Biochem. J. (1989) 262, 373–375 (Printed in Great Britain)

Properties of the superoxide-generating oxidase of B-lymphocyte cell lines

Determination of Michaelis parameters

John T. HANCOCK,* Friedrich-Ernst MALY† and Owen T. G. JONES*‡

*Department of Biochemistry, University of Bristol Medical School, University Walk, Bristol BS8 1TD, U.K., and †Institute of Clinical Immunology, Inselspital Bern, CH-3010 Bern, Switzerland

The capacity of three B-lymphocyte cell lines to generate superoxide ($O_2^{\cdot-}$) was examined. The Burkitt lymphoma lines P.3HR-1 and Jijoye gave no response to phorbol 12-myristate 13-acetate (PMA) at 100 ng/ml but produced up to 0.35 nmol of $O_2^{\cdot-}$/min per mg of protein when stimulated with 5 μg of PMA/ml; the cell line RPMI 1788 produced Nitro Blue Tetrazolium-positive responses to low PMA concentrations and approx. 0.4 nmol of $O_2^{\cdot-}$/min per mg of protein at 5 μg of PMA/ml. Each cell line contained approx. 10 pmol of low-potential cytochrome *b* (cytochrome b_{-245})/mg of protein. Homogenates of PMA-activated cells gave 10–20-fold greater rates of $O_2^{\cdot-}$ produced per mg of protein. The K_m for NADPH varied between approx. 250 μM for P3.HR-1 and RPMI 1788 cell lines and 30.5 ± 6.5 μM for the Jijoye cell line; the K_m values for NADH were higher. Determination of intracellular NADPH concentration showed that this might limit the rate of $O_2^{\cdot-}$ production since in each cell line it was at or below the K_m concentration.

INTRODUCTION

Phagocytic leucocytes (neutrophils, macrophages and eosinophils) generate superoxide ($O_2^{\cdot-}$) when stimulated with opsonized particles [1] or with a variety of soluble stimuli such as phorbol 12-myristate 13-acetate (PMA) or bacterial peptides. $O_2^{\cdot-}$ and derivatives formed from it contribute to the microbicidal activities of these leucocytes [2]. The phagocyte plasma membrane contains an oxidase complex that transfers electrons donated by reduced nicotinamide nucleotide across the cell membrane and reduces O_2 on the outer face of the plasma membrane [3]. The preferred electron donor to the oxidase is NADPH (K_m about 50 μM) rather than NADH (K_m about 500 μM), and the oxidase complex is believed to contain FAD and a low-potential cytochrome *b* ($E_{m,7} = -245$ mV) arranged in the following sequence [4]:

$$\text{NADPH} \xrightarrow{e^-} \text{FAD protein} \xrightarrow{e^-} \text{cytochrome } b \xrightarrow{e^-} O_2$$

Some Epstein–Barr-virus-transformed B-lymphocytes have the capacity to produce $O_2^{\cdot-}$ when stimulated with PMA [5]. We have shown [6] that such active lymphocytes contain a cytochrome *b* with a mid-point potential of -245 mV and a protein of molecular mass 45 kDa, believed to be the flavoprotein, which binds the NADPH oxidase inhibitor diphenyleneiodonium (DPI); both these components are characteristic of NADPH oxidase of neutrophils [7] and macrophages [8]. Recently we have described [9] $O_2^{\cdot-}$ generation by tonsillar B-cells and found that they contained cytochrome b_{-245}, detected by using an antibody to the small subunit of cytochrome b_{-245} [10] and Northern-blot analysis for the mRNA of the cytochrome *b* β-chain [11]. The oxidase was sensitive to inhibition by DPI.

It thus appeared that B-lymphocytes could assemble a $O_2^{\cdot-}$ generating oxidase with characteristics very similar to those of the NADPH oxidase of phagocytes. However, we show in the present paper that the oxidase from different B-lymphocyte cell lines varied in affinity for nicotinamide nucleotides. In two out of three lines tested, the K_m for NADPH was over 300 μM, much higher than found for neutrophils; for the cell line Jijoye the K_m for NADPH was 30.5 ± 6.5 μM. Furthermore, we show that some cell lines that yield little $O_2^{\cdot-}$ when stimulated by addition of PMA to the intact cells produce $O_2^{\cdot-}$ when NAD(P)H is supplied to isolated membranes prepared from these PMA-treated cells.

MATERIALS AND METHODS

Cell lines

The Burkitt lymphoma lines P.3HR-1 and Jijoye (obtained from the American Type Culture Collection) were maintained on RPMI medium 1640 supplemented with 20% (v/v) heat-inactivated foetal-calf serum (P.3HR-1) or with 10% (v/v) foetal-calf serum (Jijoye). Cell line RPMI 1788 (American Type Culture Collection) was grown in Iscove's modified Dulbecco's medium supplemented with 10% (v/v) foetal-calf serum. The culture media were obtained from GIBCO, Paisley, Strathclyde, Scotland, U.K.; foetal-calf serum was from Northumbria Biologicals, Cramlington, Northumbria, U.K. Cells were harvested by centrifugation and washed with phosphate-buffered saline (130 mM-NaCl/2.6 mM-KCl/8.1 mM-Na_2HPO_4/1.4 mM-KH_2PO_4, pH 7.4).

Abbreviations used: PMA, phorbol 12-myristate 13-acetate; DPI, diphenyleneiodonium; NBT, Nitro Blue Tetrazolium.

‡ To whom correspondence should be addressed.

Preparation of cell homogenates

Cells were suspended in modified Krebs buffer, pH 7.4 [10], for stimulation at 37 °C with PMA (5 μg/ml) for 5 min where appropriate. The cells were then rapidly cooled on ice, harvested by centrifugation, washed in phosphate-buffered saline and re-suspended in buffer containing 8.6% (w/w) sucrose, 5 mM-$MgSO_4$, 1 mM-NaN_3, 20 mM-NaF, 5 mM-Hepes and 2 mM-EDTA, final pH 7.4, at approx. 10^7 cells/ml. Cells were disrupted by sonication (60 W for 10 s).

Determination of lymphocyte internal volume

The method of Halestrap & Denton [12] was used, with ^3H_2O and [^{14}C]sucrose employed to ascertain excluded volume. (We are grateful to Dr. Halestrap for his help in these measurements.)

Determination of $O_2^{\cdot-}$ production

Superoxide dismutase-inhibitable reduction of added cytochrome *c* was measured by using a dual-wavelength spectrophotometer set at 550 nm minus 540 nm [7]. For intact cells PMA (5 μg/ml) was added as stimulus at 37 °C; for disrupted cells NADH or NADPH was added to initiate the reaction at 20 °C. K_m and $V_{max.}$ were determined by using a non-linear least-squares program. $O_2^{\cdot-}$ production activated on cell-culture dishes by low concentrations of PMA (100 ng/ml) was measured by using a Nitro Blue Tetrazolium (NBT) reduction assay method [9]. PMA was dissolved in dimethyl sulphoxide at 1 mg/ml.

Determination of content of cytochrome b_{-245}

Potentiometric titrations were carried out on sonicated cells by using a computer-linked scanning spectrophotometer, as described by Cross *et al.* [13] and Maly *et al.* [6].

Determination of intracellular NADPH

This was carried out by the method of Selvaraj & Sbarra [14].

RESULTS AND DISCUSSION

It has been reported that Epstein–Barr-virus-transformed B-lymphocytes secreted $O_2^{\cdot-}$ when stimulated by PMA or by cross-linking of surface immunoglobulin [6, 9]. Burkitt lymphoma cell lines did not respond in this way. The three cell lines examined in the present study all produced $O_2^{\cdot-}$ when stimulated with high doses of PMA, although rates proved variable; P.3HR-1 and Jijoye cell lines produced between 0.08 and 0.36 nmol of $O_2^{\cdot-}$/min per mg of protein when treated with PMA whereas the RPMI 1788 cell line was slightly more active (0.4 nmol of $O_2^{\cdot-}$/min per mg of protein). At a lower concentration of PMA, 100 μg/ml, an increase in NBT reduction could be seen in the RPMI 1788 cell line, but not in the Jijoye and P.3HR-1 cell lines (Table 1). The increased NBT reduction was sensitive to inhibition by superoxide dismutase, although inhibition was incomplete. This was probably due to the aggregation induced by PMA addition limiting access to dismutase. Unexpectedly, homogenates of each of the three lines produced $O_2^{\cdot-}$ when supplied with NADPH or NADH (Table 2). Cytochrome b_{-245} was found in both the Burkitt lymphoma cell lines at concentrations similar to those previously reported for Epstein–Barr-virus-transformed cells: 11.6 pmol/mg of protein and 8.0 pmol/mg of protein for the P.3HR-1 and Jijoye cell lines respectively (Table 2). This is in agreement with our finding that the P.3HR-1 cell line carried immunologically detectable cytochrome b_{-245} antigen

Table 1. $O_2^{\cdot-}$ production by B-lymphocyte cell lines induced by low concentrations of PMA

Live cells (10^5) were suspended in 500 μl of minimal essential medium with 100 μg of bovine serum albumin/ml plus 150 μl of NBT (2 mg/ml) plus 6.5 μl of PMA as indicated. After 120 min incubation and addition of 650 μl of ice-cold paraformaldehyde (1%) under agitation, at least 300 cells/sample were counted under the microscope. Data are percentages of cells that are NBT-positive (blue).

	NBT reduction (% NBT-positive)	
Cell line	No stimulus	+PMA (100 ng/ml)
P.3HR-1	2.3	1
Jijoye	0	1
RPMI 1788	28	65
RPMI 1788 + superoxide dismutase (0.1 mg/ml)	3	35

Table 2. Michaelis parameters of $O_2^{\cdot-}$-generating oxidase of disrupted B-lymphocytes

Procedures for preparation of disrupted lymphocytes, the determination of cytochrome b_{-245} and K_m and $V_{max.}$ are given in the Materials and methods section. Results were obtained from four separate cultures of each cell line. Standard errors are relatively large when NADH is used as electron donor because there is a high background rate of cytochrome *c* reduction.

		NADH as donor		NADPH as donor	
Cell line	Cytochrome b_{-245} (pmol/mg of protein)	K_m^{NADH} (μM)	$V_{max.}$ (nmol/min per mg of protein)	K_m^{NADPH} (μM)	$V_{max.}$ (nmol/min per mg of protein)
P.3HR-1	11.6±0.8	(250)*	5.6±0.2	250±24	9.1±3.4
Jijoye	8.0±0.8	341±72	4.0±0.7	30.5±6.5	6.6±2.2
RPMI 1788	8.3±0.4	746±250	9.6±1.6	250±54	4.8±1.6

* An approximate value from only one determination.

Table 3. Intracellular water-permeable volumes and NADPH concentration for B-lymphocyte cell lines

Cell line	Water-permeable space (μl/mg of protein)	[NADPH] (μM)
P.3HR-1	5.7±3.1	14.6
Jijoye	5.58±0.09	31.4
RPMI 1788	4.85±0.05	44.3

and expressed mRNA for its β-chain [9,15]. Interestingly, Pick & Gadba [16] found that membranes from lymph-node lymphocytes (which did not generate $O_2^{\cdot-}$ when intact) could be mixed with cytosol of macrophages to assemble an NADPH oxidase *in vitro*, indicating that membrane components of the oxidase were present in the lymphocytes.

The K_m for NADPH varied widely among the cell lines and, unexpectedly, was highest for the RPMI 1788 cell line. Poor responses of intact cells to PMA could not be correlated with unnaturally high K_m for NADPH. Indeed, for the Jijoye cell line the K_m for NADPH was lower than that found for the very active neutrophil NADPH oxidase (e.g. 45 μM [17]). The wide variation reported here (Table 2) suggests that the NADPH dehydrogenase component of the oxidase may not be optimally inserted into the membranes of these cell lines. NADPH appeared to be the favoured substrate.

Unlike the P.3HR-1 cell line, the poor response of the Jijoye cell line to PMA cannot be explained by an insufficient intracellular concentration of NADPH (Table 3); the concentration of 31.4 μM is close to the K_m, whereas for the RPMI 1788 and P3.HR-1 cell lines the intracellular concentration of NADPH is well below the K_m.

Our results confirm that varieties of B-lymphocytes that, when intact, produce little $O_2^{\cdot-}$ when stimulated with PMA contain components of the NADPH oxidase found in 'professional' $O_2^{\cdot-}$ generating cells, such as neutrophils and macrophages. Indeed, on disruption they gain increased (10–20-fold) capacity to produce $O_2^{\cdot-}$. The role of such a latent or low-activity oxidase is difficult to explain. However, one would expect that in cells capable of further divisions a $O_2^{\cdot-}$-generating system would be tightly controlled to minimize cellular damage due to inappropriate triggering. Below the threshold of overt cytotoxicity, several immune processes can be positively or negatively affected by oxidants. Here, B-lymphocytes could provide a locally restricted antigen-activated source of reactive oxygen species, in contrast with unspecifically triggered phagocytes. It remains to be established under which 'natural' conditions the $O_2^{\cdot-}$ generating system of B-lymphocytes becomes active and functionally relevant. It is possible that on disruption of the cell some endogenous regulator of the oxidase becomes diluted or detached, permitting activity of the enzyme complex to be manifest. It is also possible that the B-lymphocytes lack the H^+ channel that operates in neutrophils to stabilize intracellular pH and maintain a constant membrane potential when the oxidase is functioning [3].

Received 8 May 1989/19 June 1989; accepted 27 June 1989

This work has been supported by grants from the Medical Research Council and from the Swiss National Research Fund (NF 3.987-087).

REFERENCES

1. Babior, B. M. (1978) N. Engl. J. Med. **298**, 659–668
2. Klebanoff, S. J. (1980) Ann. Intern. Med. **93**, 480–489
3. Henderson, L. M., Chappell, J. B. & Jones, O. T. G. (1987) Biochem. J. **246**, 325–329
4. Cross, A. R., Parkinson, J. F. & Jones, O. T. G. (1985) Biochem. J. **226**, 881–884
5. Volkman, D. J., Buescher, E. S., Gallin, J. I. & Fanci, A. S. (1984) J. Immunol. **133**, 3006–3009
6. Maly, F.-E., Cross, A. R., Jones, O. T. G., Wolf-Vorbeck, G., Walker, C., Dahinden, C. A. & de Weck, A. L. (1988) J. Immunol. **140**, 2334–2339
7. Cross, A. R. & Jones, O. T. G. (1986) Biochem. J. **237**, 111–116
8. Hancock, J. T. & Jones, O. T. G. (1987) Biochem. J. **242**, 103–107
9. Maly, F.-E., Nakamura, M., Gauchat, J.-F., Urwyler, A., Walker, C., Dahinden, C. A., Cross, A. R., Jones, O. T. G. & de Weck, A. (1989) J. Immunol. **142**, 1260–1267
10. Nakamura, M., Murakami, M., Koga, T., Tanaka, Y. & Minakami, S. (1987) Blood **69**, 1404–1408
11. Royer-Pokora, B., Kunkel, L. M., Monaco, A. P., Goff, S.-C., Newburger, P. E., Bachner, R. L., Cole, F. S., Curnutte, J. T. & Orkin, S. H. (1986) Nature (London) **322**, 32–38
12. Halestrap, A. P. & Denton, R. M. (1974) Biochem. J. **138**, 313–316
13. Cross, A. R., Higson, F. K., Jones, O. T. G., Harper, A. M. & Segal, A. W. (1981) Biochem. J. **194**, 599–606
14. Selvaraj, R. J. & Sbarra, A. J. (1947) Biochim. Biophys. Acta **141**, 243–249
15. Segal, A. W. (1987) Nature (London) **326**, 515–517
16. Pick, E. & Gadba, R. (1988) J. Immunol. **140**, 1611–1617
17. Cross, A. R., Parkinson, J. F. & Jones, O. T. G. (1984) Biochem. J. **223**, 337–344