THE PRODUCTION OF OXYGEN-DERIVED RADICALS BY NEUTROPHILS FROM SELENIUM-DEFICIENT CATTLE

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1. Introduction

Neutrophils prepared from the blood of Se-deficient rats and cattle are known to have impaired microbicidal activity when compared to cells from animals supplemented with Se [1–3]. Histochemical tests have demonstrated that the neutrophils from Se-deficient animals are less able to reduce nitroblue–tetrazolium (NBT) when challenged with endotoxin [2,3] which suggests they may be less able to produce superoxide (O_2^-) required for microbicidal activity [4]. The neutrophils from Se-deficient cattle evolve less $^{14}CO_2$ when incubated with [1-^{14}C]glucose than the cells from Se-supplemented cattle [5]. Oxidation of glucose by the hexose monophosphate shunt in neutrophils is thought to produce NADPH necessary for O_2^- production [6]. These changes in microbicidal and metabolic activity in Se-deficient neutrophils are associated with a fall in the activity of the selenoenzyme glutathione peroxidase (GSHpx) [1–3,5] (EC 1.11.1.9). The fall in GSHpx activity may allow the peroxides produced during phagocytosis to accumulate and damage the neutrophil itself [1,2].

Thus there is indirect evidence for an impaired ability to produce O_2^- in Se-deficient bovine neutrophils. More direct evidence for the production of radicals by human neutrophils has now been obtained employing the technique of spin-trapping [7,8]. Here we describe the application of these and some indirect techniques to examine the production of radicals by bovine neutrophils.

2. Materials and methods

Neutrophils were prepared from whole blood samples from Se-deficient and Se-supplemented calves (5 and 6 animals/group) [2] using the method in [9]. Preparations contained >95% neutrophils with no evidence of differences in yield of cells between the Se-deficient and Se-supplemented animals. Viability of the neutrophils was >95% in both groups as assessed by trypan blue exclusion.

Spin-trapping experiments were carried out using 10^7 cells/ml in Hank's balanced salt solution (BSS without phenol red; Gibco Biocult Ltd), 100 mmol/l 5′,5′-dimethyl-1-pyrroline-*N*-oxide (DMPO) and 1 mmol/l diethylenetriamine pentaacetic acid (DPTA). The cells were maintained at 37°C prior to stimulation with zymosan and then transferred into the chamber of flat electron paramagnetic resonance (EPR) cells. The EPR spectra were recorded using a Varian E104 spectrometer operating in the first derivative mode [7,10].

Reduction of cytochrome *c* by the neutrophils was monitored at 550 nm using a Cecil CE505 spectrophotometer attached to a CE500 recorder (Cecil Instr., Cambridge). The cell holder in the Spectrophotometer was maintained at 37°C. Neutrophils (2×10^6) in 100 μl phosphate-buffered saline were added to a 1 ml cuvette with 730 μl BSS, 100 μl autologous cattle serum and 35 μl cytochrome *c* (30 mg/ml in BSS). The neutrophils were stimulated with 0.5 mg zymosan (Sigma) in 35 μl BSS. After a lag period of 5–7 min for both neutrophils from Se-deficient and Se-supplemented animals, absorbance at 550 nm increased. This increase was linear for up to 20 min in incubations of neutrophils from Se-deficient animals and for 30 min for incubations of cells from Se-supplemented animals. In some incubations, cytochrome *c* reduction was monitored in the presence of 35 μg superoxide dismutase (bovine erythrocyte, Miles Research Ltd, Slough) to assess the amount of

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cytochrome *c* reduction directly or indirectly due to O_2^-.

In one experiment cytochrome *c* was replaced in the reaction mixture by 35 μl 2-(*p*-iodophenol)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride (INT, Aldrich Chem. Co. 2.5 mg/ml in ethanol/BSS 1:4, v/v) which forms a coloured formazan on reaction with O_2^-. After incubation for 15 min the reactions were stopped by placing them on ice. Neutrophils were then sedimented by centrifugation at 4°C at 1000 × *g* for 5 min. Reduced INT was measured by its absorbance at 500 nm ($\epsilon_M = 19.3 \times 10^3$) in the supernatant and a cyclohexane extract of the sedimented cells.

Oxygen uptake by neutrophils was measured using a Clark oxygen electrode [7].

3. Results

3.1. *O_2 uptake*

The maximum rate of O_2 uptake by the neutrophils was measured after they had been stimulated by zymosan particles. Before zymosan addition the O_2 consumption rate of neutrophils from both Se-deficient and Se-supplemented calves was less than 0.15 nmol . 10^6 . $cells^{-1}$. min^{-1}. It was not possible with these low rates to detect any difference between the groups of cells. However after zymosan addition, the neutrophils from the Se-deficient cattle consumed O_2 at a greatly reduced rate compared to those of normal cattle. The DMPO and the DTPA used in the spin-trapping study described below did not affect the resting or stimulated rates of O_2 uptake by the neutrophils (not shown).

3.2. *Cytochrome c reduction*

There was no measurable cytochrome *c* reduction by the resting neutrophils of both groups. After zymosan stimulation, there was a lag of 5–7 min in both groups before reduction of cytochrome *c* commenced. There was also lower rate of cytochrome *c* reduction in the neutrophils from the Se-deficient animals (table 1). The period for which cytochrome *c* reduction continued was 20 min in neutrophils from low Se animals and 30 min in those from supplemented calves. In both groups the reduction of cytochrome *c* was prevented by addition of superoxide dismutase to incubations.

3.3. *INT reduction*

Reduction of INT by neutrophils was inhibited by 95% on the addition of superoxide dismutase to incubations. When superoxide dismutase was not added to the incubation, supernatants and pellets of Se-deficient neutrophils produced 45% and 50% less reduced INT, respectively, than the corresponding fractions from normal animals (table 1).

3.4. *Spin-trapping*

When neutrophils from the Se-deficient animals were stimulated with zymosan they produced 25% less hydroxyl radical adduct of DMPO than the cells from Se-supplemented animals (fig.1). These signals

Table 1
Oxygen consumption, reduction of cytochrome *c* and reduction of INT by neutrophils from Se-supplemented and Se-deficient cattle after stimulation with zymosan

	Se-Deficient	Se-Supplemented
O_2 consumption (nmol . 10^6 $cells^{-1}$. min^{-1})	1.78 ± 0.14 (5)	2.83 ± 0.27 (6)[a]
Cytochrome *c* reduction (nmol . 10^6 $cells^{-1}$. min^{-1})	0.266 ± 0.048 (5)	0.519 ± 0.035 (6)[b]
INT reduction (nmol . 10^6 $cells^{-1}$. 15 min^{-1})		
(a) Supernatant	(a) 1.89 ± 0.24 (5)	3.31 ± 0.26 (6)[b]
(b) Cell pellet extract	(b) 5.48 ± 1.05 (5)	11.07 ± 1.36 (6)[a]

Results are mean ± SEM; no. determinations in parenthesis; significance of differences between groups, [a] $P < 0.01$, [b] $P < 0.005$, Student's *t*-test

were completely abolished by inclusion of 100 μg/ml superoxide dismutase in the incubations. Mannitol, a hydroxyl radical scavenger, at a concentration (30 mM) which did not interfere with the respiratory burst reduced the amount of DMPO hydroxyl adduct formed (fig.1).

4. Discussion

The bovine neutrophils used in the spin-trapping experiments behaved in a similar fashion to human neutrophils [7] in that they produced a hydroxyl radical adduct of DMPO when challenged with a par-

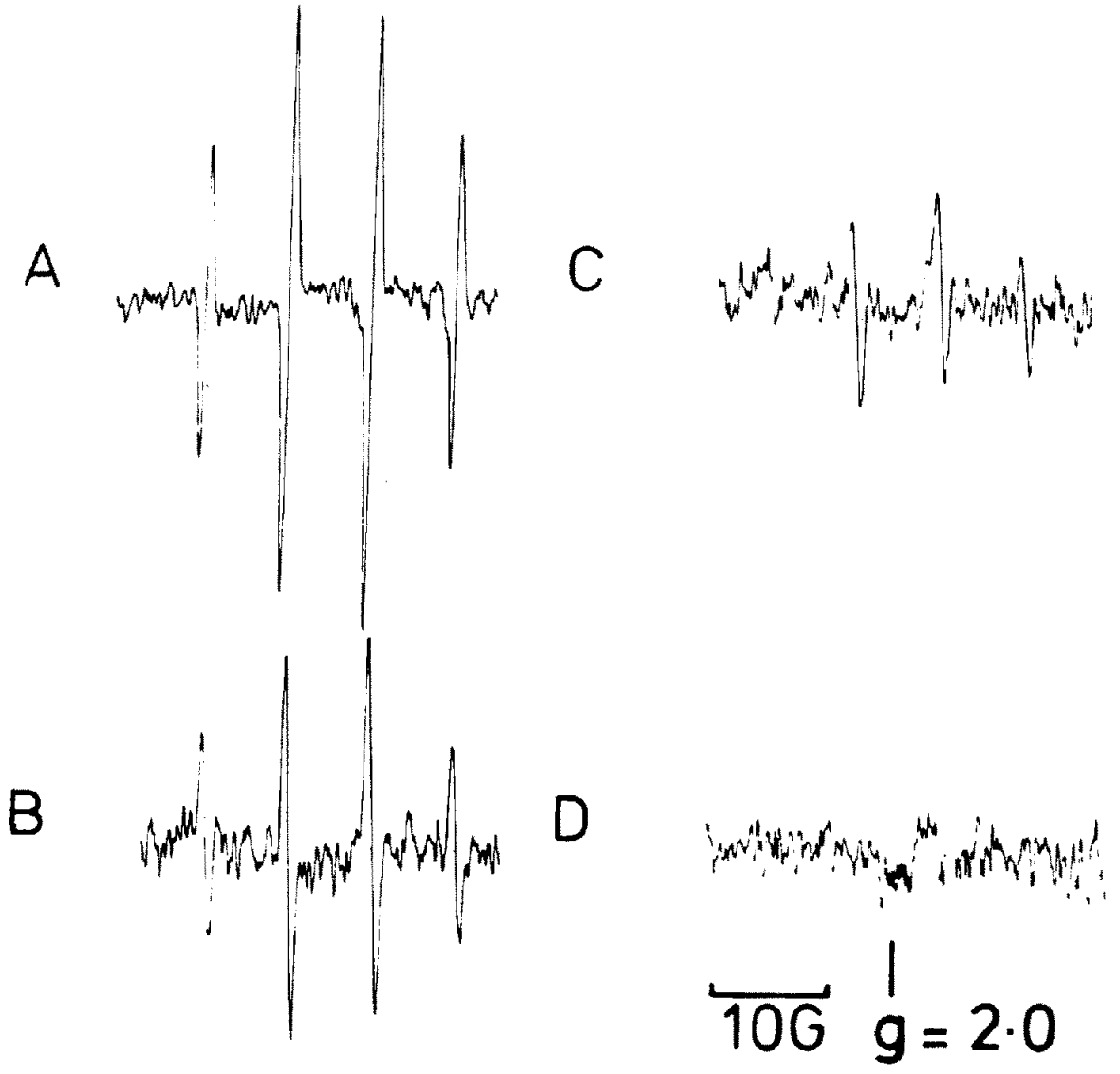

Fig.1. EPR spectra obtained after incubation at 37°C in BSS of bovine blood neutrophils (1×10^7 cells/min), DMPO (100 mM) DTPA (1 mM) with opsonised zymosan (0.5 mg/ml). Samples were removed from a thermostatted oxygen electrode chamber and transferred to an EPR flat cell 1 min after stimulation [7]: (A) normal cells + zymosan; (B) selenium-deficient cells + zymosan; (C) selenium-deficient cells + mannitol (10 mM) + zymosan; (D) selenium-deficient cells + superoxide dismutase (100 μg/ml) + zymosan (conditions field 3385 G, frequency 9.5 GHz, power 10 mW, modulation 1 G, time constant 0.1285, scan rate 0.89 s^{-1}, gain 10^5).

ticle (zymosan) and both superoxide and hydroxyl radical adducts of DMPO with phorbol myristate acetate, a soluble stimulant (not shown). However the neutrophils from Se-deficient animals produced less of the hydroxyl radical adduct of DMPO when challenged with zymosan particles than did cells from Se-supplemented animals. The Se-deficient neutrophils also displayed decreased superoxide dismutase-sensitive reduction of both cytochrome *c* and INT. The duration of cytochrome *c* reduction was also decreased in the Se-deficient neutrophils. There did not appear to be any effect of the Se deficiency on the lag time in response of neutrophils to zymosan stimulation. Assuming that superoxide dismutase-sensitive cytochrome *c* reduction and the spin-trapping experiments are a measure of the production of toxic O_2 species in the activated neutrophil, the observations above are consistent with the ability of the Se-deficient neutrophil to ingest but not kill cells of the yeast *C. albicans* [1–3]. Superoxide dismutase-sensitive reduction of INT in extracts and supernatants from incubations of stimulated neutrophils suggests that toxic oxygen metabolites are probably produced in the phagosome as well as being released into the surrounding medium. It is possible therefore that these metabolites also diffuse into the cytosol of the neutrophil. As cytosolic GSHpx activity is decreased in Se deficiency in neutrophils [1–3,5] there will be less scavenging of toxic peroxides. This could result in damage to enzymes within the neutrophil resulting in impaired microbicidal activity and metabolic activity.

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