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Role of hydrogen peroxide in the cytotoxic reaction of T lymphocytes

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SUMMARY

Evidence is presented that T lymphocyte cytotoxicity is mediated by hydrogen peroxide (H_2O_2). At a concentration of 5×10^{-4} M H_2O_2 induced ^{51}Cr release from pre-labelled P815 mastocytoma cells. H_2O_2 was generated when T lymphocytes from mouse spleen were exposed to P815 cells. The concentration of H_2O_2 produced was apparently one thousand times lower than the concentration required to lyse the P815 cells. This suggests that the H_2O_2 is produced and acts at a highly localized site on the target cell. Sulphydryl groups on the target cell were particularly sensitive both to H_2O_2 and to spleen cell attack. The activity of the spleen cells was inhibited by cyanide and azide and by reducing agents which protected the target cells. Cytotoxicity was enhanced by agents which prevented H_2O_2 breakdown.

INTRODUCTION

The cytotoxic action of T lymphocytes towards their immunizing target tumour cells has been thoroughly characterized immunologically (see Klein, 1978, for review) and ultrastructurally (Sanderson & Glauert, 1977, 1979; Matter, 1979), but the biochemistry of this killing mechanism has not yet been elucidated.

Since the killing of bacteria, viruses and certain protozoa is mediated by hydrogen peroxide and peroxidase (Klebanoff, 1975; Thorne, Svvennson & Franks, 1978) the possibility exists that a similar mechanism might operate in the cytotoxicity of T lymphocytes to target tumour cells. Clark & Klebanoff (1975) showed that an ascitic Moloney virus-induced lymphoma (LSTRA) from mice could be killed by peroxidase-H_2O_2-halide in isolation and indeed by human neutrophils which had been stimulated to secrete H_2O_2 and peroxidase by treatment with opsonized zymosan (Clark *et al.*, 1975). Mouse macrophages have also been found to be cytotoxic to lymphoma cells after artificial triggering with phorbol myristate acetate (Nathan *et al.*, 1979a). Further experiments demonstrated that it was hydrogen peroxide alone, without peroxidase, which was cytotoxic (Nathan *et al.*, 1979b).

In the present paper we describe experiments which prove that H_2O_2 is responsible for the cytotoxic action of mouse spleen T lymphocytes towards the P815 mastocytoma cells. In addition, we demonstrate that other oxidative intermediates, superoxide radicals, hydroxyl radicals and singlet oxygen are not the active agents in this system.

MATERIALS AND METHODS

Mastocytoma cells. P815-X2 mastocytoma cells (Dunn & Potter, 1957) were passaged as ascitic cells in DBA/2 mice by injecting 10^7 cells, and transferring 7 days later.

T lymphocytes. 10^7 P815-X2 mastocytoma cells harvested from a DBA-2 mouse were injected into a C57Bl/10 mouse, and cytotoxic T lymphocytes were obtained by harvesting the spleen cells (Brunner *et al.*, 1968) 11 days later. The cells were used either as harvested or were purified further by removal of inactive cells by adherence to nylon wool. For purification

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the spleen cells were incubated on a column of nylon wool in RPMI 1640 medium with bicarbonate, 20 mM HEPES, 10% foetal calf serum, 100 units penicillin per ml and 100 μg streptomycin per ml at 37°C for 1 hr. The non-adherent cells were then eluted with warm medium. Fewer than 2% of the eluted cells had membrane immunoglobulin as shown by fluorescent anti-immunoglobulin.

Cytotoxicity assay. P815 cells which had been cultured in RPMI 1640 medium with bicarbonate, 20 mM HEPES, 10% foetal calf serum, 100 units penicillin per ml and 100 μg streptomycin per ml were labelled with ^{51}Cr chromate by incubation of about 5×10^6 cells for 1 hr in 0·1 ml of medium with about 300 μCi of $Na_2{}^{51}CrO_4$ (The Radiochemical Centre, Amersham, Bucks, UK), and were then washed repeatedly until the count rate in the supernatant was less than 5% of that in the cells. All materials to be assayed were pre-warmed to 37°C before mixing, and the assay was set up, and the effector cells and target cells centrifuged together at 37°C. Quadruple samples of 5×10^3 target cells in 100 μl were mixed with 2×10^5 effector spleen cells in 200 μl, and 100 μl of inhibitor, medium or other additive was added as appropriate in small plastic tubes. They were then mixed and centrifuged at 200 *g* for 2 min to induce contact between target and effector cells, and incubated for 3 hr at 37°C in sealed humidified boxes. Tubes were then mixed and centrifuged again, and half the supernatant was removed to a second tube. Both tubes were counted in a 3 cm sodium iodide scintillation counter, and the percentage of the isotope release from the target cells was calculated.

Hydrogen peroxide determination. H_2O_2 concentration was determined by the method of Guibault, Brignac & Juneau (1968). In the presence of lactoperoxidase, H_2O_2 converts *p*-OH phenyl acetate stoichiometrically into a fluorescent dimer. Effector T cells (2 to 4×10^6 cells) were centrifuged for 10 sec at 12,000 *g* into a pellet with or without P815 cells, at a ratio of one effector to three target cells. They were incubated for 30, 60 or 90 min in 1·5 ml Hanks' balanced salt solution with 0·1% (w/v) bovine serum albumin (HBSS+BSA), 50 μg *p*-OH phenylacetate, 10 μg lactoperoxidase (Sigma Chemical Company, Kingston upon Thames, Surrey, England), with or without 4 μg beef liver catalase (Fluka AG, Fluorochem Limited, Glossop, Derbyshire, England) which had been dialysed against water before use. After incubation the pellet was resuspended briefly and then centrifuged into a pellet again and the fluorescence of the supernatant determined (excite 317 nm, read 414 nm). A calibration curve over the range 0–5 nmol H_2O_2 in 1·5 ml HBSS+BSA was made for each set of determinations.

Statistical analysis. Variance of the logarithm of isotope release was analysed initially. Tests were then made to determine which effects were significant by multiple range testing. All experiments on analysis gave significant variance ratios and the probability levels were all less than 0·05%. Indications are given in each experiment of the least significant difference in isotope release which is significant (see Thorne *et al.*, 1978 for details).

RESULTS

Cytotoxicity of hydrogen peroxide

Hydrogen peroxide is known to be toxic to tumour cells, although different target cells appear to differ in their sensitivity to this oxidizing agent (Nathan *et al.*, 1979b). Hydrogen peroxide at a concentration of 5×10^{-4} M had a significant cytotoxic activity to the P815 mastocytoma cells used in the present work (Table 1). This level of H_2O_2 was sufficient to lyse 5×10^3 P815 cells, but had a reduced cytotoxic effect when the number of cells was increased to 10^5. Dissipation of the H_2O_2 with catalase eliminated the toxicity. This cytotoxic reaction of H_2O_2 did not require the addition of peroxidase and therefore differed from the anti-bacterial (Klebanoff, 1975) and anti-trypanosomal (Thorne *et al.*, 1978) reactions of granulocytes and monocytes.

Another damaging oxidative product produced by mammalian cells is the superoxide radical (Babior, Kipnes & Curnutte, 1973). This could be the toxic agent which kills P815 cells. Superoxide was generated artificially by the action of xanthine oxidase (XO) on xanthine and on acetaldehyde. A higher concentration of acetaldehyde than of xanthine is required for superoxide generation (Rosen & Klebanoff, 1979). These radicals were indeed toxic to P815 cells (Table 2). However, superoxide is converted rapidly to H_2O_2 and it might be therefore merely acting as a precursor of H_2O_2. Addition of superoxide dismutase (SOD) to accelerate the conversion of superoxide to H_2O_2 slightly enhanced its toxic activity and the toxicity was abolished by dissipation of H_2O_2 with catalase, both in the presence and absence of SOD. This demonstrates that it is H_2O_2 and not superoxide which is cytotoxic.

Production of H_2O_2 by T lymphocytes

If T lymphocyte cytotoxicity is mediated by H_2O_2 it should be possible to demonstrate the production of H_2O_2 when the effector T lymphocytes encounter a target cell.

Peroxidase catalyses the conversion of *p*-OH phenyl acetate into a fluorescent dimer when H_2O_2 is present (Guibault *et al.*, 1968). This provides a sensitive assay for H_2O_2. However, in our previous experiments (Thorne *et al.*, 1978) we discovered that components of tissue culture medium interfere

TABLE 1. Susceptibility of P815 cells to lysis by hydrogen peroxide

H_2O_2 concentration	No. of labelled target cells	Per cent release of ^{51}Cr from P815	
		Expt 1	Expt 2
0	5×10^3	9	16
5×10^{-5} M	5×10^3	9	—
2×10^{-4} M	5×10^3	17*	—
5×10^{-4} M	5×10^3	35*	28*
5×10^{-4}+20 μg catalase	5×10^3	8	—
5×10^{-4} M	5×10^3 +10^5 unlabelled	—	16

Expt 1: Log_e l.s.d. = 0·147, corresponding to a difference of 1·5% at 10% isotope release, and 5·5% at 35% release. Expt 2: Log_e l.s.d. = 0·145, corresponding to a difference of 2·5% at 16% isotope release.

* Significantly increased isotope release (5% probability level).

with this assay, possibly as a result of the presence of an inhibitor of peroxidase in serum. For this reason all H_2O_2 assays were conducted in HBSS+BSA. Interference from other fluorescent products produced by the cells and the reagents was eliminated by measuring the fluorescence yield in the presence and absence of catalase and determining the amount of H_2O_2 as the amount of catalase-sensitive product (Thorne *et al.*, 1978). Under the conditions used in the present experiments catalase competes successfully with the added lactoperoxidase: no fluorescence was detectable when standard solutions of H_2O_2 were incubated with *p*-OH phenyl acetate in the presence of lactoperoxidase and catalase.

H_2O_2 was produced during the interaction of all of the cytotoxic spleen cell preparations tested with P815 cells, but the pattern of production was very variable (Table 3). Cells from spleens 2, 3, 5, 8 and 9

TABLE 2. Susceptibility of P815 cells to superoxide radicals

Superoxide generator	Per cent release of ^{51}Cr from P815		
	Expt 1	Expt 2	Expt 3
None	10	9	9
10 mM acetaldehyde+50 μg XO	61*	38*	34*
10 mM acetaldehyde+50 μg XO+5 μg SOD	—	50*	—
10 mM acetaldehyde+50 μg XO+20 μg catalase	—	—	8
10 mM acetaldehyde+50 μg XO+5 μg SOD+20 μg catalase	—	8	—
0·5 mM xanthine+50 μg XO	55*	—	—
10 mM acetaldehyde	7	—	—
0·5 mM xanthine	10	—	—
50 μl XO	8	—	—

Expt 1: Log_e least significant difference = 0·28, corresponding to a difference of 3% at 10% isotope release and 15% at 55% release. Expt 2: Log_e l.s.d. = 0·15, corresponding to a difference of 1·5% at 10% and 9% at 55% release. Expt 3: Log_e l.s.d. = 0·26, corresponding to a difference of 2% at 10% isotope release.

* Significantly increased isotope release.

TABLE 3. Production of hydrogen peroxide when T lymphocytes interact with P815 cells

Effector cells	nmol $H_2O_2/10^6$ cells* 30 min −P815	30 min +P815	60 min −P815	60 min +P815	90 min −P815	90 min +P815	Per cent release of ^{51}Cr from P815 cells†
1. Spleen cells	0·01	0·07	0	0·13	n.d.	n.d.	59
2. Spleen cells	0	0·07	0·01	0·07	0·03	0	56
3. Spleen cells	0	0·13	n.d.	n.d.	0	0·02	61
4. Spleen cells	0	0	0	0	0·09	0·16	70
5. Spleen cells	0·02	0·26	0	0·16	0	0·18	74
6. Spleen cells	0·06	0·05	0	0·32	0	0·26	76
7. T lymphocytes	0	0	n.d.	0·28	n.d.	0	84
8. T lymphocytes	0	0·36	0	0	0	0	88
9. T lymphocytes	0	2·0	0	0	0	0	86
10. None	—	0	—	0	—	0·1	10
11. None	—	0	—	0	—	0·05	10

* 2 to 4×10^6 effector cells were centrifuged into a pellet with or without P815, at a ratio of one effector to three target cells. Parallel samples with and without 4 μg catalase were incubated and the amount of catalase-sensitive H_2O_2 produced was determined fluorimetrically.

† Isotope release was measured at a ratio of effector to target cells of 30:1 after 3 hr.

$\log_e$ l.s.d. = 0·11, corresponding to a difference of 1% at 10% release.

had maximum levels after 30 min, while cells from spleens 1, 4, 6 and 7 responded more slowly. It is not clear why the level of H_2O_2 appeared to deteriorate after reaching a maximum, but possibly the fluorescent dimer is metabolized by the cells. The amount of free H_2O_2 produced varied, from 0·07 to 0·32 nmol per 10^6 spleen cells and from 0·2 to 2·0 nmol per 10^6 purified T lymphocytes. This is considerably less than the amount of H_2O_2 necessary to kill P815 cells *in vitro*. While 5×10^4 T cells are cytotoxic to

TABLE 4. Susceptibility of P815 cells to reagents which attack sulphydryl groups

Additions	Per cent release of ^{51}Cr from P815 Expt 1	Expt 2	Expt 3
None	8	9	9
10^{-6} M NEM	9	—	—
10^{-5} M NEM	22*	—	—
10^{-4} M NEM	86*	91*	90*
10^{-3} M *p*-Cl mercuribenzoate	—	53*	—
10^{-3} M iodoacetate	—	51*	—
10^{-4} M NEM + 2×10^{-4} M DTT	9	—	9
10^{-4} M NEM + 2×10^{-4} M DETC	—	—	11
10^{-4} M NEM + 2×10^{-4} M cysteine	—	10	—

Expt 1: $\log_e$ l.s.d. = 0·49, corresponding to a difference of 5% at 10% isotope release and 31% at 50% release. Expt 2: $\log_e$ l.s.d. = 0·15, corresponding to 1·5% at 10% and 8% at 50% release. Expt 3: $\log_e$ l.s.d. = 0·25, corresponding to 3% at 10% release and 14% at 50% release.

* Significantly higher than control.

TABLE 5. Protection of P815 cells from hydrogen peroxide by agents which protect sulphydryl groups

Additions	Per cent release of ^{51}Cr from P815 Expt 1	Expt 2
None	9	9
5×10^{-4} M H_2O_2	35*	52*
5×10^{-4} M $H_2O_2+5\times10^{-3}$ M DTT	10	—
5×10^{-4} M $H_2O_2+5\times10^{-3}$ M cysteine	—	13†
5×10^{-4} M $H_2O_2+2\times10^{-3}$ M DETC	85*	—
5×10^{-4} M $H_2O_2+10^{-2}$ M triene	—	37*
10 mM acetaldehyde+50 μg XO	38*	34*
10 mM acetaldehyde+50 μg XO+5×10^{-3} M DTT	7	—
10 mM acetaldehyde+50 μg XO+5×10^{-3} M cysteine	—	15†
10 mM acetaldehyde+50 μg XO+2×10^{-3} M DETC	81*	—

Expt 1: Log_e l.s.d. = 0·15, corresponding to a difference of 1·5% at 10% isotope release, 5·5% at 35% and 8% at 50% release. Expt 2: Log_e l.s.d. = 0·26, corresponding to a difference of 2% at 10%, 10% at 35% and 15% at 50% isotope release.

* Significantly higher than control (no additions).

† Cysteine reduces the level of isotope release from that produced by H_2O_2 and superoxide respectively, but not completely to background level.

5×10^3 P815 cells, it takes 0·4 ml of 5×10^{-4} M H_2O_2, or 200 nmol of free H_2O_2, to kill this number of cells *in vitro*. However, less than 0·1 nmol H_2O_2 was produced during interaction with 5×10^4 T cells. Both purified T cells and unpurified spleen cells were active in the generation of hydrogen peroxide and in inducing isotope release.

Target of H_2O_2 attack

Since H_2O_2 is a powerful oxidizing agent chemical groups which are sensitive to oxidation may be particularly vulnerable. One candidate for attack is the sulphydryl group. When reagents which attack sulphydryl groups were added to radioactive P815 cells the cells rapidly lost ^{51}Cr (Table 4). N-ethylmaleimide (NEM) was toxic at a concentration of 10^{-4} M and *p*-Cl mercuribenzoate and iodoacetate at concentrations of 10^{-3} M. P815 cells were protected from the cytotoxic action of NEM by 2-fold concentrations of the reducing agents dithiothreitol (DTT), cysteine and diethyldithiocarbamate (DETC). DTT and cysteine also protected P815 from killing by H_2O_2, either added directly or formed from superoxide (Table 5). This suggests that H_2O_2 acts by oxidizing sulphydryl groups in the target cell, although the possibility that the reducing agents are protecting other sensitive components of the cell

TABLE 6. Inhibition of spleen cell cytotoxicity to P815 cells by agents which protect sulphydryl groups

Concentrations of DETC	None	5×10^{-6} M	1×10^{-5} M	2×10^{-5} M	5×10^{-5} M	1×10^{-4} M
Per cent release of ^{51}Cr from P815 cells	42	26	22	14	11	9
Concentrations of DTT	None	1×10^{-3} M	5×10^{-3} M	—	—	—
Per cent release of ^{51}Cr from P815 cells	37	33	12	—	—	—

Release in the absence of spleen cells = 9%.

Expt 1: Log_e l.s.d. = 0·24, corresponding to a difference of 3% at 10% isotope release, and 11% at 42% release. Expt 2: Log_e l.s.d. = 0·16, corresponding to a difference of 2% at 10% and 6% at 37% release.

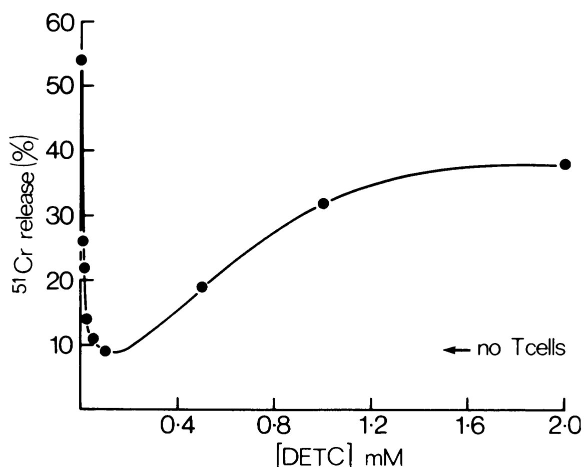

FIG. 1. Effect of DETC on spleen cytotoxicity. s.e.m. = 0·133. Log_e least significant difference = 0·37. This corresponds to a difference of 5% at 10% isotope release, and 15% at 31% release.

or might themselves dissipate the H_2O_2 cannot be excluded. The observed enhancement of the toxicity of H_2O_2 by DETC will be discussed later.

Evidence that the same mechanism is operating when spleen cells interact with target P815 cells is presented in Table 6. Not only does the sulphydryl protecting agent DTT protect P815 from the cytotoxic action of H_2O_2, it also protects P815 from spleen cells. The action of DETC is more complicated than that of DTT since in addition to being a reducing agent it is also a copper chelator, and an inhibitor of peroxidase and SOD. Nevertheless, at low concentrations it does protect P815 from cytotoxic spleen cells. At high concentrations, above 2×10^{-4} M this inhibition is partially reversed (Fig. 1).

Effect of copper chelators

The reactivation of the cytotoxic reaction of spleen cells by high concentrations of DETC could be due to the ability of DETC to chelate copper ions. This was tested by comparing DETC with another

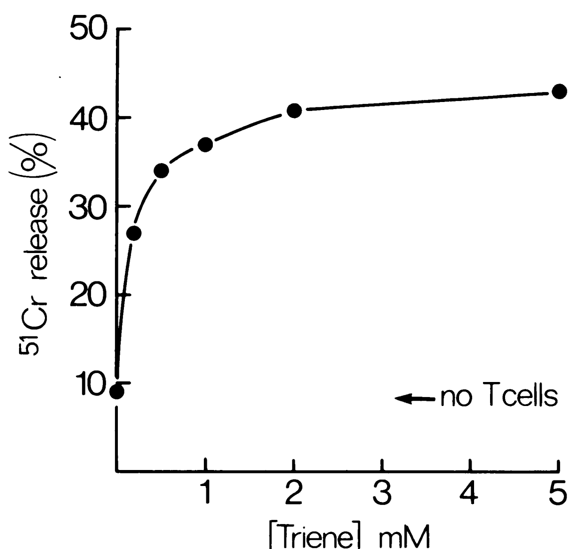

FIG. 2. Effect of triene on spleen cell cytotoxicity. Spleen cells were incubated in ^{51}Cr-labelled P815 cells in the presence of 0·1 mM DETC and increasing concentrations of triene. s.e.m. = 0·058: log_e least significant difference = 0·18. This corresponds to a difference of 2% at 10% isotope release, and 8% at 40% release.

TABLE 7. Inhibition of catalase, as measured with the oxygen electrode*

Additions	μ atoms oxygen/min (mean ± s.d.) Spontaneous	With catalase
None	0·09 ± 0·02	0·46 ± 0·02
2 mM DETC	0·02	0·19 ± 0·06
10 mM triene	0·025 ± 0·007	0·34 ± 0·07

* H_2O_2 (9·0 μmol) was incubated at 30°C in 0·1 M phosphate buffer pH 7·4, in a total volume of 3 ml in a Rank oxygen electrode (Rank Brothers, Bottisham, Cambridge). Dissolved O_2 was removed by bubbling in nitrogen. The rate of O_2 production from H_2O_2 was then measured before and after the addition of 50 ng catalase.

copper chelator, triene (Fig. 2). In the presence of a protective concentration of 0·1 mM DETC triene, at a concentration of 2 mM, mimicked high concentrations of DETC and reactivated the cytotoxic reaction of spleen cells.

In an attempt to explain the reactivation by copper chelators, the possibility that endogenous catalase, which would normally dissipate toxic H_2O_2, is being inhibited was investigated (Table 7). While 2 mM DETC did inhibit 50 ng beef liver catalase, as assayed with the O_2 electrode, triene at concentrations of up to 10 mM did not. However, both copper chelators did protect H_2O_2 from spontaneous breakdown, catalysed possibly by traces of metals. The initial reactivating effect of triene and DETC therefore appears to be a simple protection of the H_2O_2 formed from spontaneous breakdown, catalysed by heavy metals. The selective inhibition of catalase by DETC, but not by triene, explains the enhancing effect of DETC, but not triene, on H_2O_2 cytotoxic activity against P815 cells (Tables 5 and 7).

Effect of scavengers of oxidative intermediates

Evidence for the involvement of superoxide radicals, hydroxyl radicals or singlet oxygen in the killing of bacteria or in the destruction of tissue is usually obtained by demonstrating that the observed damage is prevented by a specific scavenger of the individual oxidative intermediate. Ferricytochrome *c* is used to scavenge superoxide radicals, diazabicyclooctane (DABCO) to remove singlet oxygen and mannitol or sodium benzoate to inactivate hydroxyl radicals (Nathan *et al.*, 1979b). None of these scavenging agents inhibited the cytotoxicity of spleen cells directly, which indicates that none of these oxidative intermediates are normally involved in T cell cytotoxicity. If, however, 2mM DETC was present, cytochrome *c* did inhibit cytotoxicity. Since DETC also inhibits SOD (Heikkila, Cabbat & Cohen, 1976) but not the spontaneous conversion of superoxide to H_2O_2, under these conditions superoxide may accumulate for long enough to be trapped by cytochrome *c*. When both SOD and cytochrome *c* are active all of the superoxide is converted to H_2O_2 by SOD; when SOD is inhibited by DETC cytochrome *c* can bind the superoxide and prevent its conversion to cytotoxic H_2O_2. Added catalase did not inhibit the cytotoxic activity of T cells which may be due to its inability to penetrate to the active site.

Effect of cyanide and azide

Potassium cyanide and sodium azide were tested as inhibitors of the cytotoxicity both of spleen cells and of H_2O_2 (Table 8). While 1 mM sodium cyanide and 10 mM sodium azide inhibited the cytotoxicity of spleen cells, cyanide had no effect on the toxicity of H_2O_2 or on its precursor superoxide. From this

TABLE 8. Effect of cyanide and azide on cytotoxicity of spleen cells and of hydrogen peroxide

Cytotoxic agent	Inhibitor	Per cent release of ^{51}Cr from P815
Experiment 1		
None	None	7
None	1 mM KCN	8
Spleen cells	None	60
Spleen cells	0·5 mM KCN	60
Spleen cells	1 mM KCN	11
Spleen cells	5 mM KCN	6
Spleen cells	2 mM Na azide	50
Spleen cells	10 mM Na azide	19
Experiment 2		
None	None	9
0·5 mM H_2O_2	None	36
0·5 mM H_2O_2	1 mM KCN	44
10 mM acetaldehyde + 50 μg XO	None	72
10 mM acetaldehyde + 50 μg XO	1 mM KCN	72

Expt 1: $\log_e$ l.s.d. = 0·28, corresponding to a difference of 3% at 8% isotope release and 15% at 60% release. Expt 2: $\log_e$ l.s.d. = 0·15, corresponding to a difference of 1% at 9%, 6% at 36% and 25% at 72% isotope release.

we conclude that the action of cyanide is on the effector spleen cell and not on H_2O_2 or on the target P815 cell.

DISCUSSION

The susceptibility of tumour cells to H_2O_2 is now well established. The mastocytoma P815 used in the present work was somewhat less sensitive than the many other cells described by Nathan *et al.* (1979a) since concentrations of 10^{-6} to 10^{-4} M H_2O_2 were found to be lytic under their conditions, while a concentration of 5×10^{-4} M H_2O_2 was needed for detectable cytotoxic activity against 5×10^3 P815 cells.

Little is known of the mechanism by which cytotoxic T cells kill their targets. DNA, RNA and protein synthesis are not required. The recognition phase can occur in the presence of either Mg^{2+} or Ca^{2+}, but the lethal hit phase requires Ca^{2+}. Compunds which affect microtubules or microfilaments, such as cytochalasin B, or alter cAMP or cGMP levels also affect cytotoxicity, as do very reactive compounds like azide or iodoacetate (for reviews see Golstein & Smith, 1977; Martz, 1977). All of these changes probably involve gross alterations in membrane activity of some kind, but the active material produced by the effector T cell which initiates cytotoxicity is not known.

If T lymphocyte cytotoxicity is mediated by H_2O_2 it should be possible to demonstrate that H_2O_2 is produced when T cells encounter a target cell, and to show that if production or utilization of this H_2O_2 is inhibited the cytotoxicity of the T cells is also inhibited. The demonstration of H_2O_2 formation has proved difficult since the amounts produced are so low. However, by using a sensitive fluorescent assay and by using large numbers of cells we have been able to detect the formation of small amounts of H_2O_2 when T cells interact with P815 cells. The concentration of H_2O_2 detected was less than one-thousandth of the amount necessary to kill P815 cells *in vitro*.

If this mechanism is functioning in the cytotoxic T cell the cell must presumably have a method of localizing the H_2O_2 produced to give a high concentration at particularly vulnerable points on the target.

It is therefore of considerable importance that Sanderson & Glauert (1977) showed that T cells throw out long projections which burrow into the target cell and possibly produce a local lesion in the mastocytoma cell. This could be the site of action of the H_2O_2. It should be emphasized that the levels of H_2O_2 detected by the present method represent minimum values since not only does the assay depend on catalase competing favourably with lactoperoxidase and both enzymes competing with any endogenous acceptors of the H_2O_2, but we have evidence that after formation the fluorescent product disappears.

Interpretation of the effects of inhibitors on T cell cytotoxicity is complicated by the number of separate components in the system. Inhibitors may act on the effector T cell, on the H_2O_2 produced, or by protecting the target mastocytoma cell. Inhibition by cyanide and by azide appears to act on the T cell, since these agents had no effect on *in vitro* toxicity by H_2O_2.

Inhibition of both T cell and H_2O_2 cytotoxicity by sulphydryl protecting agents suggests that H_2O_2 may attack sulphydryl groups in the target cell. Further evidence for this is that other agents which attack sulphydryl groups are also cytotoxic. However, the possibility that reducing agents protect target cells by protecting other sensitive components of the cell, or even perhaps act by dissipating the H_2O_2 cannot be excluded.

Cells which have been protected from T cell attack by low concentrations of the reducing agent DETC can be rendered sensitive again if a Cu chelating agent, such as DETC itself, or triene, is added to the system. Chelating agents appear to act by protecting the H_2O_2 from spontaneous breakdown, catalysed by metals in the medium. DETC enhances further the cytotoxicity of H_2O_2 by inhibiting endogenous, target cell catalase with which the cells are able partly to protect themselves.

The sensitivity of tumour cells to H_2O_2 *in vitro*, the production of H_2O_2 when T cells contact a target, albeit at very low and therefore perhaps highly localized concentrations, the inhibition of T cell cytotoxicity by cyanide and azide, the vulnerability of sulphydryl groups to T cells and to H_2O_2, the enhancement of cytotoxicity under certain conditions by Cu chelating agents which may act by inhibiting the target cell catalase, are all consistent with and evidence for a mechanism of T cell cytotoxicity which involves H_2O_2. We postulate that the mastocytoma interacts with the T cell inducing the production of H_2O_2, probably at a highly localized site. This H_2O_2 then attacks certain sulphydryl groups on the mastocytoma cells which initiates in the cell the series of reactions including violent blebbing or zeiosis (Sanderson & Glauert, 1977) which lead ultimately to its death.

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