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Membrane proteins are critical targets in free radical mediated cytolysis

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The hypothesis that proteins are critical targets in free radical mediated cytolysis was tested using U937 mononuclear phagocytes as targets and iron together with hydrogen peroxide to generate radicals. Those conditions which, after a lag of approx. 30 min, led to drastic lysis were also associated with very rapid membrane depolarisation. Conversely, when the early membrane depolarisation was prevented (by the addition of chelator and catalase), so was lysis. A similar correlation between early membrane depolarisation and subsequent lysis was also observed when the cells were exposed to a toxin from *Actinobacillus actinomycetemcomitans*. Those conditions of radical attack which led to lysis normally caused substantial lipid peroxidation. However, depolarisation and subsequent lysis were not prevented even when lipid peroxidation was completely suppressed by exogenous antioxidant. ATP levels were not grossly affected within the critical first 30 min period. These data exclude lipids and ATP as the target for lytic damage. We argue therefore that proteins are probably amongst the primary targets in cytolysis by radicals.

Introduction

Oxygen-centred free radicals (such as superoxide and hydroxyl) are well known toxic agents capable of killing cells relatively quickly [1–3]. Such radicals are also generated by neutrophils and macrophages [4,5] and may participate in cytolysis effected by these cells.

The mechanisms by which free radicals kill cells are presently unclear. Three major categories of mechanism of lysis by free radicals have been proposed. First, free radicals may deplete essential cellular metabolites such as GSH and ATP [6,7]. This could occur as a direct action of radicals (e.g. consumption of GSH) [6] or indirectly. An example of indirect consumption is the proposed depletion of NAD following activation of ADP-ribosylation consequent on DNA strand breaks [7]. In general, DNA damage occurs concomitantly with lysis, but in most cases, there is no evidence that it is the primary cause of cell death. In most cases it has not been shown that depletion of the relevant metabolites necessarily kills the cells. Second, free radicals may damage membrane lipids (by peroxidation) so that ionic homeostasis depending on membrane integrity is lost [8]. However, it has not been demonstrated that lipid damage can suffice to perturb a cellular membrane in this way.

The third possible mechanism is direct attack by free radicals on crucial cellular proteins [9]. Damage to even a few molecules of a catalytic

Abbreviations: HBSS, Hanks' balanced salt solution with 10 mM sodium phosphate buffer, pH 7.2; $DiSC_3(5)$, 3,3′-dipropylthiadicarbocyanine iodide; desferal, desferrioxamine mesylate; DETAPAC, diethylenetriaminepentaacetic acid; BHT, 2,6-di-*tert*-butyl-4-methylphenol; GSH, reduced glutathione; GSSG, oxidised glutathione; MEM, minimum essential medium.

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protein such as a membrane transport protein could readily lead to a catastrophic derangement of ionic homeostasis. In contrast, before lipid peroxidation can self-amplify it is necessary to consume virtually all the lipophilic antioxidant molecules present; this consumption is usually stoichiometric with radical fluxes [9].

If damage to membrane proteins is important in cytolysis, then membrane depolarisation should be an early and necessary step preceding commitment to lysis. We have tested this prediction in the present work and our data are in support of the hypothesis. In addition we have shown that membrane depolarisation can be dissociated from lipid peroxidation by the use of an antioxidant, thus supporting our argument that proteins may be critical targets.

Materials and Methods

Desferrioxamine mesylate was obtained from Ciba Geigy, Horsham, West Sussex, U.K. 3,3'-Dipropylthiadicarbocyanine iodide ($DiSC_3(5)$) was obtained from Molecular Probes Inc., Eugene, OR, U.S.A. Juvenile periodontitis toxin was obtained as a crude sonicate of *Actinobacillus actinomycetemcomitans* from Dr. N. Taichman, Department of Pathology, University of Pennsylvania, PA, U.S.A. [10]. Ferrous sulphate and cupric sulphate were obtained from BDH Ltd., Dagenham, Essex, U.K. Other chemicals were of the purest grade available and were purchased from Sigma Chemical Co. Ltd., Poole, Dorset, U.K. Tissue culture reagents were obtained from Flow Laboratories, Rickmansworth, Herts, U.K. or from GIBCO, Uxbridge, Middlesex, U.K.

Juvenile periodonitis toxin (JP_2 toxin) was dissolved in HBSS and filtered to remove insoluble matter, and was used at a final protein concentration of 25 $\mu g \cdot ml^{-1}$. Heat-inactivated JP_2 toxin was prepared in the same way, but before use was heated at 60 °C for 30 min. Iron (II) was present as ferrous sulphate and was made up at 10 mM in 10 mM sodium acetate buffer, pH 4.5. A final concentration of 100 μM was used for experimental purposes. Both copper sulphate and hydrogen peroxide were prepared in distilled water at 100-fold higher concentrations than those used experimentally.

Cell culture

U937 (human immature macrophage-like cell line) cells were obtained from Dr. S. Gordon, Oxford University and were cultured in RPMI 1640 (Flow) with penicillin and streptomycin with 10% v/v fetal calf serum (GIBCO). $P388D_1$ (murine macrophage-like cell line from Flow labs) cells were cultured in Eagle's MEM (GIBCO) with penicillin and streptomycin with 10% v/v fetal calf serum. Both cell lines were grown at 37 °C in 5% CO_2.

Cell Harvesting

U937 cells were inoculated at 10^5 cells $\cdot$ ml^{-1} and $P388D_1$ cells were inoculated at $5 \cdot 10^4$ cells $\cdot$ ml^{-1}. Both cell types were then grown up for 3 days before harvesting, to yield approx. $2 \cdot 10^7$ cells in 20 ml medium. Cells (U937 or $P388D_1$) were harvested by centrifugation at $500 \times g$ for 3 min. The pellet was then resuspended in HBSS (Flow) and the suspension was diluted to 10^7 cells $\cdot$ ml^{-1}.

Membrane depolarisation assay

$DiSC_3(5)$ was dissolved in methanol at 2 mM and diluted to 10 μM in HBSS. 1.8 ml of $DiSC_3(5)$ solution was placed in a 3 ml quartz cuvette maintained at 37 °C in a Perkin-Elmer LS3 fluorescence spectrometer and was stirred with a magnetic stirrer bar. Fluorescence was followed at 620 nm excitation and 670 nm emission [11,12], and was recorded on a servoscribe 210 chart recorder. After the addition of the fluorophore to the cuvette, there is normally a gradual loss of signal for a few minutes due to binding to the stirrer and the cuvette surfaces. When the signal was stable, 200 μl of cell suspension was added (final cell concentration 10^6 cells $\cdot$ ml^{-1}). The cells quickly took up the dye to a degree dependent on the membrane potential, quenching the fluorescence. When equilibrium was reached (approx. 15 min), the appropriate reagents were added (additions were no more than 1% of the total volume) and the fluorescence recorded continuously. In the case of hydrogen peroxide and transition metal (iron or copper), the hydrogen peroxide was added to the system and left for 4 min before the addition of the transition metal. After the chosen procedure, 20 μl of the potassium ionophore gramicidin (100

$\mu g \cdot ml^{-1}$ in methanol) was added, which completely depolarised the cells such that fluorescence was not further changed by addition of Triton X-100. Depolarisation is expressed as a percentage of the total depolarisation after gramicidin addition.

Cell lysis assay

Cells (10^6 cells $\cdot$ ml^{-1}) were incubated with the desired reagents in sterile plastic tubes, at 37°C. At appropriate times, triplicate 1 ml samples were taken from each incubation and added to small tubes containing 10 μl ethidium bromide (2.5 mM) plus stopping reagents. The metals used interfere with ethidium fluorescence and therefore whenever they were present it was necessary to chelate them before reading the fluorescence. When iron was used in these incubations, the concentration of iron was standardised in all samples and desferal was added at 1.25-times the iron concentration. The same procedure was followed when copper was present, except that DETAPAC was added at 1.5-times the copper concentration. When hydrogen peroxide was present, catalase (purified powder from bovine liver, Sigma) was added to each sample to give a final concentration of 100 $\mu g \cdot ml^{-1}$. After mixing, the fluorescence of each sample was read at 355 nm excitation and 580 nm emission [13]. 10 μl of 1% Triton X-100 was added to the remainder of each sample (0.55 ml), which lysed the cells completely, and the fluorescence was again recorded. Blanks were measured by performing the same procedures in the absence of cells.

Results are expressed as percent lysis, which is given by [100(sample − blank fluorescence in the absence of Triton) divided by (sample − blank fluorescence in the presence of Triton)], or as specific lysis (%), which is given by [100(sample % lysis − control % lysis) divided by (100 − control % lysis)] for any given time point.

Assay of lipid peroxidation

Lipid peroxidation was measured using a sensitive fluorimetric assay [14] of malondialdehyde formation as follows: U937 cells (10^6 cells $\cdot$ ml^{-1}) were incubated in the presence of 100 μM Fe^{2+} and 1 mM H_2O_2 and other reagents, such as BHT. Stock solutions of BHT were made up at 5 mM and 50 mM in ethanol, and were diluted 1000-fold into the incubations. Triplicate samples (0.265 ml) were taken at various intervals. 10 μl catalase (2.65 mg $\cdot$ ml^{-1}) and 10 μl DETAPAC (3.31 mM) was added to each sample to stop the reaction, and after 2 min, 15 μl trichloroacetic acid (final concentration 5%) was added. An equal volume of 0.67% thiobarbituric acid was then added to each sample and they were incubated for 10 min at 100°C; after a short cooling period and centrifugation, the supernatants were read for fluorescence at 537 nm excitation and 558 nm emission. In a standard curve prepared as above, malondialdehyde [12] at a final concentration in the reaction mixture of 0.5 μM gave a fluorescence of 273 arbitrary units with the fluorimeter on a sensitivity scale of 10.

Assay of cellular ATP levels

In order to assay intracellular ATP levels, U937 cells (10^6 cells $\cdot$ ml^{-1}) were incubated with the appropriate reagents at 37°C in 50 ml Falcon tubes. At the desired times, a 10 ml sample was removed from each incubation and desferal (125 μM) and catalase (100 $\mu g \cdot ml^{-1}$) were added to stop the radical reactions. The cells were then centrifuged at $500 \times g$ for 3 min. The pellet was resuspended in 0.25 ml 5% trichloroacetic acid and this suspension was spun in a microcentrifuge for 2 min. 0.15 ml of the supernatant was removed and neutralised with 0.15 ml Tris (1 M). The samples thus obtained were stored on ice until assayed.

The assay involved coupling the consumption of the ATP in the sample to the production of NADPH which could then be measured fluorimetrically with excitation at 340 nm and emission at 460 nm. The assay was a slightly modified version of the method described in Ref. 15. This method gives a one to one stoichiometry between ATP consumption and NADPH production. Glucose-6-phosphate dehydrogenase (Torula yeast enzyme type XI, Sigma) was present at 0.1 units $\cdot$ ml^{-1} reaction mixture, and hexokinase (brewer's yeast enzyme type C-130, Sigma) was present at 1.8 units $\cdot$ ml^{-1} reaction mixture. In each case, a blank was run in the absence of hexokinase to measure the rate of formation of NADPH from other sources (endogenous glucose-6-phosphate, glucose

dehydrogenase activity). The reaction was allowed to run for 15 min, by which time the ATP reaction was complete; readings were taken at $t = 0$ and $t = 15$ min for each sample. Based on the control results, we calculate that the normal resting value of ATP in U937 cells is approx. 5 nmol/10^6 cells.

Assay of cellular glutathione levels

Glutathione levels in U937 cells were measured by the method of Griffith [16] with some modifications. The buffer used was 143 mM sodium phosphate and 6.3 mM Na-EDTA, rather than 125 mM sodium phosphate and 6.3 mM Na-EDTA. Samples were prepared as follows: 10 ml of U937 cell suspension (10^{-6} cells · ml^{-1}) in HBSS were removed from the incubation and this sample was treated with 100 μl catalase (10 mg · ml^{-1}) and 100 μl desferal (12.5 mM). The sample was then centrifuged for 3 min at 500 × *g* and the supernatant discarded. The cell pellet was resuspended in 2 ml 5% trichloroacetic acid and was microcentrifuged for 5 min. The supernatant was collected and was neutralised with a small volume of 50% v/v triethanolamine in water. 1 ml of the sample was derivatised with 2-vinylpyridine as described [16], whilst the other portion was used to assay total glutathione levels.

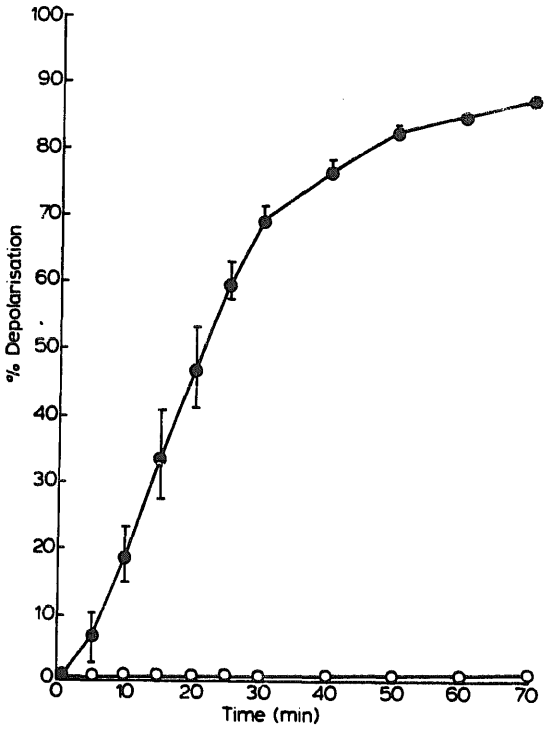

Fig. 1. Depolarisation of U937 cells by iron and hydrogen peroxide. U937 cells (10^6 cells · ml^{-1}) were incubated in HBSS with 9 μM $DiSC_3(5)$: (●) with Fe^{2+} (100 μM) and H_2O_2 (1 mM); or (○) with Fe^{2+} (100 μM), H_2O_2 (1 mM), desferal (125 μM) and catalase (100 μg · ml^{-1}).

Results

Correlation of the early depolarisation and subsequent lysis of U937 cells exposed to radical fluxes

U937 cells can be rapidly depolarised by iron and hydrogen peroxide as shown in Fig. 1. Depolarisation is detectable with little or no lag in onset after the addition of the radical generating system and is more or less complete by 70 min. No depolarisation takes place when the cells are incubated alone with the carbocyanine dye.

Cell lysis, involving the same iron and hydrogen peroxide system with the U937 cells, is shown in Fig. 2. The lysis curve is a typical sigmoidal lysis curve. In our system, H_2O_2 (1 mM) alone did not depolarise or lyse the cells. Much higher concentrations of H_2O_2, as used by some authors [7], can lyse several kinds of cells, including the $P388D_1$ cells we have used, without the addition of metal ions. The metal ion requirement for lysis depends on cell type and the medium used. The addition of Fe^{2+} or Cu^{2+} alone was not lytic.

That lysis can be prevented if the radical flux is stopped early enough is demonstrated by Fig. 3. This shows percent depolarisation achieved in 70 min and specific lysis occurring in 150 min after the addition of desferal and catalase at a range of chosen times between 0 and 60 min. These data confirm the interpretation of Fig. 2 in showing that unless desferal and catalase are added within the first 30 min, they can only prevent approx. 10% of the lysis. A variety of controls were used for the effect of catalase and desferal. Heat-inactivated catalase (100 °C, 30 min) or iron-saturated desferal had no effect on iron and hydrogen peroxide-induced depolarisation and lysis in U937 cells when used alone or together.

However depolarisation is not in itself enough to cause the extent of lysis observed when the cells are exposed to oxidative stress, for exposure of the cells to gramicidin, which completely depolarises

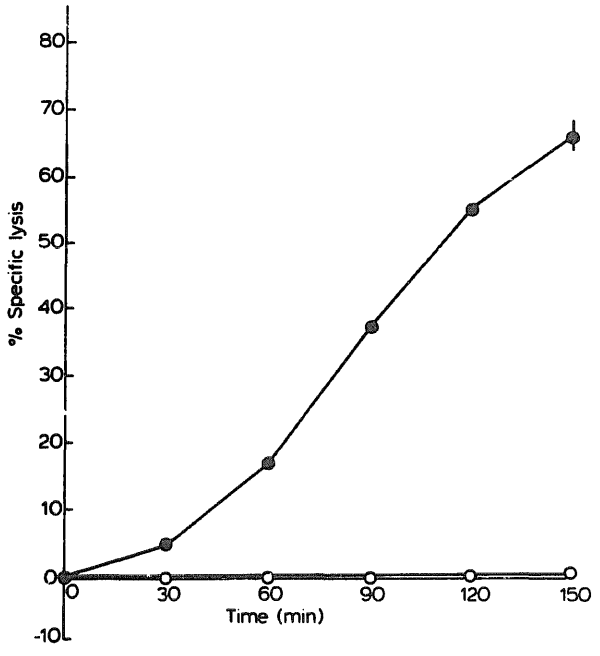

Fig. 2. U937 cells lysis by iron and hydrogen peroxide. U937 cells (10^6 cells·ml^{-1}) were incubated in HBSS: (●) with Fe^{2+} (100 μM) and H_2O_2 (1 mM); or (○) with Fe^{2+} (100 μM), H_2O_2 (1 mM), desferal (125 μM) and catalase (100 μg·ml^{-1}).

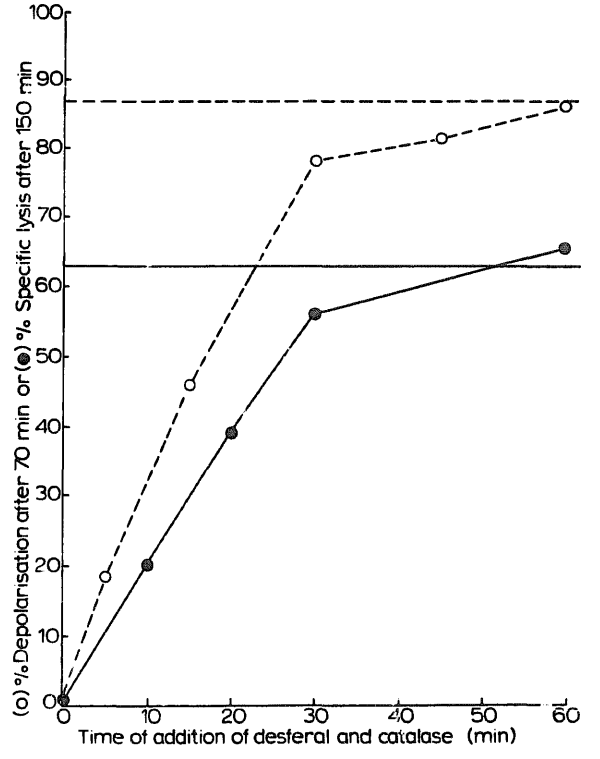

Fig. 3. Prevention of membrane depolarisation (○) and cell lysis (●) caused by iron and hydrogen peroxide in U937 cells, by the addition of desferal and catalase at different times. U937 cells (10^6 cells·ml^{-1}) were incubated in HBSS with Fe^{2+} (100 μM) and H_2O_2 (1 mM): desferal (125 μM) and catalase (100 μg·ml^{-1}) were added as shown.

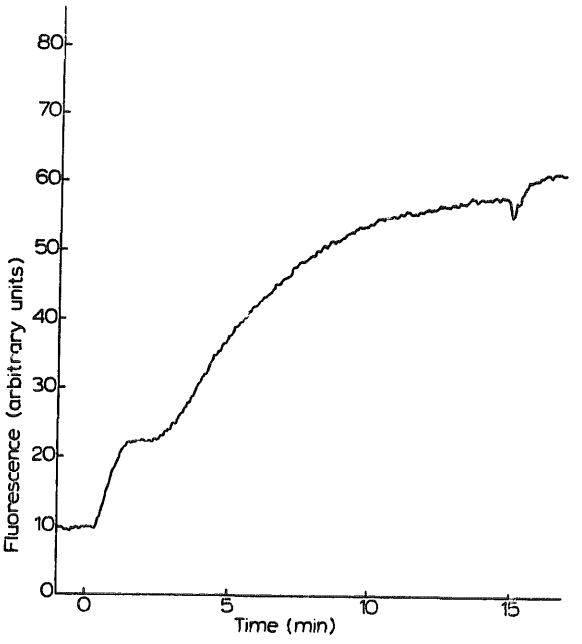

Fig. 4. Depolarisation of U937 cells by JP_2 toxin from *A. actinomycetemcomitans*. U937 cells (10^6 cells·ml^{-1}) were incubated in HBSS with JP_2 toxin (25 μg·ml^{-1} protein).

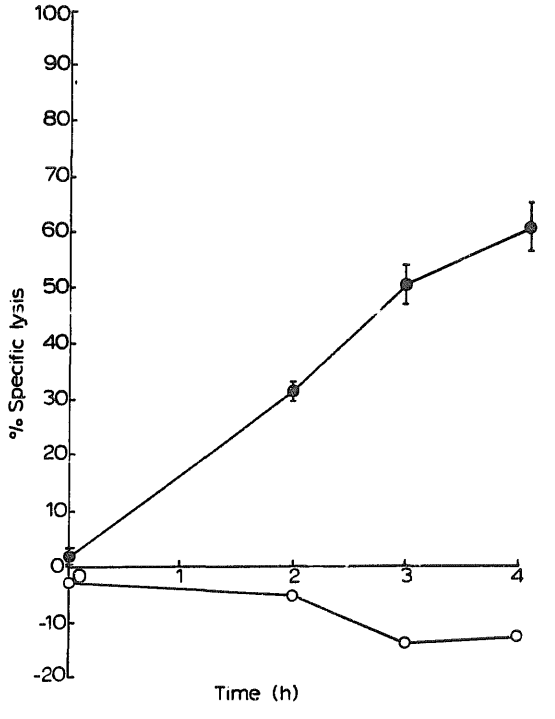

Fig. 5. Lysis of U937 cells by JP_2 toxin from *A. actinomycetemcomitans*. U937 cells (10^6 cells·ml^{-1}) were incubated in HBSS: (●) with JP_2 toxin (25 μg·ml^{-1} protein); or (○) with heat-inactivated JP_2 toxin (25 μg·ml^{-1} protein).

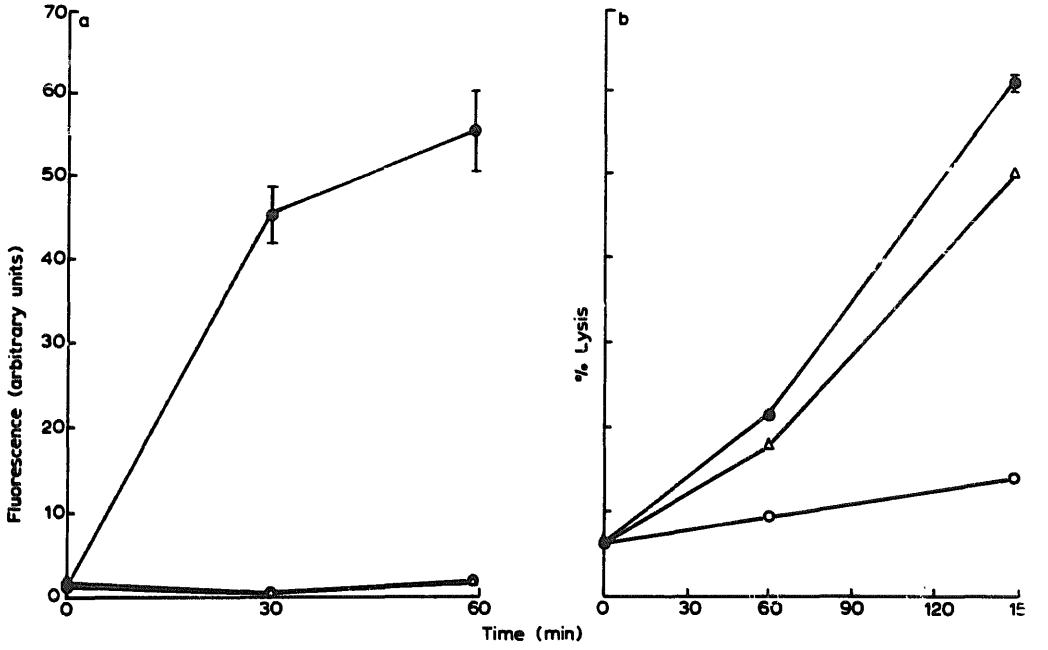

Fig. 6. (a) Lipid peroxidation and (b) cell lysis in U937 cells exposed to iron and hydrogen peroxide. (○) U937 cells (10^6 cells·ml^{-1}) were incubated in HBSS: (●) with Fe^{2+} (100 μM) and H_2O_2 (1 mM); or (△) with Fe^{2+} (100 μM), H_2O_2 (1 mM) and BHT (50 μM).

the cells very quickly, causes less than 18% specific lysis after 4 h.

Correlation of depolarisation and lysis of U937 cells due to a toxin

It was of interest to determine whether depolarisation is involved in a lytic mechanism independent of radicals. U937 cells can be lysed by a toxin obtained from the dental bacterium *A. actinomycetemcomitans* [10]. This toxin does indeed depolarise the U937 cells, again with no lag in onset, in 15–20 min (Fig. 4). The toxin also lyses the cells substantially in 4 h (Fig. 5). The toxin is heat-labile, such that heating of the toxin at 60 °C for 30 min completely destroys all its depolarising and toxin activities in this system. Comparative studies using $P388D_1$ cells showed no depolarisation or lysis by the toxin, which agrees with its specificity for primate cells [10].

Depolarisation and lysis of $P388D_1$ cells exposed to free radicals

Early depolarisation and subsequent lysis were also observed when $P388D_1$ cells were exposed to radical generating conditions. For instance, during incubation with 100 μM Fe^{2+} and 1 mM H_2O_2, depolarisation exceeded 80% after 5 min and specific lysis reached 40.6% after 2 h.

Critical targets of radical damage

In order to assess the targets of radical damage, we measured lipid peroxidation in U937 cells exposed to iron and hydrogen peroxide. Peroxidation was significant after 30 min of incubation of U937 cells with iron and hydrogen peroxide and this peroxidation could be completely inhibited by the addition of 50 μM BHT (Fig. 6a). 5 μM BHT prevented approx. 80% of the peroxidation while 0.1% ethanol had no effect (data not presented).

Although 50 μM BHT completely abolished lipid peroxidation, it only prevented 20–25% of the specific lysis caused by iron and hydrogen

TABLE I

DEPLETION OF ATP IN U937 CELLS EXPOSED TO IRON AND HYDROGEN PEROXIDE

Control cells were incubated in HBSS. Radical-exposed cells were incubated in HBSS with Fe^{2+} (100 μM) and H_2O_2 (1 mM). Values are ATP levels as a percentage of the starting value.

	Time (min)		
	0	30	60
Control cells	100	98.6	95.1
Radical-exposed cells	100	69.2	26.1

peroxide in U937 cells (Fig. 6b). 5 μM BHT had no significant effect on the extent of lysis (data not presented). Depolarisation was not significantly affected by the addition of 50 μM BHT to the incubation (data not presented).

Cellular ATP concentrations were measured in U937 cells. The concentration of ATP in unexposed cells at $t = 0$ was 5.1 ± 0.1 nmol/10^6 cells ($n = 4$). ATP was measured in cells exposed to the same conditions which lead to depolarisation and lysis of the cells. Under these conditions, ATP was only decreased by 30–40% in the first 30 min of the radical attack. After 60 min of radical exposure, ATP levels had fallen by 70–75%. Table I shows the data from a representative experiment. Since the first 30 min of exposure to radicals are critical, ATP depletion does not seem to be a primary cause of death in U937 cells. ATP levels in many cell types can be reversibly depleted by more than 80% [17].

Glutathione levels in U937 cells were also measured. Cells not exposed to radical attack showed no change in total glutathione levels (GSH + 2 × GSSG), which remained constant at approx. 6 nmol/10^6 cells. GSSG was less than 1% of this total throughout the 60 min incubation period. In cells exposed to lytic radical attack conditions, the total glutathione again remained at a constant level, comparable to that in the unexposed cells. GSSG had increased by 30 min but fell again after 60 min of incubation. Thus the percentage of total glutathione present in the oxidised form, expressed as $100(2 \times GSSG)/(GSH + 2 \times GSSG)$, was 5.5% at $t = 0$, 17.3% at $t = 10$ min, 27.6% at $t = 20$ min, 36.4% at $t = 30$ min and 14.7% at $t = 60$ min.

Discussion

The data presented demonstrate depolarisation and lysis of U937 cells by an oxygen radical generating system. A link between the two is suggested by Fig. 3, which shows prevention of depolarisation and lysis, by desferal and catalase, decreasing in an almost parallel manner for each system. Thus if one prevents depolarisation, one also prevents lysis. Further data show a similar correlation with P388D_1 cells, except that depolarisation had a much shorter time course (5–15 min), and prevention of both depolarisation and lysis was possible only up to approx. 5–10 min after the start of the incubation. Thus, during radical attack, an early depolarisation which is not reversed is predictive of subsequent lysis.

We then studied the nature of the cellular target, damage to which causes this crucial depolarisation. We found that significant lipid peroxidation was taking place inside the critical period, reaching an approximate molar ratio of oxidised lipid to plasma membrane protein of 9–10 : 1. This calculation is based on the observation that 10^6 cells yield approx. 100 μg protein, of which it is assumed that 1% is located in the plasma membrane. It is also assumed that the modal size of a plasma membrane protein is roughly 50 kDa. Lipid peroxidation, however, does not seem to be the critical membrane event which occurs during an oxygen radical attack on U937 cells. For instance, our studies showed that whilst 50 μM BHT completely inhibited lipid peroxidation, under the same conditions, it only prevented approx. 20–25% of the lysis observed. Moreover, 50 μM BHT did not have a significant effect on the depolarisation of the plasma membrane under these oxidising conditions.

The data presented show also that intracellular levels of ATP do not fall greatly during the critical period of radical attack (only 30*0% of the ATP is lost in the first 30 min), so it is unlikely that ATP depletion is the primary toxic consequence of radical attack on U937 cells. We have also shown that GSH levels in radical-exposed U937 cells do not fall drastically. After 30 min of radical attack, the majority (64%) of the cellular glutathione is still in the reduced form, and after 60 min, GSH levels have recovered to 85% of the total cellular glutathione. It is therefore unlikely that depletion of such metabolites is the primary event. Indeed, as already mentioned, it is anyway not clear that depletion of any of these metabolites suffices to kill cells.

We conclude therefore that the primary damaging target of the radical attack is not the lipid in the membrane, nor small cellular metabolites, but the membrane proteins exposed to the radical attack. We have preliminary evidence for the inactivation of the membrane Na^+/K^+-stimulated ATPase. We propose therefore that proteins of the

plasma membrane (notably ion transporters) are the primary targets.

Acknowledgements

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References

1 Clark, I.A., Butcher, G.A., Buffinton, G.D., Hunt, N.H. and Cowden, W.B. (1987) Biochem. Pharmacol. 36, 543–546.
2 Deuticke, B., Heller, K.B. and Haest, C.W.M. (1987) Biochim. Biophys. Acta 899, 113–124.
3 Schraufstatter, I.U., Hinshaw, D.B., Hyslop, P.A., Spragg, R.G. and Cochrane, C.G. (1986) J. Clin. Invest. 77, 1312–1320.
4 Andrew, P.W., Jackett, P.S. and Lowrie, D.B. (1985) in Research Monographs in Cell and Tissue Physiology, Vol. 11 (Dean, R.T. and Jessup, W., eds.), pp. 311–325, Elsevier, Amsterdam.
5 Segal, A.W. and Venge, P. (1985) in Inflammation (Venge, P. and Lindbom, A., eds.), pp. 105–119, Almqvist and Wiksel International, Stockholm.
6 Schraufsatter, I.U., Hinshaw, D.B., Hyslop, P.A., Spragg, R.G. and Cochrane, C.G. (1985) J. Clin. Invest. 76, 1131–1139.
7 Spragg, R.G., Hinshaw, D.B., Hyslop, P.A., Schraufsatter, I.U. and Cochrane, C.G. (1985) J. Clin. Invest. 76, 1471–1476.
8 Duwe, A.K., Werkmeister, J., Roder, J.C., Lauzon, R. and Payne, U. (1985) J. Immunol. 134, 2637–2644.
9 Dean, R.T. (1987) in Perspectives in Mammalian Cell Death (Potten C.S., ed.), pp. 18–38, Oxford University Press, Oxford.
10 Taichman, N.S., Dean, R.T. and Sanderson, C.J. (1980) Infect. Immun. 28, 258–268
11 Waggoner, A.S. (1979) Methods Enzymol. 55, 689–695.
12 Bashford, C.L. and Smith, J.C. (1979) Methods Enzymol. 55, 569–586.
13 Gomperts, B.D. (1983) Nature 306, 64–66.
14 Gutteridge, J.M.C. (1984) Biochem. J. 224, 761–767.
15 Lamprecht, W. and Trautschold, I. (1974) in Methods of Enzymatic Analysis (Bergmeyer, H.U., ed.), Vol. 4, pp. 2101–2110, Academic Press, New York and London.
16 Griffith, O.W. (1980) Anal. Biochem. 106, 207–212.
17 Simpson, M.V. (1953) J. Biol. Chem. 201, 143–154.