Low NADPH oxidase activity in Epstein–Barr-virus-immortalized B-lymphocytes is due to a post-transcriptional block in expression of cytochrome b_{558}

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The NADPH oxidase of phagocytes is known to be expressed in Epstein–Barr-virus-transformed B-lymphocytes, albeit at levels only approx. 5% of those found in neutrophils. We have investigated the basis of this low level of expression and find that all four specific components of the NADPH oxidase are expressed in B-lymphocytes, but only p47-*phox* protein attains levels equivalent with those found in neutrophils. This component was shown to phosphorylate and translocate to the membrane normally on activation. The other cytosolic component, p67-*phox*, did show a deficit, and by supplementing a B-cell cytosol extract with recombinant p67-*phox*, this was shown to account for the somewhat reduced activity of B-cell cytosol in a cell-free oxidase system. The cell-free analysis also clearly located the major deficiency in superoxide-generating capacity of B-lymphocytes to the membrane. Western blotting of membrane proteins revealed major reductions in the amount of cytochrome b_{558}. Analysis of the levels of mRNA for both subunits of cytochrome b_{558}, however, showed levels greater than expected. Significantly more mRNA for gp91-*phox* was present in B-cells than in undifferentiated HL60 cells, although it was not quite as abundant as in differentiated HL60 cells, which are capable of producing large amounts of superoxide. We conclude that the failure of B-lymphocytes to generate amounts of superoxide equivalent to those generated by neutrophils is primarily due to a post-transcriptionally determined block to the accumulation of cytochrome b_{558}.

INTRODUCTION

Virtually all cells have the capacity to produce superoxide anions, but only those described as the 'professional' phagocytes seem to utilize a specialized superoxide-generating system, the NADPH oxidase, to generate these highly reactive anions [1]. The NADPH oxidase is, in fact, a multicomponent electron-transfer chain capable of transferring electrons from NADPH to molecular oxygen. The components of the system consist of a membrane-bound, low-midpoint-potential cytochrome, cytochrome b_{558}[2], and two specific cytosolic factors, p47-*phox* and p67-*phox* [3,4]. The cytochrome itself has two subunits, p22-*phox* and gp91-*phox* [5,6], the latter being heavily glycosylated [7]. All the electron-transfer functions are performed by the cytochrome, which has binding sites for the NADPH substrate and FAD cofactor [8,9]. In addition, one or more molecules of haem are contained within the cytochrome, but their precise disposition is unclear; both subunits have alternatively been proposed as the haem-binding moiety [10,11]. It is possible though, that the haems are actually bound between the two cytochrome subunits. The functions of the cytosolic components are less well defined, but are assumed to have essentially a regulatory role. Despite this, the absence of either of these components, such as occurs in certain types of chronic granulomatous disease (CGD), renders the oxidase non-functional [12,13]. A third cytosolic component also essential for oxidase activity is $p21^{rac1}$ [14] or $p21^{rac2}$ [15], a small GTPase of the extended ras family. Activation of the system is accompanied by translocation of the cytosolic components to the membrane, where they interact with the cytochrome to form an active oxidase complex [16–18].

Unusually, the NADPH oxidase system would also appear to be present in certain B-cells. Subsets of tonsillar lymphocytes show evidence of oxidase activity, as demonstrated by a positive Nitroblue Tetrazolium (NBT) assay and reactivity with an anti-p22-*phox* monoclonal antibody [19]. Furthermore, B-cells immortalized by Epstein–Barr virus (EBV) similarly show evidence for oxidase activity and positive NBT results [19,20]. More recently, cell-free extracts from such cells have been shown to be capable of producing superoxide [21,22], as do similar extracts from neutrophils. Despite this, B-cells produce amounts of superoxide that are much less than those produced by myeloid cells, averaging only 5% of the activity of peripheral-blood neutrophils. It might be assumed, therefore, that the composition of the oxidase in the two cell types might differ, but evidence from CGD patients suggests that the same components are used by both cell types, as patients whose neutrophils lack a specific component also produce B-cell lines having an identical deficit [23,24]. It remains something of a puzzle, therefore, both what the precise function of the oxidase is in B-cells, especially as they are not phagocytic and can presumably only generate extracellular superoxide and, in addition, why, if all the components of the oxidase are present in these B-cells, they are only capable of producing such limited amounts of superoxide. The latter question is the one we have addressed in the present study.

Abbreviations used: NBT, Nitroblue Tetrazolium; EBV, Epstein–Barr virus; CGD, chronic granulomatous disease; PMA, phorbol 12-myristate 13-acetate.

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METHODS AND MATERIALS

Cell lines

HL60 cells and B-cell lines were cultured in RPMI medium containing 2 mM glutamine, penicillin, streptomycin and supplemented with 10% fetal-calf serum at 37 °C and 5% CO_2. For induction of HL60-cell differentiation, dimethyl sulphoxide was added to 1.25% approx. 7 days prior to cell harvest. Differentiation was monitored by acquisition of a positive NBT test. Cultures typically achieved a level of 50–60% NBT-positive cells. NBT staining was performed as described previously [13]

Cell-free superoxide

To prepare cell-free extracts capable of generating superoxide, cells were pelleted (1000 *g*, 5 min), washed twice with PBS and resuspended in ice-cold 'break buffer' [6 mM Pipes (pH 7.3)/60 mM KCl/1.8 mM NaCl/2.3 mM $MgCl_2$/6% (w/w) sucrose], containing the proteinase inhibitors di-isopropyl fluorophosphate (1 μg/ml), phenylmethanesulphonyl fluoride (1 mM) and N^{α}-*p*-tosyl-L-lysylchloromethane ('TLCK') (1 μg/ml). The cells were then lysed by sonication, the debris pelleted by centrifugation, and the supernatant separated into membrane and cytosol fractions by centrifugation on sucrose step gradients as described previously [25,26]. Superoxide generation was detected by the method of luminol-enhanced chemiluminescence [24,27] or by reduction of cytochrome *c* [25,26].

Western blotting

Western blotting of cytosolic and membrane proteins on to reinforced nitrocellulose membranes (Schleicher and Schuell) was performed by the semi-dry-transfer technique as described previously [13], using alkaline-phosphatase-conjugated goat anti-rabbit IgG (Promega) and detection with NBT/5-bromo-4-chloro-3-indolyl phosphate (Sigma) or ^{125}I-Protein A (Amersham International). All protein samples were calibrated using the Bio-Rad protein assay reagent to ensure that equal amounts of protein were loaded in each lane.

Northern blotting

RNA was prepared by guanidinium thiocyanate/acid-phenol extraction as described by Chomcynski and Sacchi [28] and separated on 4.5% formaldehyde/1.2% agarose gels [29]. The RNA was transferred to nitrocellulose membrane in 20 × SSC (1 × SSC is 0.15 M NaCl/0.015 M sodium citrate) and the blot was air-dried and baked under vacuum for 1h at 80 °C. Hybridization was performed overnight at 42 °C in standard 50% formamide hybridization solution supplemented with 10% dextran sulphate [29], with 10^6 c.p.m. of [^{32}P]cDNA/ml of hybridization solution. After hybridization, the blots were washed once at room temperature in 2 × SSC/0.1% SDS and twice for 20 min at 60 °C in 0.1 × SSC/0.1% SDS. The blots were then autoradiographed for 24–72 h at −70 °C using Hyperfilm MP (Amersham International) with intensifying screens.

RESULTS

Cell-free superoxide generation

Cell-free superoxide generation by cytosol and membrane extracts prepared from B-cell lines and neutrophils was compared for different combinations of membrane and cytosol. Using a chemiluminescence assay, B-cell cytosol and neutrophil cytosol were both shown to be capable of stimulating superoxide production in combination with neutrophil membranes (Figure 1a), whereas B-cell membranes, even when combined with neutrophil cytosol, were not detectably different in superoxide generation from neutrophil cytosol alone (results not shown). This suggested that the cytosolic components of the B-cell oxidase were nearly as efficient as those in neutrophil cytosol. A shortfall in the activity of B-cell cytosol compared with neutrophil cytosol was still present, however, and to investigate this we supplemented the B-cell cytosol extract with recombinant NADPH oxidase proteins prepared in bacteria. Addition of p21^{rac1} protein or p47-*phox* had no effect on the activity of the B-cell cytosol extract. Addition of p67-*phox*, though, did stimulate superoxide production compared with untreated B-cell cytosol (Figure 1b), giving activities comparable with those seen for neutrophil cytosol. p67-*phox* would seem, therefore, to be the limiting component of the oxidase in B-cell cytosol.

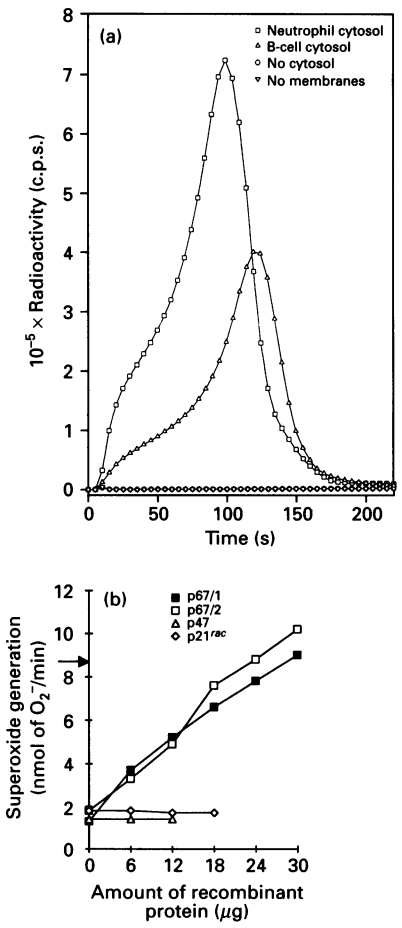

Figure 1 Addition of recombinant cytosolic factors to the cell-free-oxidase assay

Cell-free B-cell cytosol and neutrophil membrane fractions were mixed in the presence of an optimal concentration of SDS and the resulting superoxide generation measured by (**a**), luminol-enhanced chemiluminescence or (**b**) spectrophotometric estimation of reduced cytochrome *c*. The arrow on the *y*-axis indicates the level of superoxide generated using normal neutrophil cytosol and membranes. p67/1 and p67/2 represent the results from two independent experiments adding recombinant p67-*phox* to two different B-cell cytosol preparations.

Expression of oxidase protein components

This pattern was confirmed on a Western-blot analysis of NADPH oxidase components. Cytosol and membranes from

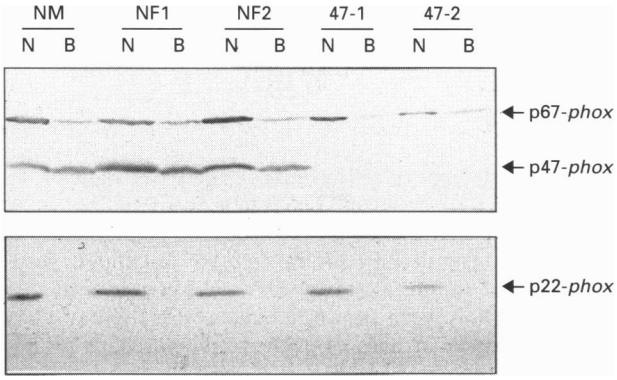

Figure 2 Western blots of neutrophils and equivalent B-cell lines

Postnuclear supernatants were prepared from neutrophils and B-cell lines; 50 μg of protein was applied to each lane of a 10%-polyacrylamide/SDS gel and, after electrophoresis, the proteins were transferred to a nitrocellulose membrane. The membrane was allowed to react with antisera to the cytosolic oxidase components (upper panel.) or to anti p22-*phox* (lower panel). Lanes NM, cells from normal male subject; lanes NF1 and NF2, cells from two normal female subjects; lanes 47-1 and 47-2, cells from two p47-*phox*-deficient patients; lanes N, neutrophils; lanes B, B-cell lines.

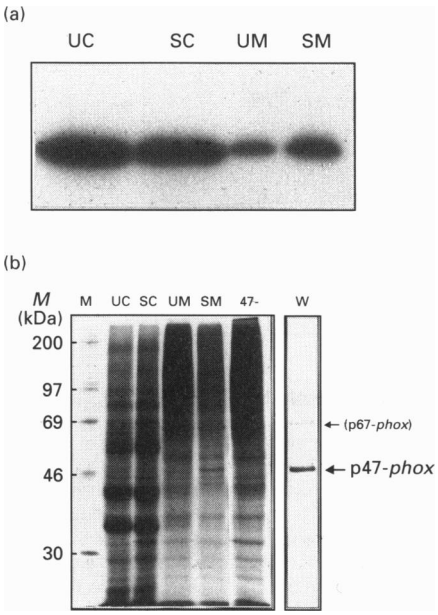

Figure 3 Translocation and phosphorylation of p47-*phox* in intact B-cells

(**a**) Translocation of p47-*phox* from cytosol to membranes. Unstimulated, and PMA-stimulated B-cells were separated into cytosol and membrane fractions run on SDS/PAGE and Western-blotted. The blots were then allowed to react with antiserum to p47-*phox* and bands were revealed with ^{125}I-Protein A. Abbreviations: UC, unstimulated cytosol; SC, stimulated cytosol; UM, unstimulated membranes; SM, stimulated membranes. (**b**) Phosphorylation of p47-*phox* in activated B-cells. B-cells were incubated with [^{32}P]P_i to metabolically label the proteins. Cytosolic and membrane fractions were separated, and proteins were separated by SDS/PAGE. The gel was dried and subjected to autoradiography. Stimulated cells were treated with PMA (1 mM) for 10 min at 37 °C prior to subcellular fractionation. Lane M, protein standard molecular-mass (*M*) markers; lane UC, unstimulated cytosol; lane SC, stimulated cytosol; lane UM, unstimulated membranes; lane SM, stimulated membranes; lane 47-, stimulated membranes from p47-*phox*-deficient B-cell line; lane W, stimulated membranes from normal B-cell line Western-blotted with anti-p47-*phox* antiserum.

neutrophils and the corresponding B-cell lines were separated by SDS/10%-PAGE, transferred to nitrocellulose membrane and allowed to react with antisera to various of the components of the oxidase (Figure 2). The two cytosolic components, p47-*phox* and p67-*phox* (upper panel), were both easily detected. The levels of p47-*phox* in neutrophils and the equivalent B-cell line were very similar. A similar pattern was observed for p67-*phox*, but, as expected from the cell-free-superoxide-generation results, somewhat reduced quantities of p67-*phox* relative to the myeloid cells was observed. Significantly though, when the same samples were allowed to react with anti-p22-*phox* (Figure 2, lower panel), a major discrepancy in the amounts present in neutrophils and B-cell lines was apparent.

Although p47-*phox* was present in B-cells in normal quantities, in neutrophils it is known also to become phosphorylated [30,31] and to translocate to the plasma membrane [16,17,30,32] on activation of the oxidase. We investigated these functions of p47-*phox* in intact B-cells, since in the cell-free system these processes appear to be regulated differently [33]. To assay translocation of p47-*phox*, cells were activated with phorbol 12-myristate 13-acetate (PMA), separated into membrane and cytosol fractions and Western-blotted on to nitrocellulose. Membranes from stimulated cells showed a significant increase in the amount of p47-*phox* (Figure 3a, lane SM). Although a concomitant decrease in the cytosolic level (lane SC) occurs, it is more difficult to reveal, since only a small fraction of the total p47-*phox* translocates from the cytosol to the membrane. To determine whether p47-*phox* was phosphorylated normally, cells were labelled with [^{32}P]P_i and the radiolabelled proteins separated by SDS/PAGE and detected by autoradiography (Figure 3b). On activation of B-cells, a new phosphorylated band is clearly visible in the membrane fraction (lane SM) that was not present in either cytosol (UC, SC) or in unstimulated membranes (UM). Moreover, this band was not observed in stimulated membranes from a B-cell line established from a p47-*phox*-deficient CGD patient ('47-'). Western blotting of normal stimulated B-cell membranes also showed that this new phosphorylated band co-migrated with anti-p47-*phox*-reactive material (lane W). This analysis indicated that p47-*phox* translocated normally to the membrane on activation and became phosphorylated. One-dimensional SDS/PAGE, however, is not capable of determining whether the specific pattern of phosphorylated residues on p47-*phox* are the same as those found in neutrophils.

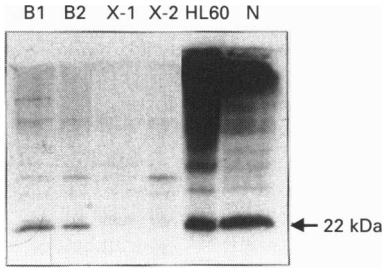

Figure 4 Western blotting of cytochrome b_{558} components

B-cell membranes were isolated and the proteins (30 μg/track) separated by SDS/PAGE followed by transfer to nitrocellulose. The blot was allowed to react with antiserum to the p22-*phox* subunit of cytochrome b_{558}. Lanes B1 and B2, normal B-cell lines; lanes X-1 and X-2, X-chromosome-linked CGD B-cell lines; lane HL60, induced HL60 cells; lane N, neutrophils

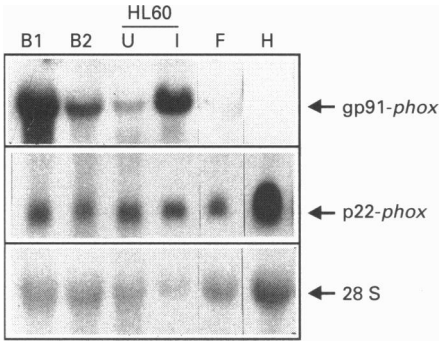

Figure 5 Northern-blot analysis of cytochrome b_{558} mRNA

Total RNA was extracted from cell lines as indicated, and 20 μg of each separated on a 1.2%-agarose/4.5%-formaldehyde gel. The RNA was transferred to nitrocellulose membrane by capillary transfer and the resulting blot hybridized sequentially with probes for gp91-*phox* (upper panel) or p22-*phox* (middle panel) or, as a control for relative amounts of RNA in the different lanes, with a probe for 28 S ribosomal RNA (lower panel). Lanes B1and B2, RNA from two B-cell lines; lanes HL60 U and HL60 I, RNA from undifferentiated and differentiated HL60 cells respectively; lane F, RNA from MC5 human fibroblast line; lane H, RNA from HeLa cells.

In order to estimate the amount of cytochrome b_{558}, Western-blot analysis on B-cell membranes was performed. As the heavy glycosylation of gp91-*phox* makes quantification difficult, we used a polyclonal antibody raised against recombinant p22-*phox*. It has been well established previously that both subunits are mutually dependent for their stability [34–38] and so are present in equal amounts. The Western-blot analysis showed a striking reduction in the amount of cytochrome b_{558} in comparison with neutrophils and induced HL60 cells (Figure 4). Although reduced in amount, the level of cytochrome protein in normal B-cell lines (B1 and B2) is distinct from that of X-chromosome-linked CGD patients (X-1 and X-2), who have no detectable cytochrome. The finding of a significantly lower quantity of cytochrome protein in B-cell lines by comparison with myeloid cells led us to investigate the mRNA levels for the two cytochrome subunits.

Expression of mRNAs encoding cytochrome subunits

Northern-blot analysis of B-cell RNA (Figure 5) showed that α-subunit (p22-*phox*) mRNA was expressed at equivalent levels in both B-cell lines (B1 and B2) and in other cell types, such as the myeloid cell line HL60 (HL60U and HL60I), as well as in fibroblasts (F). Interestingly, HeLa-cell RNA seems to express p22-*phox* mRNA very abundantly. This general pattern of expression is unsurprising, since the regulation of p22-*phox* is thought to be controlled post-translationally by its interaction with the β-subunit protein (gp91-*phox*) [37–39]. What was more unexpected was the finding that substantial levels of mRNA for gp91-*phox* were detected in B-cell lines (Figure 5, upper panel). The level of gp91-*phox* mRNA in B-cell lines (B1 and B2) was greater than in uninduced HL60 cells (HL60 U), though not as great as seen in induced HL60 cells (HL60 I). Tissue-specific expression of gp91-*phox* mRNA was indicated by its absence from a human fibroblast line and from HeLa cells. Clearly a significant discrepancy exists between the amount of gp91-*phox* mRNA that was present in B-cell lines and the amount of cytochrome b_{558} protein that they synthesize. We concluded from this analysis that the factor limiting the amount of cytochrome produced by the B-cell lines, and therefore, the major constraint on the amount of superoxide they are capable of generating, is a post-transcriptionally determined block to the synthesis of cytochrome b_{558}.

DISCUSSION

Although many types of cells produce superoxide radicals, only the 'professional' phagocytes appear to use a specialized enzyme system, the NADPH oxidase, to generate superoxide. One exception to this rule are certain types of B-lymphocytes, in particular those immortalized by EBV. The components of the B-cell oxidase have been shown to equate with those of phagocytes by a number of lines of evidence, but perhaps the most compelling is the observation that B-cell lines developed from patients with CGD also lack the same oxidase component as their phagocytic cells.

Despite this, the amounts of superoxide that B-cells are capable of generating are much lower than phagocytes at around 5% the level of production of a neutrophil, as assayed by PMA-dependent oxidase activation. We have demonstrated in the present study that EBV-transformed B-cells have, essentially, normal amounts of the two cytosolic factors of the oxidase and they appear to function normally *in vitro* in a cell-free-oxidase system. This is in accordance with the findings of Morel and co-workers [21,22]. Interestingly though, p67-*phox* is the limiting component of B-cell cytosol and this has also been found to be the case for HL60 cells, where the accumulation of p67-*phox* during induced differentiation determines the activity of the oxidase [40].

Furthermore, on activation of the B-cell oxidase *in vivo*, p47-*phox* becomes phosphorylated and translocates from cytosol to membrane in a normal fashion, with appropriate kinetics. This is somewhat unexpected, as p47-*phox* has been shown to require cytochrome b_{558} for translocation to occur [16,18]. As the amount of cytochrome b_{558} is significantly reduced in B-cells, it would be logical to assume that there would be a concomitant reduction in the amount of membrane-associated p47-*phox* in activated B-cells. That quite appreciable translocation of p47-*phox* does take place raises the possibility that there may be other membrane-associated targets to which it is capable of binding. In this context it is apparent that some cells (B-lymphoma lines for example) express p47-*phox* in the absence of any of the other NADPH oxidase components. Whether this has any physiological significance or indicates that p47-*phox* has a role in an alternative cellular pathway is unclear at present.

The small GTPases $p21^{rac1}$ or $p21^{rac2}$ are essential components of the activated oxidase complex. An attractive hypothesis, therefore, is that B-cells are limiting for $p21^{rac}$ and that this limits the rate of oxidase activation. This is especially so because B-cells appear to lack $p21^{rac2}$ (A. Abo, unpublished work), which is abundant in neutrophils. However, the addition of exogenous recombinant $p21^{rac1}$ to the cell-free oxidase system was unable to boost superoxide generation, even in the presence of neutrophil membranes. Furthermore, B-cell cytosol already functions almost equivalently to neutrophil cytosol in this assay. The stoichiometry of the oxidase components would imply that there is less than one molecule of *rac* per active oxidase complex and that $p21^{rac}$ is therefore recycled from activated oxidase complexes, but is not continuously required.

In contrast with the cytosolic compartment, the components of the cytochrome appear to tell a different story. Western-blot analysis shows that, compared with myeloid cells, cytochrome b_{558} is present in greatly reduced amounts in B-cells, and *in vitro* oxidase analysis points to the membrane as the site of the deficiency in superoxide generation.

Expression of the gene for p22-*phox*, the smaller subunit of cytochrome b_{558}, is unusual as the mRNA seems to be present ubiquitously and is regulated at the level of protein by its association with gp91-*phox*, in the absence of which it is unstable. By contrast, the gp91-*phox* gene is regulated transcriptionally and its mRNA accumulates significantly on differentiation in HL60 cells. Unexpectedly, we have found that mRNA levels for gp91-*phox* in B-cells are comparable with those found in myeloid cells, a finding normally consistent with a much greater level of cytochrome synthesis. Differentiated HL60 cells, for example, produce amounts of superoxide similar to those generated by neutrophils. This indicates that a post-transcriptional block to the accumulation of cytochrome protein is present in B-cells. The nature of this block is unclear, but one interesting explanation would be limited availability of haem. It has been shown elsewhere that blocking haem synthesis in HL60 cells interferes with the accumulation of cytochrome b_{558} [41]. Additionally, in trying to produce recombinant cytochrome b_{558} in insect cells using the baculovirus system, it was found that exogenous haem was necessary to obtain significant amounts of functional cytochrome [42].

The question still remains as to the function of the oxidase in these cells, as they are not phagocytic, and it is unlikely that they produce sufficient superoxide for it to be microbicidal. One possible explanation for the presence of the oxidase in B-cells is that it has a role in antigen presentation, particularly of intracellular proteins, which are presented in the form of short peptides in combination with Class 1 HLA molecules. This possibility is currently under investigation in our laboratory.

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