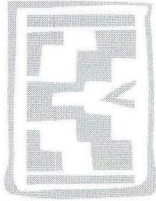

# Non-immune control of trypanosomosis: *In vitro* oxidative burst of PMA- and trypanosome-stimulated neutrophils of Boran and N'Dama cattle

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**ABSTRACT**

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An *in vitro* assay that measures the generation of superoxide anions ($O_2^-$) was used to assess the level of oxidative burst of phorbol myristate acetate (PMA)- and trypanosome-stimulated neutrophils isolated from healthy Boran and N'Dama cattle, and those infected with *Trypanosoma congolense*.

PMA stimulation of healthy bovine neutrophils resulted in between 300–400 % increase in $O_2^-$ generation. Neutrophils of Boran cattle exhibited slightly higher but insignificant $O_2^-$ generation capacity than those of the N'Dama breed. *In vitro* stimulation by trypanosomes of neutrophils isolated from *Trypanosoma congolense*-infected cattle caused significant increases in $O_2^-$ generation, especially on days 14, 28 and 42 post-infection, of both breeds of cattle. No significant differences were observed in $O_2^-$ generation capacity of the neutrophils of both breeds of infected cattle throughout the period of assay.

The results of this study have shown that PMA and trypanosomes do cause an enhanced *in vitro* oxidative burst, hence trypanosome phagocytosis and killing activity of neutrophils. Neutrophils have been shown to play very significant roles in parasite clearance, hence reduction of trypanosome parasitaemia. The rates of both *in vitro* generation of $O_2^-$ and trypanosome phagocytosis over time did not differ significantly between Boran and N'Dama breeds of cattle, even during *T. congolense* infection in this study. Hence, it may be inferred that sustained and higher parasitaemia, more pronounced neutropenia, inadequate bone marrow response and less effective trypanosome-specific immune response, rather than defective neutrophil trypanosome destruction, may be the problem of trypanosusceptible cattle breeds.

**Keywords:** Boran, cattle, N'Dama, neutrophils, phorbol myristate acetate, superoxide anions, trypanosomosis

## INTRODUCTION

It has been well established that specific anti-*trypanosoma* lytic antibodies are important in the control of successive waves of parasitaemia in African trypanosomosis (Askonas & Bancroft 1984; Pinder, Libeau, Hirsch, Tamboura, Hauck-Bauer & Roelants 1984; Pinder, Bauer, Melick & Fumoux 1988; Paling, Moloo, Scott, Getinby, McOdimba & Murray 1991). The differential roles of activated mononuclear phagocytes in immune and non-immune mediated mechanisms in the control of the pathogenesis of infection in trypanosome-infected Boran (trypanosusceptible East African Zebu) and N'Dama (trypanotolerant taurine) cattle have also been doc-

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umented (Morrison, Murray & Akol 1985; Anosa, Logan-Henfrey & Wells 1997; Taiwo & Anosa 2000).

The phagocytic activity of neutrophils and eosinophils plays an important role in host defence against foreign materials and pathogens, especially bacteria (Morrison & Ryan 1979). While little is known of their importance in the defence against trypanosomes, mammalian polymorphonuclear cells (neutrophils and eosinophils) have been reported to phagocytose *Trypanosoma cruzi* (Sanderson & de Souza 1979) and *Trypanosoma dionsisii* (Thorne, Glauert, Souvennsen & Franks 1979). In addition, little is known about the roles of neutrophils and eosinophils in the differential control of trypanosome parasitaemia in susceptible and tolerant cattle breeds in Africa.

Kissling, Karbe & Freitas (1982) reported that the trypanotolerant N'Dama cattle possessed a significantly higher number of circulating neutrophils and neutrophils with *in vitro* latex phagocytic activity than the more susceptible Baoule (Zebu) and the crossbred M75 cattle. Neutropenia and eosinopenia have both been described as part of the clinical manifestations of trypanosomosis in various animal species, including cattle (Anosa 1983, 1988; Paling *et al.* 1991). Because neutrophils have a half-life ranging from 6–12 (average 9) hours before being removed randomly from circulation (Jain 1986), and because their long-term *in vitro* culture is difficult, if not impossible to maintain (Slauson, Clifford, Zwahlen & Nielsen 1985), it is very difficult to assess the *in vivo* trypanosome activity of these phagocytes in order to elucidate their differential roles in controlling parasitaemia in trypanosome-infected animals.

This study was designed to investigate the generation of $O_2^-$ and trypanosome-phagocytosing capability of neutrophils of stimulated and unstimulated neutrophils, and those obtained from *Trypanosoma congolense*-infected Boran and N'Dama cattle.

## MATERIALS AND METHODS

### Animals and trypanosomes

Twenty of the cattle used in this study, ten Boran and ten N'Dama (five males and five females of each breed), all aged between 2 and 4 years, were the same animals used for *in vitro* erythrophagocytosis assays by cultured macrophages during experimental trypanosomosis (Taiwo & Anosa 2000). Additionally, two Boran and two N'Dama cattle (one cow and one steer of each breed) were used as uninfected sources of unstimulated and stimulated neutrophils. *Trypanosoma congolense* ILNat 3.1, a double clone derivative from a stock (STIB 212) isolated from a lion in the Serengeti area of Tanzania (Geigy & Kauffmann 1973; Nantulya, Musoke, Rurangirwa & Moloo 1984) was used. Neutrophils were isolated from the cattle before *T. congolense* infection (Taiwo & Anosa 2000) and thereafter on 14, 21, 28, 35, 42, 49 and 56 days post-infection (DPI). Similar isolations were carried out from the uninfected cattle once a week for 5 consecutive weeks.

### Chemical reagents used

Ferricytochrome C (Horse heart, Type IV), superoxide dismutase (SOD) and phorbol myristate acetate (PMA) were purchased (Sigma Chemical Co., St. Louis, Mo). Hank's buffered saline solution (Gibco, UK) containing 0.1 % bovine serum albumin (HBSS-A) at pH 7.3 was also used. Solutions of both ferricytochrome C and SOD were prepared at 50 mg/mℓ concentrations in phosphate buffered saline (PBS) at pH 7.2, while a PMA solution was prepared at a concentration of 10 µg/mℓ, initially by dissolving it in dimethyl sulphoxide (DMSO; Sigma Chemical Co.), which was then diluted with PBS to a final DMSO concentration of 0.88%. All the chemicals were aliquoted into 1 mℓ plastic vials and stored at –70 °C before thawing for use. The final concentration of ferricytochrome C used for the assay was 5 mg/mℓ (0.4 mM).

### Isolation of bovine neutrophils from peripheral blood

Eighteen ml of blood were collected from each animal on each day of assay into plastic tubes containing 2 mℓ of acid-citrate-dextrose (ACD) solution as anticoagulant. The blood-ACD mixture was carefully layered onto 7 mℓ of fortified Ficoll-paque solution. The fortified Ficoll was made by mixing 2.5 g Ficoll 400 powder (Pharmacia, UK) to 100 mℓ of commercial Ficoll-paque solution and sterilized by filtration through 0.22 µ nalgene filter before use. The tubes were centrifuged on a Beckman J-6B (Avis Zur Beachtung, Beckman, USA) at 700 *g* for 15 min. After the removal of Ficoll-paque interface-peripheral blood mononuclear cells for use in *in vitro* macrophage phagocytosis assays (Taiwo & Anosa 2000), the plasma and top three-quarters of the erythrocyte pellet were gently pipetted off and discarded. Hypotonic lysis of the erythrocytes in the remaining pellet was carried out by gently mixing

with 20 ml of cold distilled water for 30 s. This was followed immediately by the addition of 2.7 % NaCl with gentle mixing and bringing up of the total volume to 50 mℓ with cold Hank's balanced salt solution (HBSS; pH 7.3) in order to restore isotonicity. Washing of the remaining cells was carried out by centrifugation at 1000 *g* for 10 min. Both hypotonic lysis and washing procedures were repeated. Five millilitre of HBSS-A (HBSS, pH 7.3 plus 0.1 % BSA) was used to resuspend the cell pellet (neutrophils) and its concentration determined by counting on a modified Neauber haemocytometer (Hawksley, UK). The final concentration was adjusted to $10^6$ neutrophils per mℓ in HBSS-A.

### *In vitro* neutrophil phagocytosis/oxidative burst assays

A slightly modified measurement of neutrophil superoxide anion generation method described by Slauson *et al.* (1985) was used. Briefly, frozen reagents were thawed, final concentrations prepared and thereafter incubated at 37 °C. Similarly, all reaction cuvettes (rectangular type) and spectrophotometer (SP6-500 UV; PYE UNICAM Ltd, Cambridge, England) were warmed to 37 °C before use. One mℓ of HBSS-A containing $10^6$ neutrophils was placed in a reaction cuvette and incubated for 5 min. Thereafter 0.5 mℓ ferricytochrome C was added and placed in the light chamber of the spectrophotometer set at 550 nM, while ensuring that there was no spontaneous reduction of cytochrome.

For *in vitro* stimulated neutrophils, 0.5 mℓ of PMA was added to the reaction cuvette in the spectrophotometer, allowing for a 3 min lag time before taking the final absorbance reading (optical density; OD) which was read against blanks consisting of cytochrome C solution from cuvettes without neutrophils. In the unstimulated neutrophil assays, 0.5 mℓ of HBSS-A replaced PMA.

Neutrophils isolated from trypanosome-infected cattle at the various DPI were stimulated by diethylaminoethylether (DEAE)-separated and washed 6 x $10^6$ *T. congolense* ILNat 3.1 in HBSS. Each assay step was carried out four times, twice without SOD, and twice substituting 0.5 mℓ of SOD for 0.5 mℓ of HBSS-A. The quantity of inhibitive reduction of ferricytochrome C by SOD is equivalent to the quantity of $O_2^-$ produced by neutrophils in the reaction cuvettes as calculated below.

The nanomoles (nM) of $O_2^-$ produced by $10^6$ neutrophils per 3 min were calculated using the following formula (Slauson *et al.* 1985):

X = A/bk, where X = amount of $O_2^-$ per ℓ

b = light path (diameter of cuvette), which is 1 cm

k = extinction coefficient of ferricyto-cytochrome C at 550 nM, which is 21 $mM^{-1}cm^{-1}$

A = absorbance (OD reading)

Therefore, X = A/21 mM/ℓ or (A/21) x $10^3$ nM/mℓ

Since 1 mℓ contains $10^6$ neutrophils, the amount of $O_2^-$ produced by $10^6$ neutrophils (in 3 min) equals:

A x 47.619 without SOD minus A x 47.619 with SOD (nM)

Means of data obtained from replicate assays (with or without SOD) were calculated and subjected to the student *t*-test (Snedecor 1965) for comparisons between stimulated and unstimulated neutrophils and between those isolated from trypanosome-infected Boran and N'Dama cattle.

Parallel assays using cuvettes placed in water bath at 37 °C, with properly mixed cell suspensions harvested on clean glass slides by cytospin (Shandon Equipment, UK) were carried out every 30 s. The cells were stained with Diff-quik, examined by light microscopy and photomicrographs taken as described by Taiwo & Anosa (2000).

## RESULTS

Table 1 shows the trend of $O_2^-$ generated by PMA-stimulated and unstimulated neutrophils isolated from uninfected cattle, while Fig. 1 shows the pattern of generation of $O_2^-$ by neutrophils isolated from *T. congolense*-infected Boran and N'Dama cattle. PMA caused significant increases ($P < 0.01$; between 300–400 %), above the unstimulated, in $O_2^-$ generation by neutrophils isolated from uninfected cattle, with neutrophils of Boran exhibiting slightly higher but insignificant ($P > 0.05$) $O_2^-$ generation potential than those of the N'Dama breed (Table 1). *In vitro* stimulated neutrophils isolated from trypanosome-infected cattle by trypanosomes caused significant increases ($P < 0.001$) in $O_2^-$ generation by neutrophils, especially on 14, 28 and 42 DPI, of both breeds of cattle (Fig. 1). No significant differences ($P > 0.05$) were observed in $O_2^-$ generation capacity of the neutrophils of both breeds of infected cattle throughout the period of assay.

The technique adopted for the separation of neutrophils from peripheral blood proved very effective as a > 95 % monoculture of isolated unstimulated N'Dama cattle neutrophils was achieved (Fig. 2), with occasional contamination by few eosinophils.

TABLE 1 *In vitro* superoxide anion generation (nM/$10^6$ cells) by non-stimulated (NS) and PMA-stimulated (PMA-S) bovine neutrophils within 3 min

| Week | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| N'Dama | | | | | |
| NS | 19.3 ± 3.2* | 18.3 ± 3.1 | 20.1 ± 2.2 | 19.1 ± 1.1 | 18.2 ± 1.3 |
| PMA-S | 69.3 ± 4.1 | 65.0 ± 5.6 | 60.5 ± 2.3 | 68.6 ± 2.0 | 74.2 ± 1.9 |
| Boran | | | | | |
| NS | 23.6 ± 2.3* | 22.3 ± 3.6 | 20.3 ± 2.1 | 18.1 ± 1.9 | 19.9 ± 1.7 |
| PMA-S | 72.5 ± 4.5 | 75.2 ± 4.0 | 67.4 ± 5.2 | 70.2 ± 3.5 | 72.3 ± 2.8 |

* Superoxide anion generation by PMA-S neutrophils was significantly higher ($P < 0.001$) than NS neutrophils at every stage of assay in both breeds of cattle

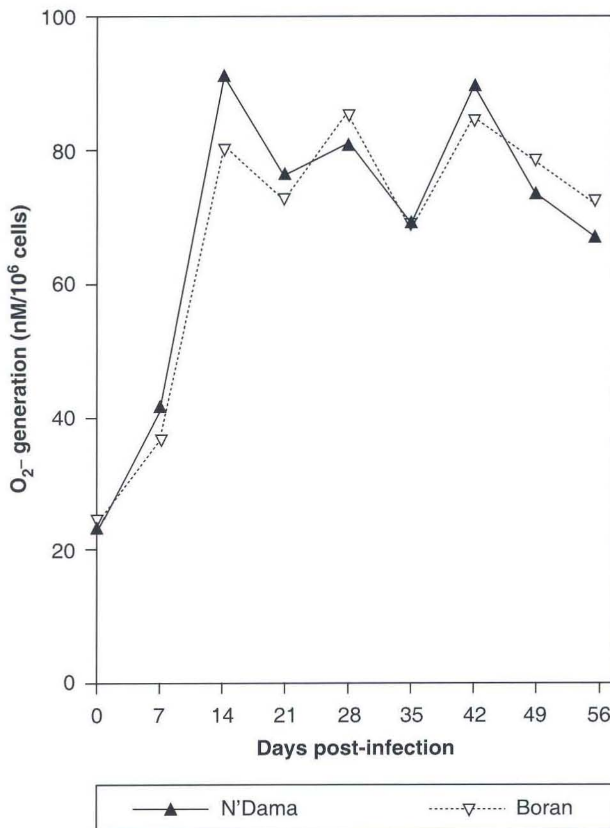


FIG. 1 *In vitro* superoxide anion ($O_2^-$) generation by trypanosome-stimulated neutrophils isolated from *Trypanosoma congolense*-infected Boran and N'Dama cattle

Photomicrographs of neutrophils from infected cattle co-incubated with trypanosomes for 30 s and 2 min revealed the presence of free trypanosomes (Fig. 3), and both attached and phagocytosed trypanosomes (Fig. 4), respectively. Some of the trypanosomes were found in various stages of division (Fig. 3 and 4). A few eosinophils contained phagocytosed trypanosomes (Fig. 5). Similar findings were observed in the assays involving neutrophils of both breeds of cattle. A time-dependent more drastic reduction in the number of neutrophils was observed in PMA- and trypanosome-stimulated neutrophils than in unstimulated neutrophils during the assays.

## DISCUSSION

Mammalian polymorphonuclear neutrophils have been shown to play important roles in the defence against bacteria, viruses and fungi, and notably by phagocytosis (Kissling *et al.* 1982). However, comparatively little is known regarding their effectiveness against protozoa. This may have been due to their low numbers and the very short lifespan of these versatile cells in the circulation of domestic animals (Jain 1986). The assays described in this study have shown that neutrophils, and probably less so, eosinophils not only phagocytose trypanosomes *in vitro*, but exhibited increased oxidative burst resulting in the release of reactive oxygen reduction products, similar to what is observed with elicited or activated macrophages (Pick & Keisari 1981; Adams & Hamilton 1984). The latter activity has also been demonstrated when phagocytes are exposed to stimulants such as bacterial endotoxin, lipopolysaccharide (LPS), phorbol myrisytate acetate (PMA), concanavallin A (Con-A), protozoa and even helminths (Morrison & Ryan 1979; Klebanoff 1980; Kissling *et al.* 1982; James & Hibbs 1990). In this assay, phagocytosis was preceded by attachment of the trypanosomes to the surface of neutrophils. Once inside the cytosol, a very rapid change in the structure of the trypanosome was observed with resultant rounding up, vacuolation and disintegration within the phagolysosome. Many neutrophils were observed to show degenerative and necrotic changes such as swollen nuclear segments

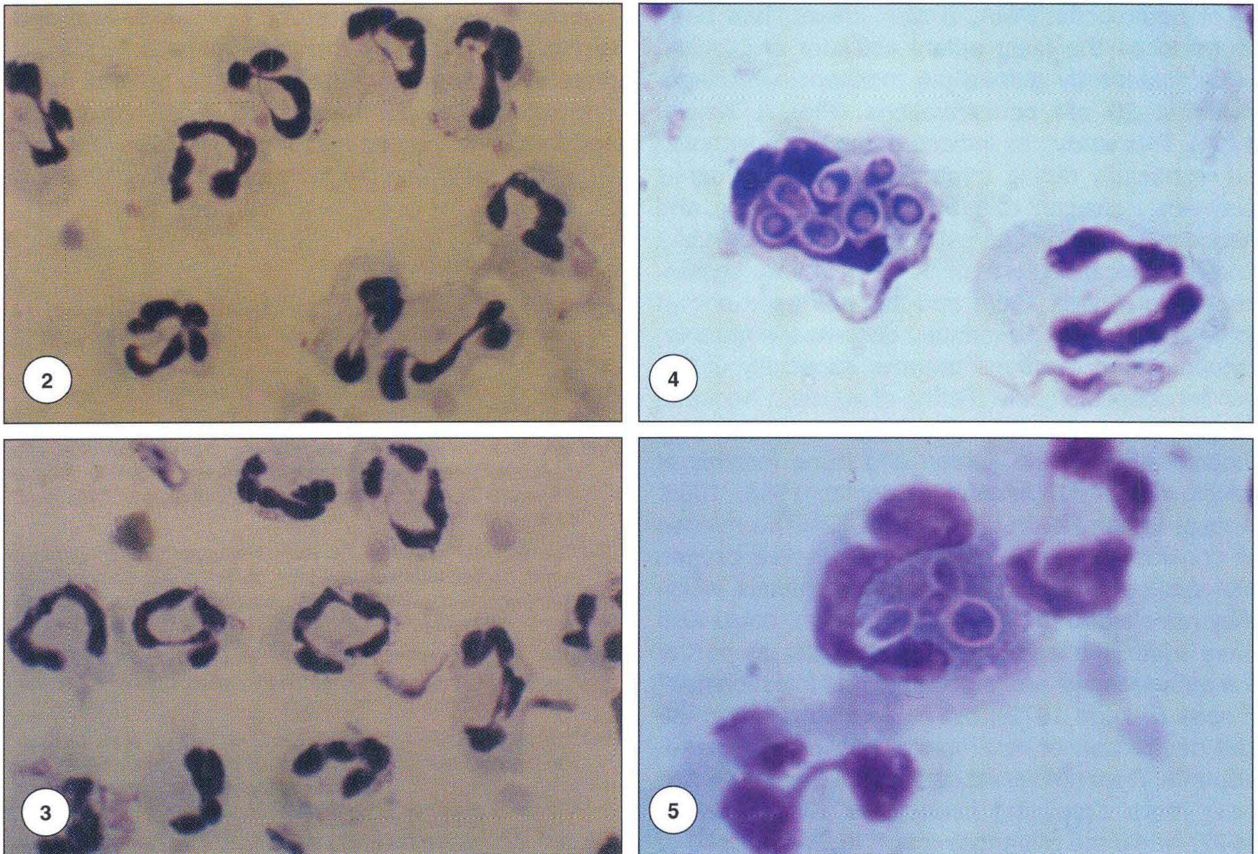


FIG. 2 Freshly isolated neutrophils of a *Trypanosoma congolense*-infected Boran cow (7 DPI) (H & E; X450)

FIG. 3 Neutrophils of the Boran cow (in Fig. 2) 30 s in co-culture with trypanosomes. Note the dividing trypanosome (top left) (H & E; X450)

FIG. 4 Neutrophils of a trypanosome-infected (28 DPI) N'Dama steer with attached (dividing) and engulfed trypanosomes (H & E; X600)

FIG. 5 An occasional eosinophil from an infected Boran cow (14 DPI) with engulfed trypanosomes (H & E; X600)

and karyolysis between 1–2 min after trypanosome phagocytosis.

The sequence of biochemical events arising from a sharp increase in the uptake of oxygen and its subsequent reduction is known as an oxidative burst (Fantone & Ward 1982; James & Hibbs 1990). It involves a one-electron reduction of molecular oxygen to $O_2^-$ in the presence of NADPH or NADH oxidase (Pick & Keisari 1981). When molecules of $O_2^-$ interact, hydrogen peroxide ($H_2O_2$) is formed. Both of these products, as well as hydroxyl radical ($OH^-$) and singlet oxygen ($^1O_2$), all by-products of the process of oxidative burst, have been shown to be involved in both intracellular and extracellular destructive processes of bacteria, fungi, viruses, tumour cells, collagen (Adams & Hamilton 1984; Klebanoff, Verdas, Harlan, Sparks, Gamble, Agosti & Waltersdorph 1986), and other cells in the vicinity of the phagocytes, including the phagocytes themselves (Pick & Keisari 1981; Fantone & Ward 1982). Apart from products of oxidative burst, potent hydrolytic enzymes such as acid phosphatase, lysozyme, arylsuphatase, cathepsins B, D and G, among many others are present within the primary (azurophilic) granules of neutrophils (Marmont, Damasio & Zucker-Franklin 1988), all of which when released into the external milieu have a deleterious effect on both neutrophils, parasites and host tissues.

The present study has shown that PMA and trypanosomes do cause enhanced an oxidative burst, hence trypanosome phagocytosis and killing activi-

ty of neutrophils. PMA, a skin irritant, has been found to be the most potent activator of $O_2^-$ and $H_2O_2$ release by guinea pig mononuclear phagocytes at 20 nM concentrations (Pick & Keisari 1981). This study has indicated that when stimulated, especially during trypanosomosis, neutrophils play very significant roles in parasite clearance, and hence a reduction of parasitaemia. Neutropenia is a known clinical feature of African animal trypanosomosis (Anosa 1988) and this has been shown to be due to cytokine-induced decrease in granulocytopoiesis in the bone marrow, especially of trypanosusceptible cattle (Paling *et al.* 1991; Anosa *et al.* 1997) and neutrophil phagocytosis by activated macrophages in the spleen and bone marrow of infected animals (Anosa & Kaneko, 1983, 1989; Anosa, Logan-Henfrey & Wells 1992). The release of considerably large amounts of reactive oxygen metabolites into the blood of infected animals, which may have aided in parasite destruction, could also have adverse effects on the host's cells, as part of the pathogenesis and outcome of the infection itself (Losos & Ikede 1972), but could also result in the self destruction of neutrophils, with resultant neutropenia. Trypanotolerant cattle have been shown to have more circulating neutrophils (Kissling *et al.* 1982), while the trypanosusceptible Boran breed of cattle was shown to suffer more pronounced neutropenia than the tolerant N'Dama during *T. congolense* infection (Paling *et al.* 1991; Williams, Naessens, Scott & McOdimba 1991). In a previous study (Taiwo & Anosa 2000), it was determined that Boran cattle suffered a more severe anaemia than their N'dama counterparts during *T. congolense* infection. Other studies (Paling *et al.* 1991; Williams *et al.* 1991) and unpublished data have revealed that Boran cattle had consistently higher and more prolonged parasitaemias than N'Dama cattle in *T. congolense* infections. It is to be noted, however, that the rates of both *in vitro* generation of $O_2^-$ and trypanosome phagocytosis, over time, did not differ significantly between the Boran and N'Dama breeds of cattle in this study. It could thus be inferred that higher and sustained parasitaemia, more pronounced neutropenia, inadequate bone marrow response and less effective trypanosome-specific immune response, rather than defective neutrophil trypanosome destruction, may be the problem of trypanososusceptible cattle breeds.

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