Immunology 1990 **71** 213–217

Superoxide generation by EBV-transformed B lymphocytes. Activation by IL-1β, TNF-α and receptor independent stimuli

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Accepted for publication 25 June 1990

SUMMARY

The generation of superoxide by Epstein–Barr virus (EBV)-transformed human B lymphocytes can be stimulated by a range of compounds; receptor-dependent stimuli include tumour necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β) and lipopolysaccharides (LPS), and independent stimuli include AlF_3, A21387 and ionomycin. The stimuli suggest that the activation pathway for the lymphocyte oxidase is similar to that proposed for the neutrophil oxidase. Although the rate of superoxide production was lower than that by neutrophils, the respiratory burst was much prolonged. It is possible that this superoxide generation by lymphocytes may have a biological function.

INTRODUCTION

The production of superoxide ($O_2^{\cdot -}$) by phagocytic leucocytes is a component of their microbicidal and tumouricidal activities (Badwey & Karnovsky, 1980). The $O_2^{\cdot -}$ is generated by a NADPH oxidase present in the plasma membrane of these cells (Rossi, 1986). The oxidase is normally quiescent, being activated by bacterial peptides, opsonized bacteria and a number of compounds. It is now apparent that this activity is not restricted to phagocytes. Epstein–Barr virus (EBV)-transformed B lymphocytes have been shown to produce $O_2^{\cdot -}$ in response to phorbol-12-myristate-13-acetate (PMA), a soluble activator of the leucocyte oxidase (Volkman *et al.*, 1984; Melinn & McLaughlin, 1987; Maly *et al.*, 1988; Hancock, Maly & Jones, 1989). There is some evidence that this $O_2^{\cdot -}$-generating activity is present in non-transformed B lymphocytes isolated from tonsils (Maly *et al.*, 1989) and also in a variety of cell types, including fibroblasts (Meier *et al.*, 1989), platelets (Marcus, 1979) and endothelial cells (Matsubara & Ziff, 1986).

It has previously been shown that there are similarities in the components, the cytochrome *b*-245 and the suggested flavoprotein (Yea, Cross & Jones, 1990), between the neutrophil and lymphocyte oxidase (Maly *et al.*, 1988). In this paper we have studied the ability of a range of compounds to activate or inhibit the generation of $O_2^{\cdot -}$ by EBV-transformed B lymphocytes. We suggest that the signal transduction pathway is similar to that proposed for the neutrophil oxidase (Badwey & Karnovsky, 1986).

Abbreviations: DPI, diphenylene iodonium; EBV, Epstein–Barr virus; GTP, guanosine triphosphate; HAGG, heat-aggregated immunoglobulin; IL, interleukin; LPS, lipopolysaccharide; PMA, phorbol-12-myristate-13-acetate; TNF, tumour necrosis factor.

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MATERIALS AND METHODS

Materials

The RPMI-1640 medium was obtained from Gibco, Uxbridge, Middlesex, U.K. and the foetal calf serum (FCS) from Northumbria Biological, Cramlington, Northumberland, U.K. The antibiotics penicillin and streptomycin were obtained from Flow Laboratories, Rickmansworth, Herts, U.K. Amphotericin B, gentamycin and Nutridoma-SP were obtained from Boehringer Mannheim, Lewes, East Sussex, U.K. Staurosporine was obtained from Calbiochem, Nottingham, U.K.; a stock solution of 2 mM was prepared in 20% DMSO, 80% ethanol and diluted prior to use. Horse heart cytochrome c (type III) and superoxide dismutase were obtained from Sigma, Poole, Dorset, U.K.

Cell line

The EBV-transformed B lymphocytes were a kind gift from Mrs S. Finerty, Dept. of Pathology, University of Bristol (Moss, Rickinson & Pope, 1978; Finerty *et al.*, 1982). The cell line was maintained on RPMI-1640 supplemented with 10% FCS, 100 IU/ml penicillin, 100 μg/ml streptomycin, 2·5 μg/ml amphotericin B and 100 μg/ml gentamycin. The lymphocytes for the lymphokine concentration dependence studies were grown on RPMI-1640 medium supplemented with Nutridoma-SP and so were free of FCS factors. The cells were harvested from the culture flasks by centrifugation at 400 ***g***, for 10 min, at 4°. The cells were washed in Krebs–Ringer buffer, pH 7·4, to remove remaining traces of phenol red, pelleted at 400 ***g*** for 10 min, resuspended in Krebs–Ringer buffer and stored on ice.

Determination of superoxide generation

Superoxide generation was determined as the superoxide dismutase (100 μg/ml) inhibitable reduction of 100 μM horse heart cytochrome c. The rate of reduction was monitored using a dual-wavelength spectrophotometer (550 nm–540 nm) at 37°, in Krebs–Ringer buffer (Cross *et al.*, 1982). All experiments were repeated a minimum of three times.

Consumption of oxygen

The rate of oxygen consumption was monitored in Krebs–Ringer buffer, at 37°, using a Clark oxygen electrode.

Protein determination

The protein concentrations were determined using the Bradford assay (Bradford, 1976) with bovine serum albumin (BSA) as the standard.

RESULTS

An outline of the signal transduction pathway for the neutrophil oxidase is shown in Fig. 1. It is considered to contain specific receptors linked to a phosphatidylinositol-specific phospholipase C (PLC) via a GTP-binding protein. PLC generates inositol (1,4,5) triphosphate (IP_3) and diacylglycerol (DAG) from phosphatidyl inositol 4,5 bisphosphate (PIP_2). DAG and the Ca^{2+} released by IP_3 activate protein kinase C (PKC) (Badwey & Karnovsky, 1986). The kinase phosphorylates a number of proteins (Gennaro, Florio & Romeo, 1986), which may directly result in the activation of the oxidase.

In order to be able to study the activation pathway for the EBV-transformed B lymphocyte oxidase, we investigated the ability of a range of compounds to stimulate $O_2^{\cdot-}$ generation. Table 1 lists the compounds which were found to activate the lymphocyte oxidase and those which did not.

Activation of $O_2^{\cdot-}$ generation by TNF-α and IL-1β were both found to be concentration dependent (Fig. 2). Both response curves were sigmoidal, as was that for PMA (Fig. 2, insert). For these experiments the lymphocytes were grown on a serum-free medium to eliminate background levels of the cytokines. The EC_{50} values for activation of the oxidase by PMA, TNF-α and IL-1β were 5×10^{-8} M, 140 ng/ml and 130 ng/ml, respectively. The sensitivity to PMA was similar to that for neutrophils. However, higher concentrations of both TNFα and IL-1β were required to stimulate the oxidase than to promote their cytokine effects.

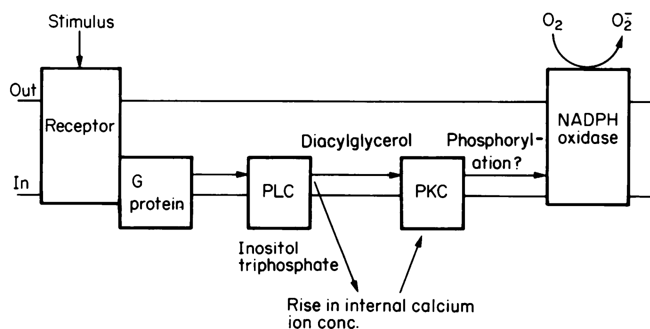

Figure 1. Diagram of the proposed activation pathway for the NADPH oxidase of neutrophils. Receptor-dependent stimuli acting upon their specific receptor activate a GTP-binding protein. This activates a phosphatidylinositol-specific PLC-generated release of IP_3. The resulting rise in internal Ca^{2+} with diacylglycerol activates PKC. Activation may result from a direct phosphorylation of the NADPH oxidase.

The cytokines TNF-α and IL-1β are reported to regulate proliferation and maturation of B lymphocytes acting through a specific plasma membrane receptor (De Franco, 1987). The IL-1

Table 1. Stimulators of superoxide generation by EBV-transformed B lymphocytes

Stimulus	Superoxide production nmol/min/mg protein
Receptor mediated	
TNF-α (250 ng/ml)	1·2
IL-1β (150 ng/ml)	1·2
Protein A (pansorbin) (10 mg/ml)	1·7
Heat-aggregated Ig (1·5 mg/ml)	0·77
Serum opsonized zymosan (1·5 mg/ml)	1·25
LPS (Re mutant) (10–20 μg/ml)	
Salmonella minnesota Re595	0·75
Escherichia coli EH100	0·75
Shigella flexnen	0·75
Receptor independent	
PMA (50 nM)	3–5
AlF_3^- (10 μM $AlCl_3$, 25 mM NaF)	1·7
A23187 (10 μM)	1·1
Ionomycin (2 μM)	0·43
Ineffective as activators	
FMLP (5 μM)	
IL-2 ($2·5 \times 10^3$ U/ml)	
IL-4 (500 U/ml)	
IL-1α (125 ng/ml)	
INF-α ($8·5 \times 10^3$ U/ml)	
INF-γ (3×10^3 U/ml)	

Superoxide generation was monitored as described in the Materials and Methods section. A variety of different stimuli was found to activate superoxide generation at the final concentrations shown in the table. A number of compounds which were unable to stimulate the oxidase are also listed.

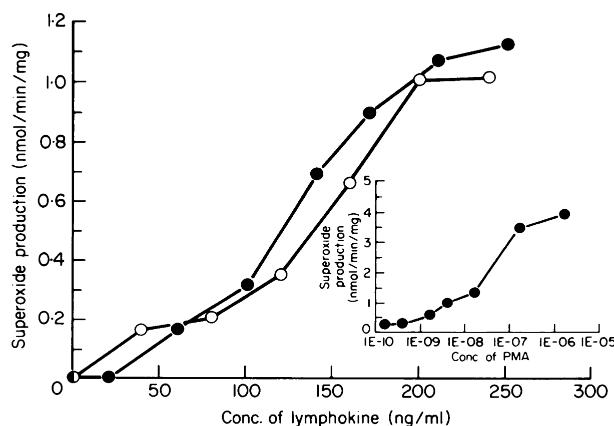

Figure 2. Concentration dependence of the stimulation of superoxide generation by EBV-transformed B-lymphocytes. Superoxide generation by the lymphocytes was measured as described in the Materials and Methods section, following the addition of varying concentrations of TNF-α (○) and IL-1β (●) and PMA (insert).

Table 2. Priming of the PMA-stimulated oxidase activity by ionomycin HAGG and pansorbin.

Treatment	Rate of superoxide (nmol/mg protein/min)	% PMA rate
Cells + PMA (1·6 μM)	4·71	100
Cells + ionomycin (1 nM) for 2 min then + PMA (1·6 μM)	6·92	147
Cells + PMA (1·6 μM)	3·05	100
Cells + 0·15 mg/ml HAGG (2 min) then + PMA (1·6 μM)	4·15	136
Cells + 0·45 mg/ml HAGG (2 min) then + PMA (1·6 μM)	4·38	144
Cells + 2 mg/ml pansorbin (2 min) then + PMA (1·6 μM)	4·1	134
Cells + 6 mg/ml pansorbin (2 min) then + PMA (1·6 μM)	5·0	163

The generation of superoxide was monitored as described in the Materials and Methods section. Sub-stimulatory concentrations of each of the stimuli (given in the table) were added 2 min prior to the addition of 1·6 PMA. The rate is compared with that for 1·6 M PMA alone.

receptor has been shown to be present on EBV-transformed B lymphocytes (Matsushima *et al.*, 1986), although the B-cell receptor differs from that present on T cells, fibroblasts and macrophages (Tanaka *et al.*, 1989; Chizzonite *et al.*, 1989; Scapigliati *et al.*, 1989). The B-cell receptor has been shown to have a higher affinity for IL-1β than IL-1α (Scapigliati *et al.*, 1989), and only the IL-1β response is coupled to PKC (Bomsztyk *et al.*, 1989). Therefore the lower affinity and inability to stimulate PKC may explain why IL-1α failed to stimulate $O_2^{\cdot-}$ generation. It is interesting that two molecules acting through the same receptor can stimulate different responses.

The ability of pansorbin (protein A-bearing staphylococci) to stimulate the oxidase agrees with that previously published and with the ability of anti-IgG to stimulate $O_2^{\cdot-}$ generation in B-cell lines (Maly *et al.*, 1988). It is therefore possible that the activation by pansorbin may result from its ability to cross-link lymphocyte surface immunoglobulins. Both heat-aggregated immunoglobulins (HAGG) and zymosan, opsonized with human serum, activated $O_2^{\cdot-}$, suggesting that the Fc receptor and possibly the complement factor receptors are also linked to the activation pathway.

Bacterial lipopolysaccharides (endotoxins) from Re mutants have been found to contain less carbohydrate and to stimulate at much lower concentrations than the native LPS (Brade, Brade & Reitschel, 1988). LPS have been shown to be capable of causing the proliferation and differentiation of B cells (Anderson *et al.*, 1977). The biologically active component of LPS is the lipid A. As a number of detergents and fatty acids (particularly arachidonic acid) have been shown to be activators of $O_2^{\cdot-}$ generation in neutrophils (Bellavite, 1987), it is at present uncertain whether the LPSs activate $O_2^{\cdot-}$ generation following binding to a specific plasma membrane receptor or as a result of a non-specific perturbation of the membrane.

Aluminium fluoride is considered to activate the neutrophil oxidase by acting through the GTP-binding protein (Gilman, 1984). Therefore the ability of AlF_3 to stimulate the $O_2^{\cdot-}$ generation by EBV-transformed B lymphocytes suggests that the activation pathway may contain a similar G protein. However, this requires confirmation.

The stimulation by PMA agrees with results previously published (Volkman *et al.*, 1984; Melinn & McLaughlin, 1987; Maly *et al.*, 1988; Hancock *et al.*, 1989) and suggests that protein phosphorylation by PKC is involved in the activation of the lymphocyte oxidase. Interestingly, kidney mesangial cells produce $O_2^{\cdot-}$ in response to stimulation by IL-1α and TNF-α but not PMA (Radeke *et al.*, 1990), suggesting that activation pathways vary between different cell types.

It has been reported previously that A23187 does not stimulate the lymphocyte oxidase (Volkman *et al.*, 1984). However, in contrast with this report, we have found that both A23187 and ionomycin, in the presence of external Ca^{2+}, stimulated $O_2^{\cdot-}$ generation. Therefore Ca^{2+} may be involved in the activation pathway (Sullivan *et al.*, 1989). Cells contain a number of Ca^{2+}-sensitive enzymes, including PLC and PKC, which are involved in neutrophil NADPH oxidase activation. The site(s) of action of Ca^{2+} in EBV-transformed B lymphocytes is at present unknown.

A number of receptor-linked stimuli failed to activate the lymphocyte oxidase (Table 1). The receptors for these compounds are either not linked to the activation of the oxidase or are not present on the membrane of the cells. These results suggest that the response to both TNF-α and IL-1β is a specific activation.

Priming

The ability of a submaximal concentration of an activator to stimuate $O_2^{\cdot-}$ generation has been shown to be greatly enhanced by preincubation of the neutrophils with a substimulatory concentration of a stimulus (Bender, McPhail & Van Epps, 1983). As this may be a property of the activation pathway we sought evidence for priming in EBV-transformed B lymphocytes. Ionomycin, HAGG and Pansorbin were each found to enhance the ability of PMA to stimulate $O_2^{\cdot-}$ generation by B lymphocytes (Table 2). Not only were the final rates of $O_2^{\cdot-}$ generation enhanced, but the lag period in achieving that rate was much diminished. The ability of HAGG and pansorbin to enhance the PMA response was found to be concentration dependent.

Inhibitors of superoxide generation

The two compounds which were found to inhibit $O_2^{\cdot-}$ generation by the EBV-transformed B lymphocytes are shown in Table 3. The ability of DPI to inhibit $O_2^{\cdot-}$ generation agrees with previously published results (Cross & Jones, 1986; Hancock & Jones, 1987; Maly *et al.*, 1988). As all activators are sensitive to DPI it is likely that they all stimulate the same oxidase.

Staurosporine is a microbial alkaloid that has anti-fungal activity. It has been reported to be a potent inhibitor of PKC, having an IC_{50} 2·7 nM for the soluble enzyme from rat brain (Tamaoki *et al.*, 1986). As predicted from our proposed involvement of PKC, staurosporine inhibited $O_2^{\cdot-}$ generation stimulated by a number of compounds. This suggests that the activation pathways for these stimuli must all involve PKC.

The inability of CN^- to inhibit the $O_2^{\cdot-}$ generation stimulated by PMA confirms that the response is independent of the mitochondrial respiratory chain (Sbarra & Karnovsky, 1959).

Table 3. Inhibition of superoxide generation by EBV-transformed B lymphocytes

Compound tested	Stimulus	Effect
Staurosporine (1 μM)	PMA (50 nM)	100% inhibition
	LPS (10 μg/ml)	90% inhibition
	IL-1β (250 μg/ml)	96% inhibition
	Heat-aggregated Ig (0·75 mg/ml)	87% inhibition
	Serum opsonized zymosan (1·5 mg/ml)	100% inhibition
	AlF_3 (10 μM $AlCl_3$ 25 mM NaF)	85% inhibition
Diphenylene iodonium (10 μM)	All stimuli	>90% inhibition
Cyanide (1 mM)	PMA	No inhibition

The generation of superoxide was monitored as described in the Materials and Methods section. The inhibitors and stimulators tested were added at the final concentrations given in the table.

Time course respiratory burst

The rate of $O_2^{\cdot-}$ generation by lymphocytes stimulated with PMA, LPS of AlF_3 was maximal approximately 10 min after stimulation. The lymphocytes continued to generate $O_2^{\cdot-}$ for more than 60 min after stimulation, although the rate had started to decline (not shown). This is in contrast to the respiratory burst of neutrophils, which terminates 15–20 min after stimulation (Curnutte & Babior, 1974). The prolonged respiratory burst suggests that there are differences in the down-regulation/termination of the respiratory burst between lymphocytes and neutrophils.

Activation pathway

The results we have presented in this paper suggest that stimulation of the lymphocyte oxidase involves a GTP-binding protein, Ca^{2+} and PKC. It is therefore possible that the signal transduction pathway is the same as that proposed for the neutrophil oxidase (Fig. 1) (Badwey & Karnovsky, 1986). The results also suggest that a number of specific receptors are linked to the activation pathway.

CONCLUSIONS

There are strong similarities in both the activation and inhibition characteristics of the oxidase expressed by EBV-transformed B lymphocytes and that found in neutrophils. The rate of $O_2^{\cdot-}$ generation by lymphocytes is lower than that of neutrophils. However, the respiratory burst of lymphocytes was found to be much more prolonged compared with that of neutrophils.

The reduced pyridine nucleotide requirement for the generation of $O_2^{\cdot-}$ by a number of other B-cell lines has been investigated previously (Hancock *et al.*, 1989). In disrupted cells the oxidase activity was found to be dependent upon the addition of either NADH or NADPH. The K_m (Michaelis constant) for NADPH was found to be lower than that for NADH in the cell lines studied. This was similar to the pattern determined for the neutrophil oxidase, although the actual K_m values in lymphocytes and neutrophils differed (Cross, Parkinson & Jones, 1984; Rossi, 1986; Hancock *et al.*, 1989). The similarities suggest that the lymphocyte oxidase may also be an NADPH-dependent oxidase.

Endothelial cell-derived relaxing factor (EDRF), which promotes vasodilation, has recently been shown to be nitric oxide (NO), a radical (Marletta, 1989; Palmer, Ashton & Moncada, 1988). NO is considered to stimulate vasodilation by activating guanylate cyclase, by liganding to its haem group, resulting in a rise in the intracellular concentration of cGMP. The capacity to generate $O_2^{\cdot-}$ is an important contributor to the bactericidal activity of neutrophils and other phagocytes. At the present time the function of the $O_2^{\cdot-}$ generated by the B lymphocytes is uncertain. It has been reported that oxygen radicals stimulate fibroblast proliferation (Murrell, Francis & Bromley, 1989) and anti-oxidants inhibit the proliferation of T lymphocytes (Chaudhri *et al.*, 1986, 1988). It is apparent that the capacity to produce $O_2^{\cdot-}$ is present in a variety of cell types, including fibroblasts (Meier *et al.*, 1989), mesangial cells (Alder *et al.*, 1986), platelets (Marcus, 1979) and endothelial cells (Matsuban & Ziff, 1986). It is therefore possible that, like nitric oxide (Marletta, 1989), $O_2^{\cdot-}$ may act as a secondary messenger, possibly initiating proliferation or transformation (Saran & Bors, 1989).

ACKNOWLEDGMENTS

The work was supported by grants from the Medical Research Council and the Wellcome Trust. The authors wish to thank Mrs S. Finerty (University of Bristol) for the original gift of the B lymphocytes, Dr R. Rees, University of Sheffield Medical School, for the kind gift of IL-2 and IL-4, and Dr C. Elson, University of Bristol, for the TNF-α.

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