The effect of iron and agar on production of hydrogen peroxide by stimulated and activated mouse peritoneal macrophages

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The effect of iron on H_2O_2 production by mouse peritoneal macrophages exposed to opsonised zymosan has been investigated. Macrophages elicited with thioglycollate broth produced less H_2O_2 than macrophages activated by *Corynebacterium parvum*, and levels were not affected by prior incubation of the cells with 0.1 mM iron nitrilotriacetate. However, preincubation with the iron chelator desferrioxamine (1 mM) reduced H_2O_2 production by both types of macrophages. Incubation of macrophages with agar, a component of thioglycollate broth, also reduced H_2O_2 production, particularly by *C. parvum*-activated macrophages. The results indicate that although iron appears to be necessary for H_2O_2 production by macrophages, the low level of production by thioglycollate-elicited macrophages is not due to an inadequate level of metabolically utilisable iron, but may be a result of prior ingestion of agar present in the broth.

Macrophage *Macrophage activation* *Hydrogen peroxide* *Iron* *Agar*

1. INTRODUCTION

It is well known that mouse peritoneal macrophages elicited with thioglycollate broth produce only low levels of reactive oxygen compounds when triggered with agents such as phorbol esters or opsonised zymosan. In contrast, activated macrophages obtained following intraperitoneal injection of mycobacteria or *Corynebacterium parvum* (also known as *Propionibacterium acnes*) produce much greater quantities of these compounds and it is thought that this may account for their tumoricidal activity [1]. The reason for this difference is, however, not well understood.

As part of a study of the mechanisms involved in the anaemia of chronic disease, we have recently investigated the way in which iron is handled by resident, thioglycollate-stimulated and *C. parvum*-activated mouse peritoneal macrophages. These investigations suggested that thioglycollate-elicited macrophages divert intracellular iron to a functionally inert pool, their metabolically-active iron pool being consequently reduced [2]. Since iron may be involved in oxygen metabolism, e.g. as a component of cytochromes it seemed possible that insufficient metabolically-active intracellular iron might account for the reduced production of reactive oxygen compounds by thioglycollate-elicited macrophages. However, it has also been suggested that agar present in thioglycollate broth may be taken up by macrophages and inhibit H_2O_2 production [3,4], though this has not been shown experimentally. This work has therefore sought to determine whether either intracellular iron or agar can affect production of H_2O_2 by mouse peritoneal macrophages, and whether any effect of agar is mediated by a disturbance of intracellular iron metabolism.

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2. MATERIALS AND METHODS

2.1. *Macrophages*

Thioglycollate-elicited mouse peritoneal macrophages were obtained from adult BALB/C mice 4 days after stimulation as described by Esparza and Brock [5]. *C. parvum*-activated macrophages were harvested 4 days after intraperitoneal injection of 2 mg of freeze-dried *C. parvum* strain 4982, kindly provided by Professor P.C. Wilkinson. Monolayer cultures (1 ml) were prepared in multiwell plates from 5×10^6 cells in 1 ml of RPMI 1640 medium containing 10% fetal calf serum (both from Flow Laboratories, Irvine, Scotland). Non-adherent cells were removed after 2 h incubation at 37°C in 10% CO_2/90% air in a humidified incubator.

2.2. *H_2O_2 production*

The procedure of Ruch et al. [6] was used, which utilises the oxidation of homovanillic acid to a fluorescent dimer. This method was found in preliminary experiments to have excellent sensitivity and reproducibility when used with adherent macrophage cultures. Cells were triggered as described by Ruch et al. [6] by incubating for 1 h in a total volume of 2 ml with zymosan (2 mg per culture: Sigma, Poole) which had been opsonised by incubating for 15 min with 50% (v/v) fresh mouse serum. The supernatants were centrifuged at $700 \times g$ for 10 min to remove the zymosan and any non-adherent cells before reading in a Perkin-Elmer 1000 Fluorimeter. The adherent cells were dissolved in 2% (w/v) SDS and total cell protein estimated by the Lowry method. Results were expressed as nmol H_2O_2 released per μg of cell protein.

2.3. *Iron release*

The ability of macrophages to release iron was determined using a method described previously [5] in which cells are pulsed with ^{59}Fe transferrin-antitransferrin immune complexes, and subsequent release of soluble ^{59}Fe to the culture supernatant is measured. Each well received 10 μg of labelled transferrin, as immune complexes.

2.4. *Addition or depletion of iron*

Following removal of non-adherent cells, the macrophages were either loaded with iron by including 0.1 mM Fe as the nitrilotriacetate complex (FeNTA) or depleted of iron by including 1 mM desferrioxamine (DFO: Desferal, Ciba-Geigy, Horsham, Sussex) in the culture medium. Cultures were incubated for 2 h or overnight, washed twice with Hanks buffered salts solution and reincubated for a further 1 h in fresh medium to allow any internalised DFO or nitrilotriacetate to be released before washing, triggering the cells with opsonised zymosan and assaying for H_2O_2 production as described above. To test the effect of DFO on iron release, cells were pulsed with ^{59}Fe-transferrin-antitransferrin immune complexes as described above prior to overnight incubation with DFO, after which ^{59}Fe in the culture supernatant was determined.

2.5. *Addition of agar*

A solution (5 mg/ml) of agar (Oxoid) was prepared in boiling phosphate-buffered saline, cooled to 45°C and diluted 1 : 10 in culture medium pre-heated to 45°C. The medium was then cooled to 37°C before addition to macrophage cultures. To allow for any possible effects of temperature on the culture medium, control medium without agar was also heated to 45°C and then cooled before use. Cultures were incubated overnight with the agar before washing and assaying for H_2O_2 production.

2.6. *Cell viability*

Initial cell viability, assessed by eosin exclusion was >98%. Adherent cultures were observed by phase-contrast microscopy, and in no case did any of the treatments described above cause an alteration in morphology relative to the controls. Furthermore, none of the treatments caused a decrease in total cell protein relative to the control, which indicates that cells were remaining adherent.

2.7. *Reproducibility of results*

The results are means of readings from 2 duplicate wells, in which the variation from the mean did not exceed 20%. All experiments were performed at least twice, on different days with different cell cultures.

3. RESULTS AND DISCUSSION

3.1. *Effect of FeNTA on H_2O_2 production*

In the absence of additional iron, production of H_2O_2 was, as expected [1], less for thioglycollate-stimulated macrophages than for *C. parvum*-activated macrophages (table 1). Addition of FeNTA to the cultures for both short (2 h) and long (16 h) periods had no effect on either cell type. Since it has previously been shown that this concentration of FeNTA enhances ferritin production in both types of macrophages, and that iron is taken into the cells [2], it must be concluded that in neither cell type is iron a limiting factor for H_2O_2 production.

3.2. *Effect of iron depletion on H_2O_2 production*

Overnight incubation of the macrophage cultures with 1 mM DFO prior to triggering for H_2O_2 production showed that in both cell types the chelator decreased H_2O_2 production, particularly from *C. parvum*-activated macrophages (table 2). To confirm that DFO was indeed removing intracellular iron from the macrophages, iron release from macrophages previously pulsed with ^{59}Fe-transferrin-antitransferrin immune complexes was determined in the presence and absence of DFO. It was found (table 3) that DFO caused a 7-fold increase in iron release from thioglycollate-stimulated macrophages, and a 3-fold increase from *C. parvum*-activated macrophages. It may thus be concluded that while iron is not normally a limiting factor for H_2O_2 production by either type of cell, it is nevertheless required as depletion of cellular iron by DFO causes reduced H_2O_2 production. Clearly, though, the reduced H_2O_2 production by thioglycollate-elicited macrophages cannot be explained by a lack of metabolically utilisable iron.

3.3. *Effect of agar on H_2O_2 production*

To determine whether agar ingested in vivo by thioglycollate-stimulated macrophages might, as suggested by Eichner and Smeaton [3], be responsible for reduced H_2O_2 production, both thioglycollate-stimulated macrophages and *C. parvum*-activated macrophages were preincubated in the presence of 0.5 mg/ml agar (the same concentration as is present in thioglycollate broth). It was found (table 4) that agar caused a marked reduction of H_2O_2 production by both cell types. However, the effect was much greater on *C. parvum*-activated macrophages which had not

Table 1

Effect of preincubation with 0.1 mM FeNTA on H_2O_2 production by thioglycollate-elicited and *C. parvum*-activated mouse peritoneal macrophages

Type of macrophage	FeNTA	Pre-incubation time (h)	H_2O_2 release (nmol/mg cell protein)
Elicited	–	2	46
	+	2	46
Activated	–	2	160
	+	2	174
Elicited	–	16	114
	+	16	98
Activated	–	16	214
	+	16	177

Table 2

Effect of DFO (1 mM) on H_2O_2 production by mouse peritoneal macrophages

Type of macrophage	DFO	H_2O_2 release (nmol/mg cell protein)
Elicited	–	45
	+	31
Activated	–	191
	+	58

Table 3

Effect of DFO on iron release by mouse peritoneal macrophages pulsed with ^{59}Fe-transferrin-antitransferrin immune complexes

Type of macrophage	DFO	^{59}Fe uptake (% of complexes)	^{59}Fe release (% of uptake)
Elicited	–	27	5.1
	+	33	35.7
Activated	–	35	10.1
	+	39	29.8

Table 4

Effect of agar on H_2O_2 production by mouse peritoneal macrophages

Type of macrophage	Agar	H_2O_2 release (nmol/mg cell protein)
Elicited	–	26
	+	7
Activated	–	182
	+	12

previously been exposed to agar (16-fold reduction) than on thioglycollate-elicited macrophages which had already been exposed to agar in the mouse peritoneum (4-fold reduction). It thus seems likely that agar ingestion is at least a contributory factor to the poor production of reactive oxygen species by thioglycollate-elicited macrophages. Incubation of *C. parvum*-activated macrophages with agar following a pulse of ^{59}Fe transferrin-antitransferrin immune complexes showed that agar did not inhibit iron release (not shown), indicating that agar did not act at the level of sequestering intracellular iron. It was also noted that the amount of H_2O_2 produced by thioglycollate-elicited macrophages, while always less than that produced by *C. parvum*-activated macrophages, varied considerably from one experiment to another (tables 1, 2 and 4), and also tended to increase if the cells were first incubated overnight (cf. table 1). The first observation could be explained by a variable degree of in vivo agar ingestion in different mice, depending upon the size of the cellular influx, and the second by excretion of agar during in vitro culture.

It may thus be concluded that although iron is necessary for H_2O_2 production by macrophages, it is not normally a limiting factor. It is also worth noting that an alternative hypothesis, namely that additional iron might enhance catalysis of the Haber-Weiss reaction between H_2O_2 and superoxide [7] also appears to be untenable as no decrease in H_2O_2 release occurred. The effect of agar on H_2O_2 production suggests that caution may need to be exercised in extrapolating from experiments with thioglycollate-stimulated macrophages, as the broth itself may affect macrophage properties. The reason for this effect of agar is not immediately obvious, but it could be related to the fact that agar can act as a free radical scavenger [3]. A study of the effect of agar on the production of other reactive oxygen species might help to elucidate the mechanism.

While the effect of agar reported here may be considered essentially artifactual, there are wider implications with regard to the effect of environment on macrophage properties. Ingestion of damaged erythrocytes and other cell debris by macrophages in inflammatory lesions doubtless occurs, and it has been demonstrated that ingestion of erythrocytes reduces the tumoricidal activity of activated macrophages [8]. In such situations, production of reactive oxygen compounds may also be affected, and this could reduce the ability of activated macrophages to kill invading microorganisms. Preliminary experiments (L.S. Stewart and J.H. Brock, unpublished) indicate that ingestion of erythrocytes inhibits H_2O_2 production by activated macrophages, and further studies on the effect of environmental factors on production of reactive oxygen species by macrophages would be of interest.

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