below the $H_2O_2$ threshold for schistosomula killing. This resulted in lower levels of cell-mediated killing of schistosomula* in vitro *by S-PEC than by BCG or THGL PEC. Superoxide levels, however, were similar between the three cell populations. The efficiency of PEC to kill schistosomules* in vitro *correlated with $H_2O_2$ rather than superoxide levels. The lower tolerance of schistosomula, compared to adult* S. mansoni *to GSH depleting agents increases their sensitivity to oxidative attack and resulted in higher levels of cell-mediated killing* in vitro.

The role of macrophages as effector cells for killing of schistosomula of *Schistosoma mansoni* has been demonstrated *in vitro* (Mahmoud et al., 1979; Bout et al., 1981; James et al., 1982a, b; McLaren & James, 1985; Scott et al., 1985). Their cytotoxicity, either as a result of antibody-dependent mechanisms or due to non-specific lymphokine activation, can be accompanied by a respiratory burst which heralds the production and release of reactive oxygen species. The role of reactive oxygen species as the main effector mechanism in macrophage-mediated killing of schistosomula has been questioned (Scott et al., 1985; McLaren & James, 1985). These authors reported effective killing of schistosomula, in the absence of a detectable respiratory burst, utilizing either a lymphokine-stimulated macrophage cell line IC-21 or macrophages from patients suffering from acute granulomatous disease. Both these cell populations are defective producers of reactive oxygen species.

*Non-specific and antibody mediated killing of schistosomula and the effect of the addition of exogenous antioxidants* – Schistosomula exposed to cell-free generators of oxygen metabolites can be protected by the addition of exogenous antioxidants or the presence of adult worms (Mkoji et al., 1988a). Schistosomula were incubated with peritoneal exudate cells (PEC) to determine if a similar level of protection could be obtained. PEC from uninfected CD-1 mice stimulated with secretions (SCS) obtained from spleen cells stimulated with phytohemagglutinin and concanavalin A (U-PEC + SCS), and PEC from mice chronically infected with *S. mansoni* (S-PEC) in the presence of heat-inactivated immune serum (IMS) were incubated with newly transformed schistosomula in the presence or absence of catalase, superoxide dismutase (SOD) or glutathione + glutathione peroxidase (GPO/GSH). The U-PEC + SCS killed a higher percentage ($P < 0.05$) of schistosomula than did the S-PEC in the presence of IMS (Table I). However, in neither case did the addition of any of the antioxidants produce a significant reduction in the mortality of the schistosomula. This indicates that in the case of PEC from infected mice, oxygen metabolites are probably not important for schistosomula cytotoxicity. When PEC from Bacillus Calmette-Guerin (BCG) injected mice (BCG-PEC) were incubated with schistosomula in the presence of opsonized zymosan particles, a significant level ($P < 0.05$) of schistosomula were killed compared to unstimulated cells. BCG-PEC stimulated with opsonized zymosan

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TABLE I

Effect of peritoneal exudate cells and antioxidants on the schistosomula mortality (Mean % ± S. E. n = 3)

| Conditions | % Killed | Conditions | % Killed |
|---|---|---|---|
| U-PEC | 7.5 ± 5.8 | S-PEC | 3.7 ± 1.0 |
| U-PEC + SCS | $61.3^a \pm 10.3$ | S-PEC + IMS | $26.9^b \pm 2.0$ |
| U-PEC + SCS + CAT. | $61.7^a \pm 7.5$ | S-PEC + IMS + CAT. | $29.2^b \pm 2.2$ |
| U-PEC + SCS + SOD | $46.8^a \pm 6.1$ | S-PEC + IMS + SOD | $20.1^b \pm 3.3$ |
| U-PEC + SCS + GPO/GSH | $74.1^a \pm 5.5$ | S-PEC + IMS + GPO/GSH | $18.7^b \pm 5.3$ |
| BCG-PEC | $18.2^a \pm 6.5$ | | |
| BCG-PEC + Zymosan | $56.3^b \pm 5.9$ | | |
| BCG-PEC + Zymosan + CAT. | $26.3^a \pm 3.9$ | | |

*Conditions* 100 schistosomula and 2 x $10^6$ PEC were incubated in MEM medium + faetal calf serum (FCS), (A) alone, (B) with SCS (50 $\mu$l), (C) with heat inactivated IMS (50 $\mu$l), (D) opsonized zymosan (2 mg/ml), to a final volume of 250 $\mu$l for 24 hours at 37°C in an atmosphere containing 5% $CO_2$ + 95% air. Catalase 5000 U/ml, GPO 20 mU/ml, GSH 0.1 mM, SOD 100 U/ml.
U-PEC stimulated with SCS were significantly more effective in killing schistosomula than S-PEC + IMS ($P < 0.05$). Schistosomula killing was significantly reduced when catalase was added to BCG-PEC stimulated with zymosan ($P < 0.01$). Values with the same superscripts are not significantly different.

are known to produce significant levels of $H_2O_2$ and superoxide (Nathan & Root, 1977; Nathan et al., 1979; Johnston et al., 1978; Pick & Keisari, 1980; Cohen et al., 1982). Addition of catalase (5000 U/ml) under these conditions significantly reduced ($P < 0.01$) schistosomula mortality (Table I).

*Threshold $H_2O_2$ levels for killing of schistosomula by cell-free generating systems* – The threshold $H_2O_2$ level for schistosomula killing in medium containing glucose (5.5 mM) and different concentrations of glucose oxidase (GO) and the level of $H_2O_2$ produced at each concentration of GO was determined (Fig. 1). The threshold for $H_2O_2$ killing of schistosomula was determined to be between 8 and 11 nmoles $H_2O_2$/ml/90 min. Superoxide and $H_2O_2$ can be generated in the presence of xanthine (0.5 mM)-xanthine oxidase (XO). It was determined that the threshold for schistosomula killing was obtained at between 4-5 mU/ml of XO, which generated superoxide at a maximal rate of 2.4 nmoles/min. However, as shown in Fig. 2 schistosomula mortality was significantly reduced ($P < 0.001$) with the addition of catalase to the incubation mixture containing this or higher levels of xanthine oxidase. This indicates that $H_2O_2$ levels rather than superoxide levels were responsible for the schistosomula killing. These results highlight the fact that even though $H_2O_2$ is being released, a critical $H_2O_2$ level might be required before toxicity, associated with the respiratory burst, occurs. A similar sharp threshold in $H_2O_2$ killing has also been reported for trypanosomes (Nathan et al., 1979).

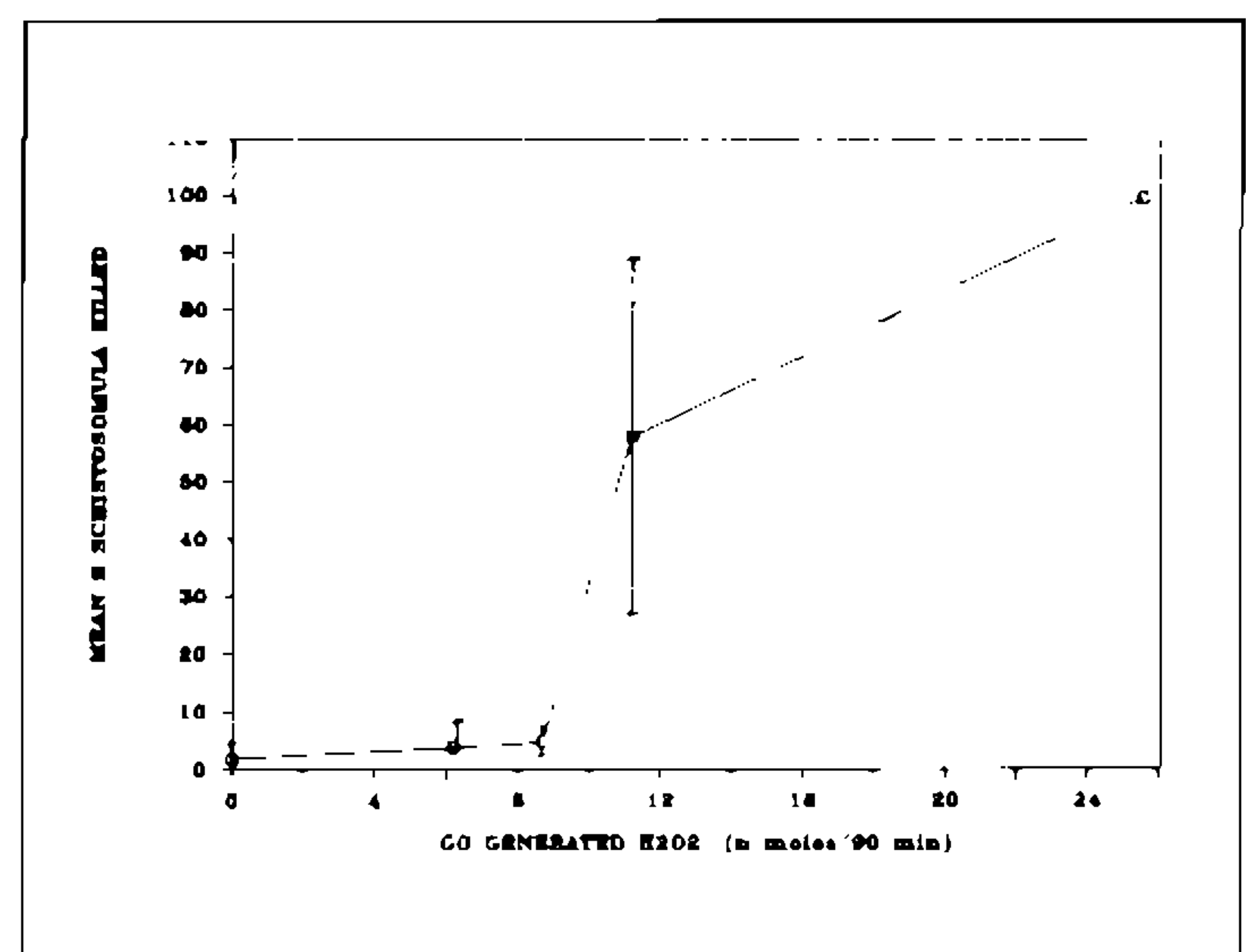


Fig. 1: threshold level for killing of schistosomula by glucose (G) + glucose oxidase (GO) generated $H_2O_2$. Mortality was assessed when 100 schistosomula were incubated in 250 $\mu$l of MEM medium + 10% FCS containing G (5.5 mM) + GO (0.5-10 mU/ml). $H_2O_2$ production was determined by monitoring the oxidation of phenol red (0.28 mM) in the presence of horseradish peroxidase (8.5 U/ml) at 610 nm (Pick & Keisari, 1980). Means ± SEM, n = 3.

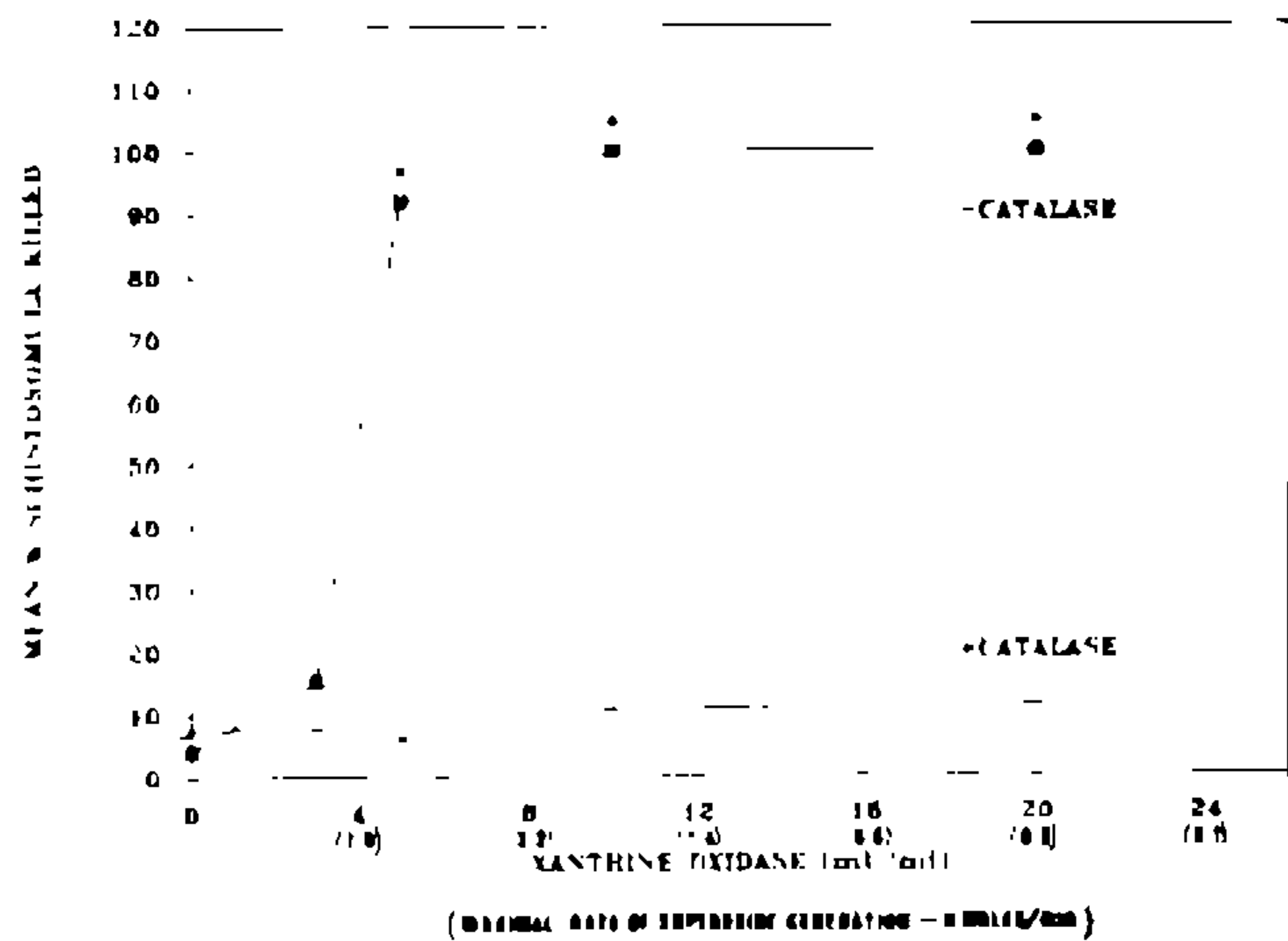

Fig. 2: threshold level for killing of schistosomula by xanthine (X) + xanthine oxidase (XO) generated $H_2O_2$ and superoxide. Mortality was assessed when 100 schistosomula were incubated in 250 µl of MEM medium + 10% FCS containing X (0.5 mM) + XO (3-20 mU/ml). A significant increase in schistosomula killing was obtained between 4-5 mU/ml of XO. The addition of catalase (5000 U/ml) significantly reduced schistosomula mortality. Means ± SEM, n = 3. The maximal rate of superoxide production by XO was determined by measuring the SOD-inhibitable superoxide reduction of cytochrome c (Cohen, 1985).

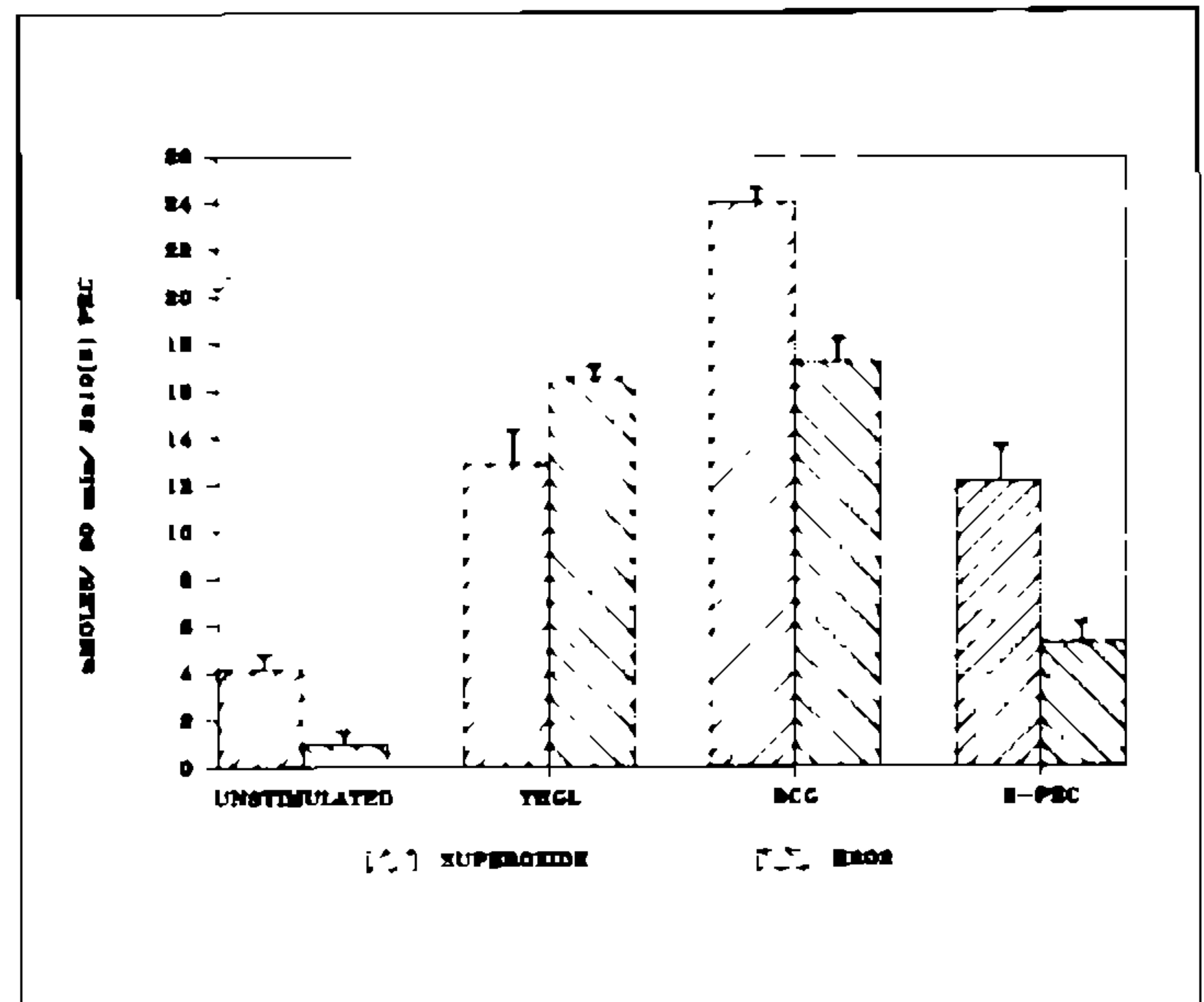

Fig. 3: superoxide and $H_2O_2$ levels produced by U-PEC, THGL-PEC, BCG-PEC and S-PEC when stimulated with opsonized zymosan (2 mg/ml). PEC (2 x $10^6$) were incubated in a final volume of 1 ml of Hanks' balanced salt solution, for superoxide determinations (Johnston et al., 1978), or phenol red solution, for $H_2O_2$ (Pick & Keisari, 1980), at 37°C for 90 min. Means ± SEM, n = 3.

*Comparative levels of $H_2O_2$ and superoxide produced by different PEC populations* – The levels of $H_2O_2$ and superoxide produced by three cell populations. PEC from thioglycollate elicited mice (THGL-PEC), BCG-PEC and S-PEC were compared after stimulation with opsonized zymosan (0.5 mg/ml) and results are expressed in nmoles/2 x $10^6$ PEC/90 min (Fig. 3). Levels of $H_2O_2$ produced by both thioglycollate and BCG elicited PEC were similar but significantly higher ($P < 0.001$) than those obtained from S-PEC. However, superoxide levels were similar between S-PEC and THGL-PEC but lower ($P < 0.05$) than those obtained from BCG-PEC. This suggests that PEC from mice with chronic *S. mansoni* infections have a reduced capacity to produce $H_2O_2$.

*Schistosomula killing and $H_2O_2$ release* – The ability of zymosan stimulated S-PEC and THGL-PEC to (a) kill schistosomula, and (b) to release $H_2O_2$ were compared. THGL-PEC stimulated with zymosan produced significant mortality of schistosomula, compared with S-PEC ($P < 0.001$) exposed to similar conditions (Fig. 4a). This correlated with a correspondingly higher level of $H_2O_2$ produced by THGL-PEC (Fig. 4b). The level of $H_2O_2$ produced by S-PEC lies below the threshold level of $H_2O_2$ for schistosomula killing.

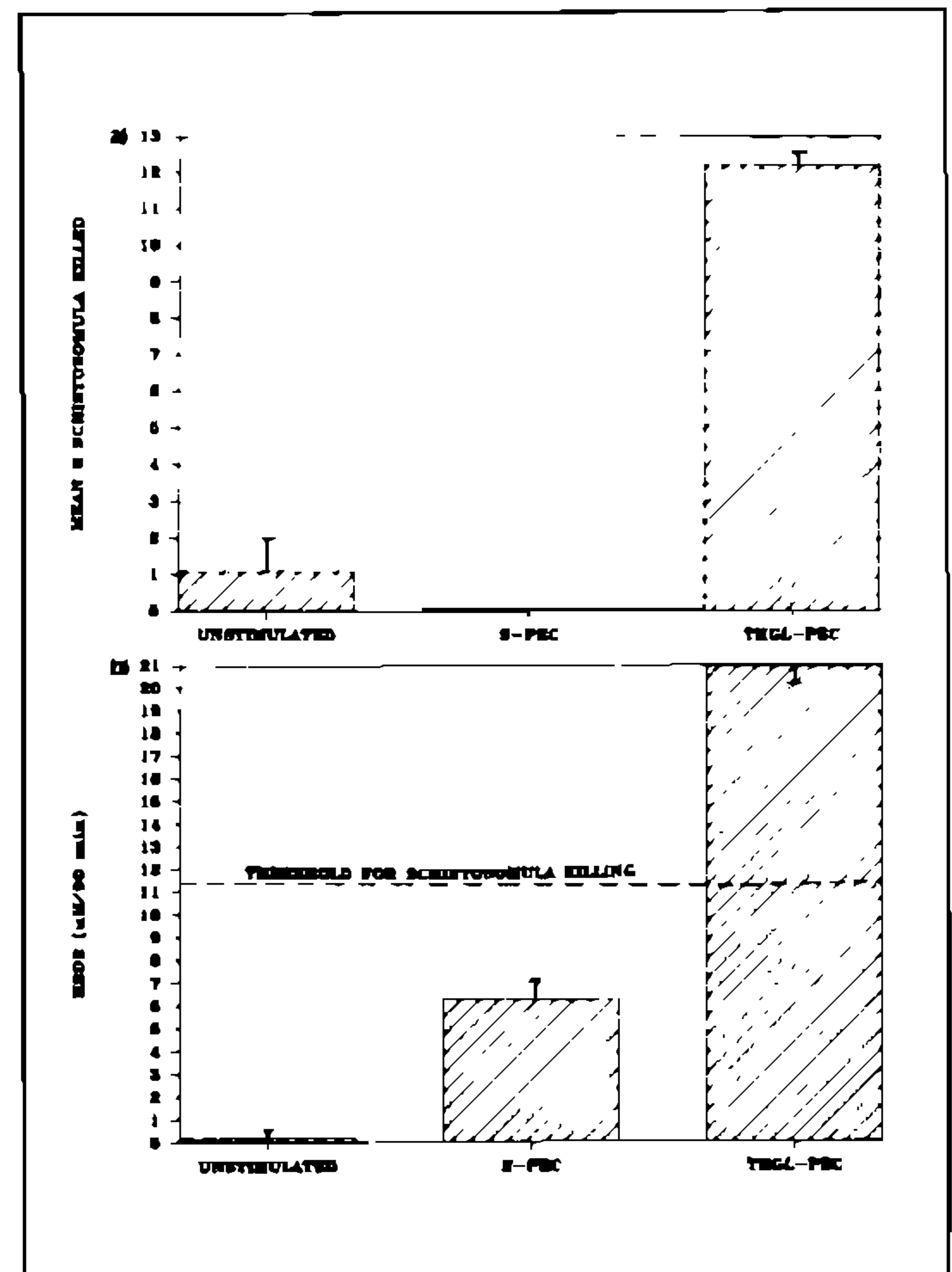

Fig. 4: comparison of a) schistosomula mortality, b) $H_2O_2$ production by S-PEC and THGL-PEC stimulated with zymosan. a) 2 x $10^6$ PEC, 100 schistosomula + zymosan (2 mg/ml) incubated in 250 µl of MEM + 10% FCS for 24 h. at 37°C. b) 2 x $10^6$ PEC + zymosan (0.5 mg/ml) incubated in 1 ml of phenol red solution for 90 min at 37°C. Means ± SEM, n = 3.

*$H_2O_2$ release from specifically and non-specifically stimulated S-PEC* – $H_2O_2$ production was measured from S-PEC either unstimulated, stimulated with zymosan, or stimulated with the membrane fraction of 100, 300, or 500 homogenized schistosomula in the presence of IMS and compared to zymosan stimulated THGL-PEC from uninfected mice. The level of $H_2O_2$ produced by S-PEC was significantly lower ($P < 0.05$) than THGL-PEC irrespective of the mode of stimulation (Table II). There was no significant difference in the level of $H_2O_2$ produced when S-PEC were stimulated with different concentrations of the schistosomula membrane fraction. However the level was significantly higher ($P < 0.05$) than unstimulated S-PEC but lower ($P < 0.05$) than the zymosan stimulated S-PEC.

TABLE II

$H_2O_2$ produced when S-PEC are stimulated with zymosan or IMS + membrane fractions of schistosomula, compared with THGL-PEC stimulated with zymosan (Mean ± SEM, n = 3)

| Conditions | $H_2O_2$ production nmoles/2 x $10^6$/90 min |
|---|---|
| S-PEC | $0.6^a \pm 0.3$ |
| S-PEC + Zymosan | $7.9^c \pm 0.1$ |
| S-PEC + IMS + 100 SM | $2.5^b \pm 0.3$ |
| S-PEC + IMS + 300 SM | $2.2^b \pm 0.1$ |
| S-PEC + IMS + 500 SM | $2.1^b \pm 0.1$ |
| THGL-PEC + Zymosan | $21.4^d \pm 0.4$ |

*Conditions* 2 x $10^6$ PEC incubated in 1 ml of PRS at 37°C. Zymosan 0.5 mg/ml, IMS 50 μl. Membrane fractions prepared from the equivalent of 100, 300, or 500 schistosomula. Values with the same superscript are not significantly different ($P < 0.05$).

*Effect of thioglycollate induction on chronically infected mice* – In order to determine whether the mode of activation of PEC affected production of $H_2O_2$ and whether chronic infections impaired the ability of PEC to produce $H_2O_2$, mice with 10 week old *S. mansoni* infections were divided into two groups, one injected with thioglycollate solution, the other with sterile saline. Four days later PEC were recovered from the two groups of mice and the $H_2O_2$ production after zymosan stimulation, was measured and compared with PEC from uninfected mice also treated with thioglycollate. There was no significant difference between the levels of $H_2O_2$ produced by the two cell populations from infected mice (Fig. 5), both were below the threshold for schistosomula killing and markedly lower than the $H_2O_2$ produced from the THGL-PEC from uninfected mice. These results suggest that mice chronically infected with *S. mansoni* have a suppressed ability to respond to inflammatory agents. Whether this decrease in $H_2O_2$ production is a result of a modulation of an anabolic or catabolic process in host immune cells has yet to be determined. Freund & Pick (1985) reported a marked increase in spontaneous release of $H_2O_2$ from lymphokine treated guinea-pig macrophages. This increase in $H_2O_2$ release was correlated with a decrease in macrophage catalase activity which may result in a decrease in intracellular degradation of $H_2O_2$. Szuro-Sudol & Nathan (1982) reported a tumor factor which suppressed the ability of macrophages to release $H_2O_2$ and superoxide in response to non-specific stimulation and suggested that the factor resulted in an increase in catabolism of these products. A decreased production of reactive oxygen species is quite common in protozoan infections (Li & Li, 1987) and can be linked to modulation of host cell antioxidant enzyme levels (Etkin & Eaton, 1975; Cox, 1983). Brinkmann et al. (1984) recorded depressed levels of oxygen metabolites from splenic macrophages in mice infected with *Plasmodium berghei* compared to mice infected with *P. yoelii.* Li & Li (1987) reported elevated levels of oxygen products in zymosan stimulated PEC from *P. berghei*-immunized mice compared with levels produced by PEC from infected mice, suggesting some type of immunodepression during infection. The mechanisms responsible for the depression of $H_2O_2$ production in S-PEC are currently being investigated.

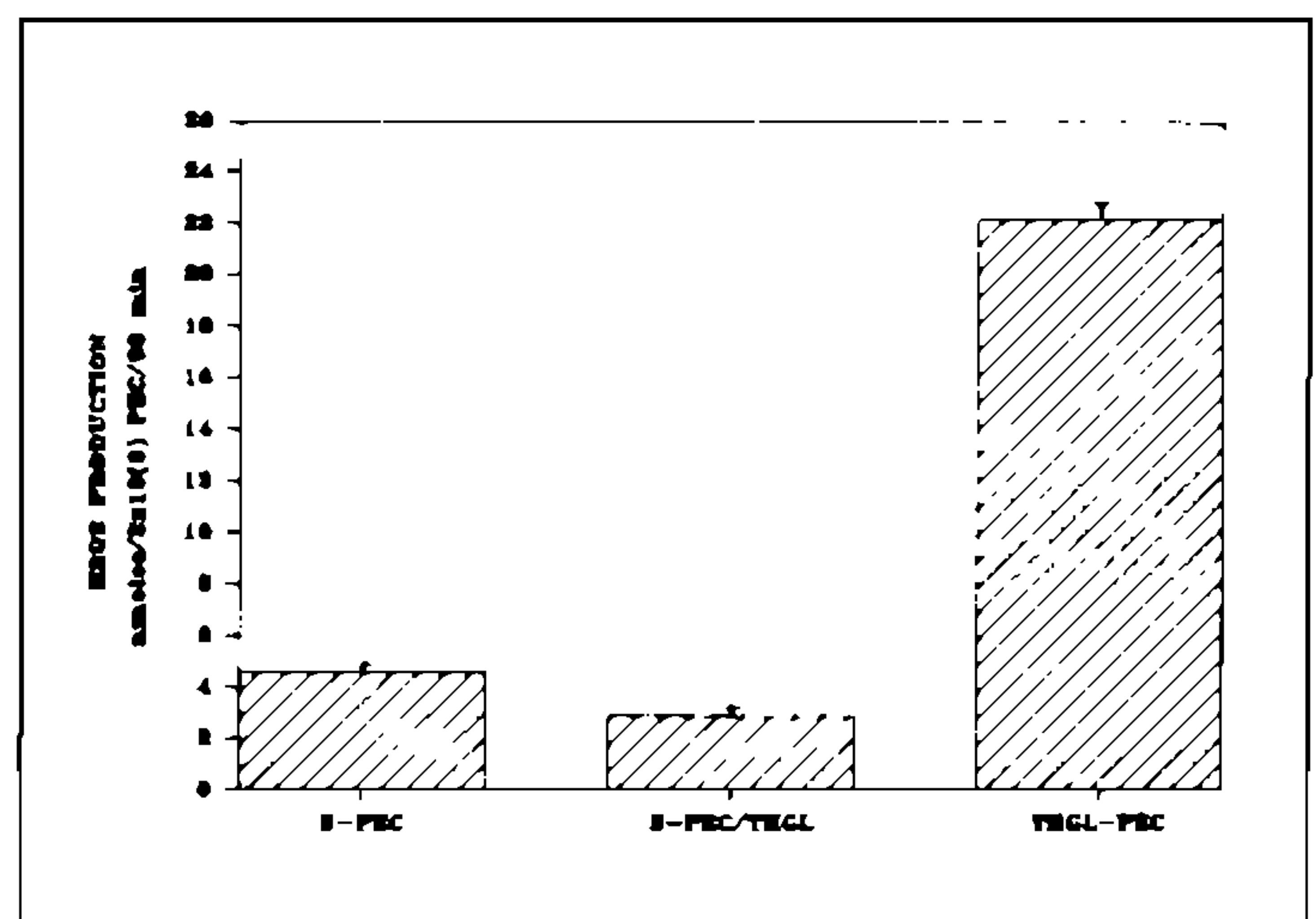

Fig. 5: reduced capacity of S-PEC, and PEC from chronically infected mice injected with 3% thioglycollate solution (S-PEC/THGL) to produce $H_2O_2$ compared with THGL-PEC stimulated with zymosan (0.5 mg/ml). Means ± SEM, n = 3.

*The effect of glutathione depleting agents on the susceptibility of schistosomula to oxidative killing* – The difference in susceptibility between adult schistosomes and schistosomula to killing, in the presence of cell-free generators of oxygen products can be demonstrated *in vitro.* This correlates with significantly higher levels of glutathione and antioxidant enzymes SOD, GPO, and cytochrome c peroxidase in adult worms (Mkoji et al., 1988b). Adult schistosomes treated with GSH-depleting agents have increased susceptibility to killing by oxidants *in vitro* (Mkoji et al., 1988a). Results reported above demonstrate that although S-PEC produce $H_2O_2$, the levels are below the threshold for schistosomula killing. The possibility of lowering the threshold at which levels of $H_2O_2$ would kill schistosomula by interfering with glutathione metabolism was examined. 1-chloro-2,4-dinitrobenzene (CDNB) is known to deplete GSH through conjugation reactions and inhibits the enzyme glutathione reductase of the GSH cycle in tumor cells (Arrick et al., 1982). When schistosomula were incubated in 2 $\mu$M concentrations of CDNB in the presence of normally sublethal levels of cell-free generated $H_2O_2$, there was a significant ($P < 0.05$) increase in mortality (Fig. 6). Oltipraz has been observed to deplete schistosome GSH by mechanisms not yet clear (Beuding et al., 1982). We have found (Fig. 7) that schistosome mortality was significantly increased by oltipraz when incubated in the presence of either $H_2O_2$ (0.1 mM), glucose (5.5 mM) + glucose oxidase (2 mU/ml) or S-PEC (2 x $10^6$) + IMS (20%).

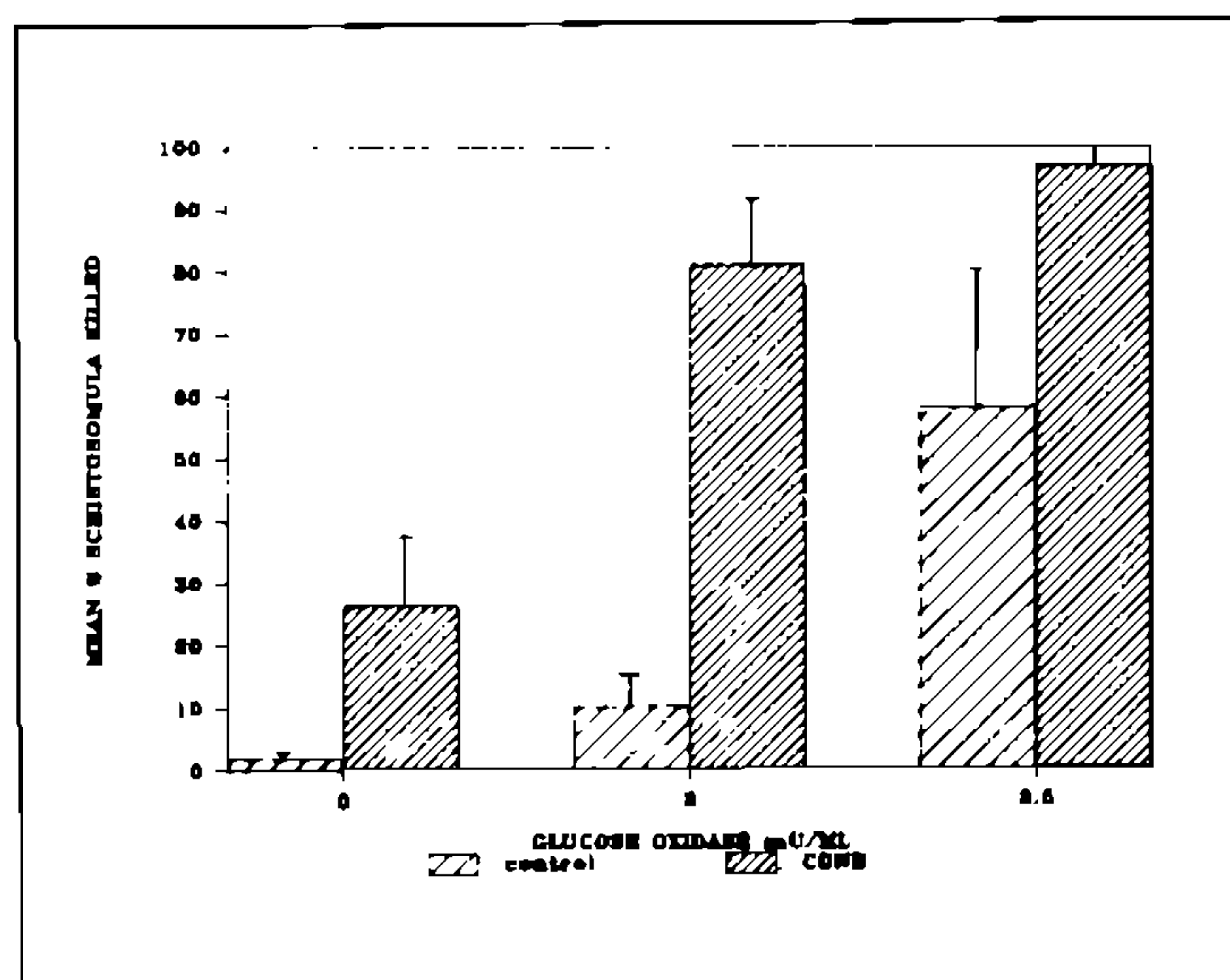

Fig. 6: the effect of CDNB (2 $\mu$M) on the susceptibility of schistosomula to GO generated $H_2O_2$. 100 schistosomula were incubated in 250 $\mu$l of MEM + 10% FCS containing CDNB and GO, at 37°C and % killed recorded after 24 h. Means ± SEM, n = 3.

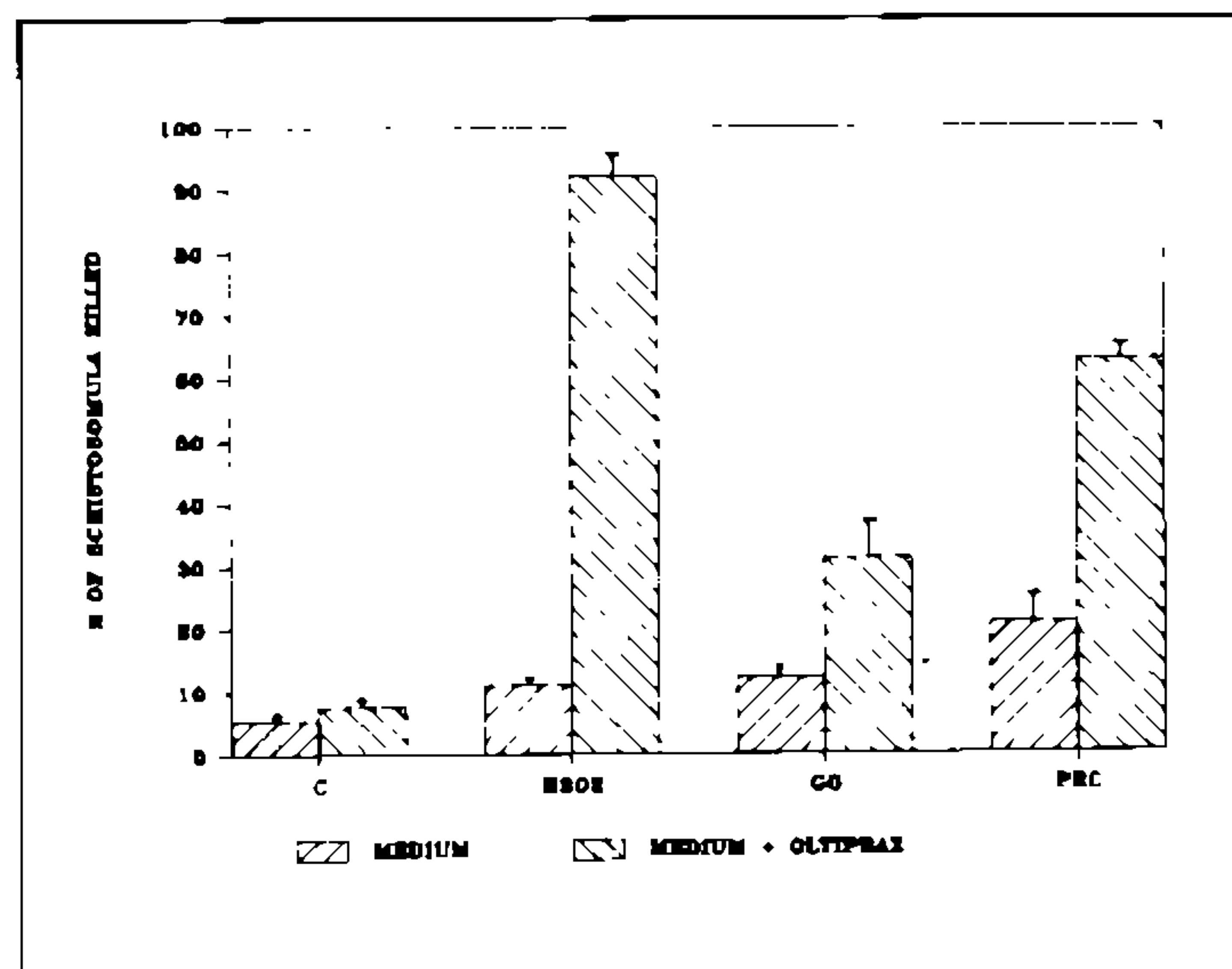

Fig. 7: the effect of oltipraz (4.0 $\mu$g/ml) on the susceptibility of schistosomula to reagent $H_2O_2$ (0.1 mM), GO (2 mU/ml) generated $H_2O_2$ or S-PEC (2 x $10^6$) + IMS (20%). Means ± SEM, n = 3.

## CONCLUSIONS

PEC from mice chronically infected with *S. mansoni,* when either specifically or non-specifically stimulated, have a reduced capacity to produce $H_2O_2$ at levels which are toxic to schistosomula *in vitro.* Levels of superoxide, however, are similar to those obtained from BCG or THGL-induced populations of PEC. This suggests that although respiratory burst activity may be detected in activated cells, the ratio of different oxygen products produced, and the relative susceptibility of the organism to those products, may be of greater importance in terms of potential cell cytotoxicity. These data confirm that $H_2O_2$-, or superoxide-dependent killing is unlikely to play a prominent role in antibody-dependent macrophage-mediated killing of schistosomula. However, in the case of BCG or thioglycollate elicited PEC elevated production of $H_2O_2$ may contribute significantly to an enhancement of schistosomula killing. Drug induced increased susceptibility of schistosomula to killing by low levels of $H_2O_2$, also enhances killing by immune cells.

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