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The Effect of Organophosphorus Inhibitors, *p*-Nitrophenol and Cytochalasin B on Cytotoxic Killing of Tumour Cells by Immune Spleen Cells, and the Effect of Shaking

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Summary. Organophosphorus (OP) agents are irreversible inhibitors of OP sensitive esterases. Their effect on the cytotoxic killing of ^{51}Cr-labelled mastocytoma cells by immune spleen cells was assessed by the release of ^{51}Cr. OP fluoridates inhibited cytotoxic killing whether used as a pretreatment before the addition of the labelled target cells or as a concurrent treatment. The effect of pretreatment was time, temperature and concentration dependent and was abolished by hydrolysis. It occurred at concentrations which did not inhibit the incorporation of [^{14}C] leucine and uridine. It is tentatively suggested that the OP agents inhibited an activated esterase present in the spleen cell before the cytotoxic reaction.

In contrast, phosphonates inhibited cytotoxic killing when used concurrently but had little effect as a pretreatment. The chemically more reactive *p*-nitrophenyl phosphonates and the much less reactive phenyl phosphonates inhibited cytotoxic killing to a similar degree. It was concluded that the phosphonates did not *irreversibly* inhibit activated esterase.

The effect of *phenyl* phosphonates was virtually abolished by hydrolysis. However the effect of the *p*-nitrophenyl phosphonates was increased by hydrolysis. This was due to the *p*-nitrophenol formed.

Cytochalasin B, which is known to interfere with cell movements, and *p*-nitrophenol, both of which inhibited spleen cell migration from a capillary tube, inhibited cytotoxic killing when used concurrently. This inhibition was partly reversed by shaking. It is suggested that movements of the cell surface may be essential, in the absence of shaking, to provide the contact needed for cytotoxic killing.

INTRODUCTION

The organophosphorus (OP) agents specifically and irreversibly inhibit esterases with serine in their active site (Aldridge, 1969). These esterases are sometimes called B esterases or serine esterases but the term OP sensitive esterases will be used in this paper. The OP agents only inhibit esterases in their active and not in their precursor form. For instance, the OP sensitive esterase trypsin, but not its precursor trypsinogen, is inhibited by di-isopropylphosphorofluoridate (DFP).

Becker and his co-workers (see Pearlman, Ward and Becker, 1969; Ward and Becker,

1970) used OP agents to define the role of esterases in chemotaxis, phagocytosis and histamine release from mast cells. Some OP agents inhibit chemotaxis when the cells are pretreated before exposure to the chemotactic factor. This inhibition is attributed to *activated* esterase, i.e. esterase present in an active form before the cell is exposed to chemotactic factor. Other OP agents are more effective when used concurrently so that the cells are exposed to the agent and the chemotactic factor simultaneously. This inhibition is attributed to an activatable esterase, i.e. esterase which becomes active on exposure to the chemotactic factor. The activation of an esterase may be an early step in initiating the sequences whereby certain cells respond to changes in their environment. This paper presents evidence for the role of activated esterase in cytotoxic killing of tumour cells by immune spleen cells but leaves open the question of the role of activatable esterase.

MATERIALS AND METHODS

Cytotoxic killing by immune spleen cells

Target cells: DBA/2 mouse mastocytoma cells (P-815-X2) kindly supplied by Dr K. T. Brunner were maintained in RPMI 1640 medium (Grand Island Biological Company) with 10 per cent foetal bovine serum and antibiotics. The cultures were always fed the day before cytotoxic experiments. It is necessary periodically to use a new batch of mastocytoma cells preserved by freezing as after continued passage *in vitro* the rate of cytotoxic killing is reduced.

Immunization: C57/Bl female mice about 8 weeks old were given 3×10^7 mastocytoma cells intraperitoneally and used 11–13 days later. The cells were prepared by intraperitoneal passage weekly in DBA/2 mice.

Medium: Eagle's minimum essential medium (Wellcome Reagents Ltd) containing cloxacillin and ampicillin (125 μg/ml) with 0·11 per cent sodium bicarbonate and 5 per cent heat inactivated foetal bovine serum (Flow Lab.) gassed with 5 per cent CO_2/air.

Cytotoxicity assay: Immune spleen cells and target cells labelled with ^{51}Cr were prepared following Brunner, Mauel, Rudolf and Chapuis (1970) except that the cells were labelled in the presence of 10 per cent foetal bovine serum. The cytotoxic killing was undertaken in 1·25 × 9-cm disposable flat bottomed test tubes (Stayne Laboratories Ltd) containing 0·5 ml medium with 5×10^6 spleen cells and 0·1 ml medium with $0{\cdot}5–1{\cdot}0 \times 10^5$ chromium labelled mastocytoma cells at 37° for 2 hours, unless otherwise stated. At the end of the experiment 1·5 ml medium was added and after centrifugation 1 ml supernatant was removed and counted in a Panax gamma counter. All experimental tubes were set up in duplicate. The OP agents did not kill spleen cells as judged by dye exclusion with 0·1 per cent eosin, in 1·2 per cent foetal calf serum at room temperature for 2 minutes. In some experiments the tubes were agitated by a horizontal platform with a rotary movement transmitted by 4·4 cm diameter wheels rotating at 140 rev/min at 37° in the hot room ('Rotatest', Luckham Ltd).

Cytotoxic killing by normal mouse spleen cells

Cytotoxic assay with antibody coated chicken red cells: chicken red cells were labelled following Perlmann, Perlmann and Holm (1968). Rabbits were immunized intravenously on several occasions with chicken red cells. After appropriate pretreatment, 0·5 ml con-

taining 5×10^6 of normal spleen cells, 0·5 ml 1/500 antibody and 0·1 ml containing 2×10^5-chromium-labelled chicken red cells were incubated and the chromium release assessed 24 hours later. $5–10 \times 10^6$ mouse red blood cells were added to all the tubes to reduce non-specific lysis in the control tubes without spleen cells.

Cytotoxic assay with phytohaemagglutinin (PHA): normal spleen cells (2×10^7/ml) were mixed with an equal volume of OP agents, incubated for 1 hour, washed twice and left at 37° for 20 hours with 1 μg/ml final concentration of purified PHA (Wellcome Reagents Ltd). Then ^{51}Cr-labelled mastocytoma cells (1×10^5 in 0·1 ml) and 3 μg PHA were added to 1×10^7 spleen cells in 1 ml and chromium release assessed 4 hours later. The final volume was 1·125 ml.

Organophosphorus agents and other chemicals

OP agents were prepared following Becker, Fukuto, Boone, Canham and Boger (1963). DFP (di-isopropylphosphorofluoridate) and *p*-nitrophenol (spectrophotometric grade) were from British Drug Houses and cytochalasin B was from Imperial Chemical Industries Ltd. OP agents are extremely toxic and contact with skin and inhalation was avoided. Nonvolatile OP agents were dissolved in acetone and added to tissue culture medium without serum. Glass tubes were usually used to minimize adsorption. Immediately these solutions were mixed with an equal volume of spleen cells. The final concentration of acetone was 0·25 or 0·125 per cent. DFP, which was volatile, was handled in a fume cupboard. It was weighed in a glass stoppered flask and was dissolved in added tissue culture medium. Further operations were carried out on an open bench. Glassware and plastic containers were decontaminated by soaking for 1 day or longer in 10 per cent alcoholic solution of caustic soda.

The OP agents were hydrolysed in two ways:

(a) solution in methanolic N/10 sodium hydroxide, heating at 60° for 30 minutes, followed by neutralization with hydrochloric acid, evaporation *in vacuo* which was facilitated by two additions of ether, and final solution in acetone.

(b) solution in aqueous N/50 sodium hydroxide, leaving at 20° for 18 hours, neutralization with hydrochloric acid and addition of the appropriate volume of ×10 concentrated Eagle's medium. Tubes to which unhydrolysed OP agents were to be added were treated in parallel.

Pretreatment with OP agents: spleen cells (1×10^7 ml) were incubated at 37° with the agent for 1 hour unless otherwise stated, diluted, spun down, and washed once. Labelled target cells were then added for cytotoxic assay. Spleen cells preincubated with acetone alone and then washed were used to measure the chromium release caused by immune spleen cells in the absence of inhibitor.

Concurrent treatment with OP agents: spleen cells were incubated with the labelled target cells and the agent concurrently. Spleen cells incubated with acetone but without the agent were used to measure the chromium release caused by immune spleen cells in the absence of inhibitor.

The results are given as a per cent inhibition of cytotoxicity which expresses the ability of the agent to inhibit the cytotoxicity of spleen cells.

$$\text{Percentage inhibition} = 100 \times \frac{^{51}\text{Cr released by immune spleen cells} - ^{51}\text{Cr released by immune spleen cells treated with OP agent}}{^{51}\text{Cr released by immune spleen cells} - \text{spontaneous release of } ^{51}\text{Cr}}.$$

Controls

The following controls were included:

(1) Total releasable radioactivity: this was the supernatant count after freezing and thawing and was about 85 per cent of the radioactivity of the target cells. The actual counts ranged from 6300–18400 in different experiments.

(2) Chromium release caused by immune spleen cells in the absence of inhibitors: a separate control was used in the pretreatment, concurrent treatment and shaking part of each experiment.

(3) Chromium release in medium alone or the presence of normal spleen cells: this is virtually unaffected by the presence or absence of normal spleen cells and the mean difference of these two observations is 0·1 per cent. Separate controls were used for the pretreatment, concurrent treatment and shaking part of each experiment. OP agents did not inhibit the release of chromium from labelled target cells which occurs either spontaneously at 37°, or on exposure to hypotonic medium or on freezing and thawing. The chromium release from target cells in the presence of immune spleen cells and in their absence (spontaneous release) is shown as a percentage of the total releasable radioactivity in the table of control data.

Statistics

All observations were performed in duplicate and the standard deviation calculated for each experiment using all the pairs of figures within that experiment. The standard error of the mean of each pair was then found by dividing the standard deviation by $\sqrt{2}$ and is shown after the first set of figures in the tables. It applies to all the data in the same column.

Incorporation of radioactive precursors into spleen cells

After pretreatment with OP agents and washing, 0·25 μCi DL-[1-^{14}C] leucine- (55·2 mCi/mM) or 0·1 μCi [U-^{14}C] uridine (502 mCi/mM; Radiochemical Centre, Amersham) was added to 5×10^6 spleen cells in 0·5 ml and incubated for 3 hours at 37°.

Two millilitres medium or phosphate buffered saline was added to each tube, the cells were spun down and resuspended in 0·5 ml; 0·1 ml was placed on filter paper discs (Whatman, grade 3MM size, 1·9 cm, W. & R. Balston Ltd). The discs were dried, washed in cold 10 per cent trichloroacetic acid for 30 minutes, and then four times in alcohol and twice in ether. They were finally dried, placed in counting pots, and scintillation fluid (3 G PPO, 0·3 G POPOP, Nuclear Enterprises Ltd, in 1L Analar toluene) was added. A Philips liquid scintillation analyser was used. (See Festenstein, 1968.)

Capillary cell migration

See Friedman, Sanz, Combe, Mills and Lee (1969). Chambers were made by attaching teflon plastic rings (Richard Klinger Ltd) with silicone grease (M 494, Imperial Chemical Industries Ltd) to microscope slides. Spleen cells were mixed with the inhibitor placed in 100-μl capillary tubes (Drummond Scientific Co.), sealed with Cristaseal (Hawksley & Sons Ltd) and centrifuged at 600 rev/min for 5 minutes in MSE Mistral centrifuge. The tubes were cut at the cell fluid interface, placed in the chamber and secured with silicone grease. The chambers were filled with medium containing the inhibitor and 10 per cent foetal calf serum and sealed with a cover slip. At 18 hours the chambers were projected

on paper and the outline of the cell migration area traced, cut out and weighed. Each observation was based on eight tubes in four chambers.

RESULTS

EFFECT OF PRETREATMENT WITH OP AGENTS ON CYTOTOXICITY AND LEUCINE AND URIDINE INCORPORATION BY IMMUNE SPLEEN CELLS

Pretreatment with OP fluoridates

Immune spleen cells kill mastocytoma cells (target cells) *in vitro*. This cytotoxic killing can be assayed by the release of ^{51}Cr from labelled tumour cells. In the pretreatment experiments immune spleen cells were pretreated with varying concentrations of OP agents. The cells were then washed and cytotoxic killing assessed (in the absence of OP agents) by adding chromium-labelled target cells.

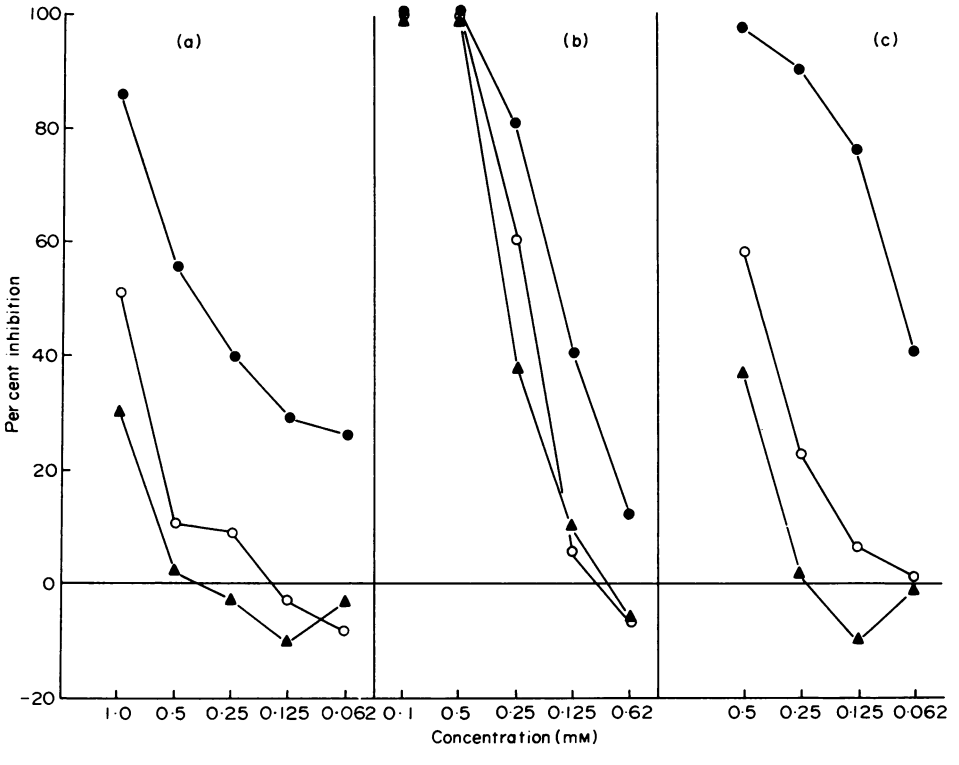

FIG. 1. The effect of pretreatment with OP fluoridates on immune spleen cells. The ordinate shows the per cent inhibition of (●) cytotoxic killing (as measured by ^{51}Cr release with 3-hours incubation); (○), leucine incorporation (▲), uridine incorporation caused by pretreatment of immune spleen cells with OP fluoridates. The cells treated with 1·0 and 0·5 mM cyclohexyl hexylphosphonofluoridate were dead by eosin dye exclusion. All the other cells were unaffected by the OP agents as judged by this test. (a) Cyclohexyl butylphosphonofluoridate; (b) cyclohexyl hexylphosphonofluoridate, (c) cyclohexyl phenylphosphonofluoridate.

Fig. 1 shows that the cytotoxic activity of immune spleen cells was reduced by pretreatment with fluoridates for 1 hour before adding ^{51}Cr-labelled target cells. The three

fluoridates tested depressed cytotoxic killing by 40 per cent or more at 0·25 mM. The most active fluoridate caused 40 per cent inhibition of cytotoxic killing at 0·06 mM. Fig. 1 also shows the effect of pretreatment with fluoridates on the incorporation of [^{14}C]leucine and uridine which were added after the fluoridates had been removed by washing. The three fluoridates tested depressed leucine and uridine incorporation when used at 0·125 mM or greater. However they caused no depression at 0·06 mM although this concentration caused 13–41 per cent inhibition of cytotoxic killing.

There are several criteria which should be met before the effect of an OP agent is provisionally attributed to the irreversible inhibition of an OP-sensitive esterase. These criteria are: (1) increase of effect with increasing concentration of OP agent. (2) decrease of effect at lower temperature. (3) increase of effect with increasing time of exposure. (4) abolition of effect by hydrolysis of the OP agent.

Fig. 1 shows that the inhibition of cytotoxic killing by fluoridates is dose dependent.

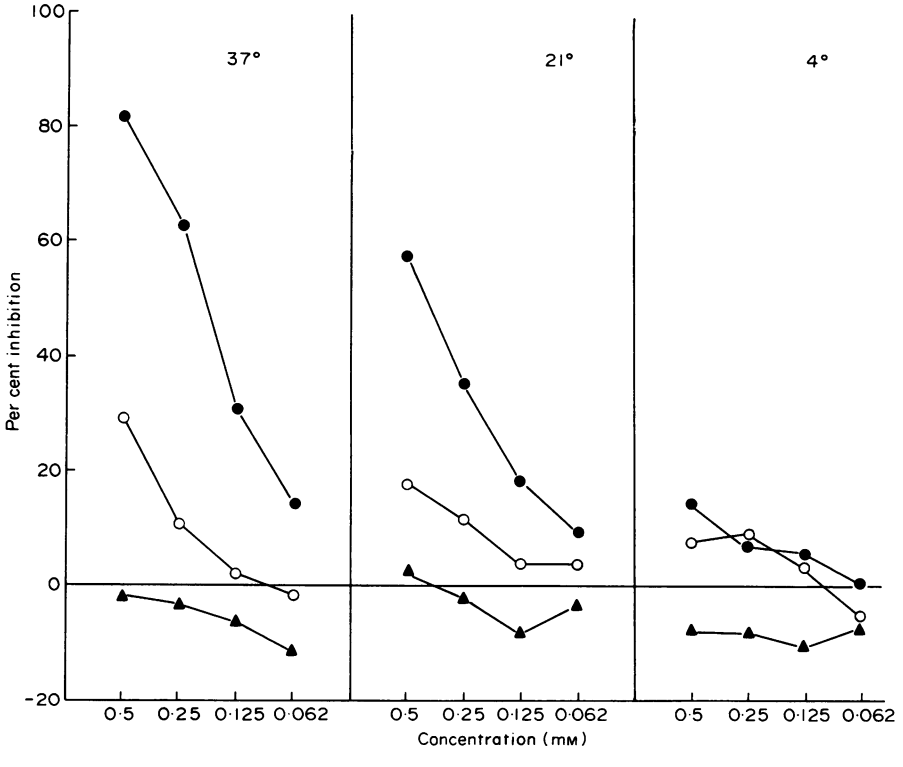

FIG. 2. The temperature dependence of the effect of pretreatment with an OP fluoridate. Per cent inhibition of (●) cytotoxic killing; (○) leucine incorporation; (▲) uridine incorporation. Immune spleen cells were pretreated with cyclohexyl phenylphosphonofluoridate at the temperature indicated. After washing cytotoxic killing was assessed after 4-hour incubation.

Fig. 2 shows that the action of one fluoridate tested was temperature dependent and pretreatment at 4° had little effect on cytotoxic killing and leucine and uridine incorporation. Fig. 3 shows that the effect of pretreatment with fluoridate increased linearly with time between 15 and 60 minutes. Table 1 shows that the one fluoridate tested almost completely lost its activity after hydrolysis in N/50 NaOH. It was concluded that pretreat-

ment with fluoridates depressed cytotoxic killing by immune spleen cells and that the characteristics of this inhibition were compatible with the fluoridates irreversibly inhibiting an OP sensitive esterase.

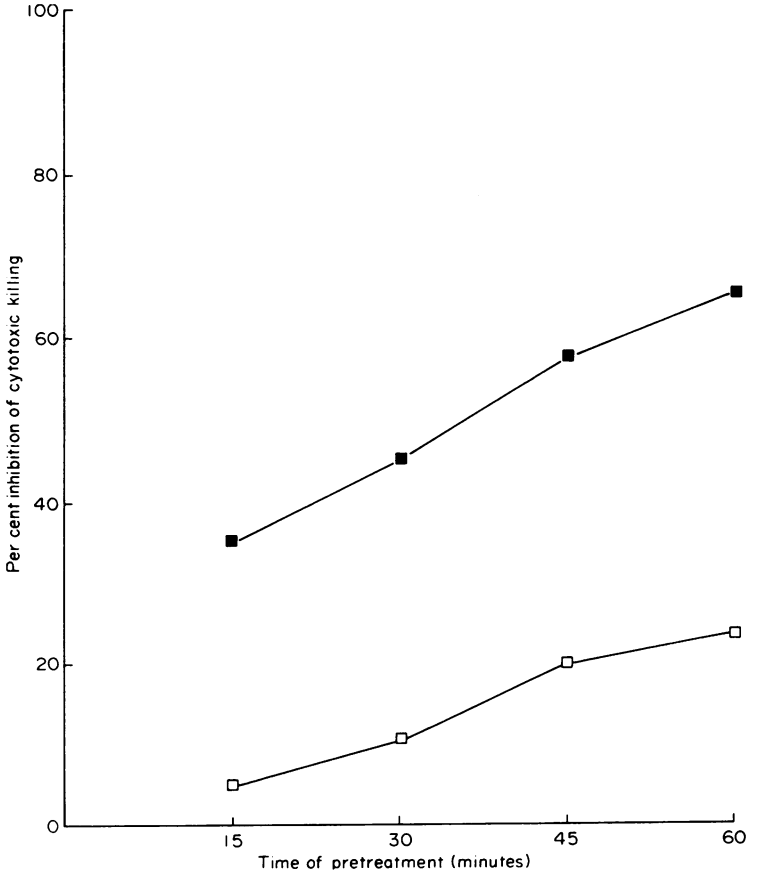

FIG. 3. The effect of time of pretreatment with OP agents on immune spleen cells. (■) Cyclohexyl butylphosphonofluoridate 0·66 mM; (□) *p*-nitrophenyl ethyl phenylphosphonate 0·5 mM.

Pretreatment with phosphonates

Fig. 4 shows that pretreatment with four of the five phosphonates tested had little or no effect on cytotoxicity at 0·5 mM and three of these agents had little effect even at 1·0 mM. The phosphonates only depressed cytototoxic killing at concentrations which also depressed leucine incorporation. In contrast the fluoridates depressed cytotoxic killing at concentrations which had no effect on [^{14}C]leucine and uridine incorporation. Fig. 3 shows that the effect of pretreatment with one phosphonate tested increased with time of incubation. It was concluded that pretreatment with phosphonates only depressed cytotoxic killing when present at high concentrations (1 mM) while the fluoridates caused depression at much lower concentrations (0·06 mM).

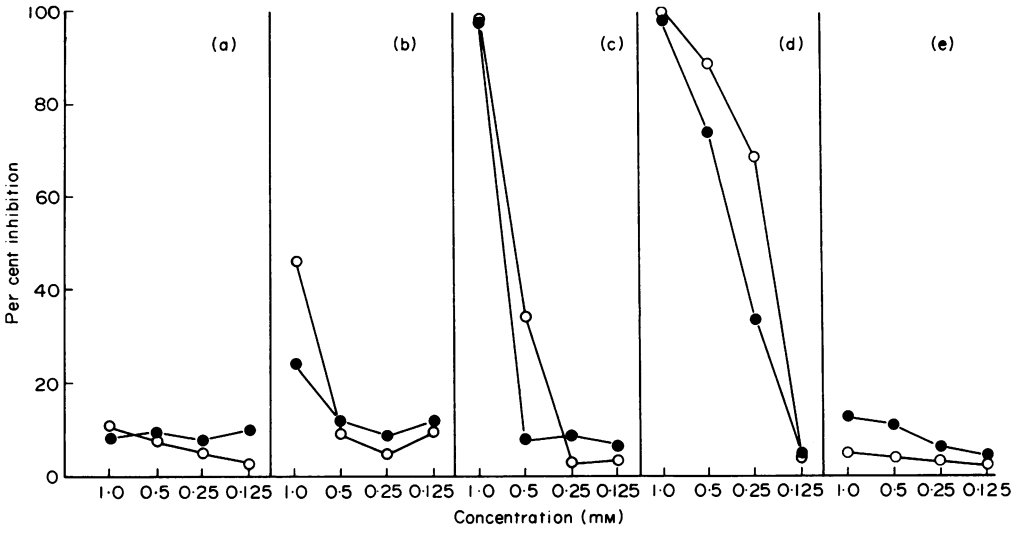

FIG. 4. The effect of pretreatment with phosphonates on immune spleen cells. Per cent inhibition of (●) cytotoxic killing; (○), leucine incorporation. (a) *p*-Nitrophenyl ethyl propylphosphonate, (b) *p*-nitrophenyl ethyl butylphosphonate, (c) *p*-nitrophenyl ethyl pentylphosphonate, (d) *p*-nitrophenyl ethyl hexylphosphonate, (e) *p*-nitrophenyl ethyl 6-aminohexylphosphonate.

TABLE 1

EFFECT OF HYDROLYSIS OF OP AGENTS ON THEIR ABILITY TO DEPRESS CYTOTOXIC KILLING BY IMMUNE SPLEEN CELLS

Exp.	Agent	Concentration (mM)	Inhibition of cytotoxic killing following (per cent): Pretreatment of the spleen cells Agent absent during reaction		Concurrent treatment Agent present during reaction	
			Unhydrolysed	Hydrolysed	Unhydrolysed	Hydrolysed
1*	Cyclohexyl butyl-phosphonofluoridate	1·0	90 (3·6)‡	8 (3·6)‡	93 (6·1)‡	0 (6·1)‡
2†	Phenyl ethyl butylphosphonate	1·0	0 (2·4)	4 (2·4)	52 (1·8)	14 (1·8)
		1·5		3		31
	p-Nitrophenyl ethyl butylphosphonate	1·0	17	12	43	91
		1·5		20		97
3†	*p*-Nitrophenyl ethyl butylphosphonate	0·5	8 (1·5)	3 (1·5)	41 (1·6)	93 (1·6)
	Phenyl ethyl butylphosphonate	0·75	0	2	25	12
	p-Nitrophenyl ethyl pentylphosphonate	0·5	2	4	75	93
	Phenyl ethyl pentylphosphonate	0·75	8	0	78	14
	p-Nitrophenyl ethyl phenylphosphonate	0·5	12	2	70	95
	p-Nitrophenyl ethyl 6-aminohexylphosphonate	1·0	16	8	62	98
	p-Nitrophenol	0·5	1		95	
		0·166	5		78	

In Experiments 1 and 3 the agents were hydrolysed in N/50 aqueous NaOH and in Exp. 2 in methanolic N/10 NaOH.
* The spleen cells were pretreated with the agent for 90 minutes and the cytotoxic killing assessed after 90 minutes.
† The spleen cells were pretreated with the agents for 2 hours.
‡ This standard error of the mean refers to all the data in the same experiment.

EFFECT OF OP AGENTS ON CYTOTOXICITY BY IMMUNE SPLEEN CELLS WHEN PRESENT DURING THE REACTION

In the following experiments OP agents were added to the immune spleen cells at the same time as the labelled target cells and were present during the cytotoxic killing. This is referred to as concurrent treatment with OP agents. Table 2 shows that the three fluoridates

TABLE 2

EFFECT OF PRETREATMENT AND CONCURRENT TREATMENT WITH OP AGENTS ON CYTOTOXIC KILLING BY IMMUNE SPLEEN CELLS

Exp.	Agent	Concentration (mM)	Inhibition of cytotoxic killing (per cent)	
			Pretreatment	Concurrent treatment
			Agent absent during reaction	Agent present during reaction
1*	Di-isopropyl phosphonofluoridate	10·0	89 (1·2)	94 (1·3)
		5·0	45	20
		1·6	38	18
		0·74	20	21
		0·4	12	16
2†	Cyclohexyl butyl-phosphonofluoridate	0·67	76 (3·9)	83 (4·5)
		0·44	63	51
		0·29	47	43
3*	Cyclohexyl phenyl-phosphonofluoridate	0·2	73 (1·8)	80 (2·4)
		0·1	47	56

* The spleen cells were pretreated for 2 hours.
† The cells were pretreated for 35 minutes and the cytotoxic killing was assessed after 35 minutes.

tested depressed cytotoxic killing to a similar extent whether used for pretreatment or concurrent treatment. Table 1 shows that the six phosphonates tested depressed cytotoxic killing when used for concurrent treatment but had little or no effect when used for pretreatment.

The effect of hydrolysis on the activity of the phosphonates depended upon the class of phosphonate. The phenyl phosphonates which liberate phenol on hydrolysis lost most of their activity after exposure to N/50 sodium hydroxide. In contrast, the *p*-nitrophenyl phosphonates caused greater inhibition of cytotoxic killing following hydrolysis (concurrent treatment). This paradoxical result was due to the liberation of *p*-nitrophenol following hydrolysis. The last line of Table 1 shows that this agent was a more effective inhibitor of cytotoxic killing when used for concurrent treatment than any of the OP agents.

EFFECT OF SHAKING ON INHIBITION OF CYTOTOXIC KILLING BY OP AGENTS AND *p*-NITROPHENOL

The possibility that OP agents acted by interfering with cell movement prompted a study on the effect of shaking the cells on the ability of various agents to inhibit cytotoxic killing. Table 3 shows that cytochalasin B, which is known to inhibit cell movement, inhibits cytotoxic killing about 85 per cent when present at 2 μg/ml. However when the tubes are shaken during the reaction only 40 per cent inhibition occurs. Table 3 also shows that the inhibition caused by *p*-nitrophenol (depending on its concentration) is partially or completely reversed by shaking. Direct evidence that these agents reduce cell

movement was provided by the finding that cytochalasin B (1·6 μg/ml) and *p*-nitrophenol (0·2 mM) depressed the migration of spleen cells from capillary tubes by 28 and 25 per cent. Depression of mobility may be the chief mode of action of cytochalasin B as even at 2·5 μg/ml it caused only 6 per cent depression of incorporation of [^{14}C]leucine while *p*-nitrophenol (0·2 mM) has other effects on the cell as it depressed leucine incorporation by 36 per cent.

TABLE 3

THE EFFECT OF SHAKING ON THE INHIBITION OF CYTOTOXIC KILLING CAUSED BY CONCURRENT TREATMENT WITH OP AND OTHER AGENTS

Exp.	Agent	Concentration (mM)	Inhibition of cytotoxic killing (per cent) Unhydrolysed Unshaken	Unhydrolysed Shaken	Hydrolysed Unshaken	Hydrolysed Shaken
1	Cyclohexyl butyl-phosphonofluoridate	0·5	69 (2·0)	77 (2·3)		
		0·25	49	48		
	p-Nitrophenyl ethyl phenylphosphonate	0·5	73	64	93 (2·0)	41 (2·3)
	p-Nitrophenyl ethyl pentylphosphonate	0·5	77	63	93	36
	Phenyl ethyl penthylphosphonate	0·5	52	41	4	5
	p-Nitrophenol	0·5	94	50		
		0·16	64	4		
	Cytochalasin B	2 μg/ml	85	40		
2	*p*-Nitrophenyl ethyl butylphosphonate	0·25	14 (3·6)	12 (2·3)	82 (3·6)	26 (2·3)
	Phenyl ethyl butylphosphonate	0·5	4	3	13	13
		0.25	0	0	8	8
	p-Nitrophenyl ethyl pentylphosphonate	0·25	11	14	83	22
	Phenyl ethyl pentylphosphonate	0·5	23	22	5	11
		0·25	0	0	6	12
	p-Nitrophenyl ethyl phenylphosphonate	0·25	30	25	84	24
	p-Nitrophenol	0·25	86	19		
		1·125	49	12		
	Cytochalasin B	0·62 μg/ml	54	38		
		0·31 μg/ml	39	19		
3	Cyclohexyl phenyl-phosphonofluoridate	0·2	80* (2·4)	71 (4·0)		
		0·1	56*	52		
	p-Nitrophenol	0·16	57	0		

The agents were hydrolysed in N/50 aqueous NaOH.
* These figures are also shown in Exp. 3, Table 2.

The inhibition caused by *p*-nitrophenyl phosphonates was not reversed by shaking. (Table 3). This showed that their action was not due to the formation of *p*-nitrophenol by hydrolysis. In contrast the action of hydrolysed *p*-nitrophenyl phosphonates was reversed by shaking suggesting that the responsible agent was *p*-nitrophenol.

The inhibition caused by fluoridates was little affected by shaking (see Table 3). Pretreatment with cyclohexyl phenylphosphonofluoridate at 0·3, 0·15 and 0·075 mM inhibited cytotoxicity by 81, 57 and 33 per cent. Shaking reduced these figures to 74, 55, and 28 per cent. It was concluded that shaking had a different effect on the inhibition

TABLE 4

EFFECT OF PRETREATMENT WITH OP AGENTS ON CYTOTOXIC KILLING BY NORMAL SPLEEN CELLS

Exp.	Cytotoxic system	Target	Agent	Concentration (mM)	Inhibition of cytotoxic killing (per cent)
1	Normal spleen cells + PHA	Mastocytoma cells	Cyclohexyl butyl-phosphonofluoridate	0·75	81 (2·0)
				0·25	72
2	Normal spleen cells + PHA	Mastocytoma cells	Cyclohexyl butyl-phosphonofluoridate	0·5	82 (1·8)
			p-Nitrophenyl ethyl 4-chlorobutylphosphonate	1·0	42
3	Normal spleen cells + PHA	Mastocytoma cells	*p*-Nitrophenyl ethyl 6-aminohexylphosphonate	1·0	8 (4.0)
			Cyclohexyl hexyl-phosphonofluoridate	1·0	35
			*Cyclohexyl butyl-phosphonofluoridate	1·0	45
4(a)	Normal spleen cells	Chicken RBC coated with antibody	*Cyclohexyl butyl-phosphonofluoridate	1·0	28 (2·9)
			Cyclohexyl phenyl-phosphonofluoridate	1·0	58
(b)	Immune spleen cells	Mastocytoma cells	*Cyclohexyl butyl-phosphonofluoridate	1·0	29 (0·9)
			Cyclohexyl phenyl-phosphonofluoridate	1·0	89

* The Cyclohexyl butyl phosphonofluoridate was from an old batch.

TABLE OF CONTROL DATA

Experiment	Percentage of chromium release from mastocytoma cells			
	In presence of immune spleen cells		In absence of immune spleen cells	
	Pretreatment	Concurrent treatment	Pretreatment	Concurrent treatment
Table 1. Exp. 1	27	18	8	7
Exp. 2	42	56	10	10
Exp. 3	67	63	9	8
Table 2. Exp. 1	100	92	10	8
Exp. 2	23	18	9	6
Exp. 3	62	47	11	8
Table 3. Exp. 1	36	35	7	9
Exp. 2	47	73	6	10
Exp. 3	47	34	8	11
Table 4. Exp. 1	24	—	12	—
Exp. 2	41	—	17	—
Exp. 3	27	—	14	—
Exp. 4a	39	—	16	—
4b	82	—	9	—
Fig. 1.	42	—	8	—
Fig. 2.	97 (37°)	—	13	—
	93 (21°)	—	—	—
	92 (4°)	—	—	—
Fig. 3.	72	—	12	—
Fig. 4.	64	—	9	—

In experiments in Table 3 the percentages under the pretreatment and concurrent treatment columns refer to without and with shaking respectively.

In Experiments 1, 2 and 3 of Table 4 the cytotoxic percentage killing by normal spleen cells was measured using spleen cells preincubated with PHA and mixed with the target cells in the presence of PHA. The percentage spontaneous release was measured using spleen cells preincubated alone and then mixed with target cells in the presence of PHA.

The spontaneous release by target cells alone or with PHA, or target cells in the presence of normal spleen cells and the absence of PHA varies from 7–8 per cent.

caused by cytohalasin B and *p*-nitrophenol on the one hand and the OP agents on the other. This presumably reflects different modes of action of these inhibitors.

EFFECT OF OP AGENTS ON CYTOTOXICITY OF NORMAL SPLEEN CELLS

Cytotoxicity by immune spleen cells may involve two steps—the production of receptor or antibody against the target cell, followed by the actual cytotoxic step. The first step is presumably absent when normal spleen cells kill antibody coated target cells or when phytohaemagglutinin is used to render spleen cells cytotoxic. Table 4 shows that pretreatment with OP fluoridates inhibited cytotoxic killing by normal spleen cells in the presence of PHA. It also inhibited cytotoxic killing of chicken red cells coated with antibody.

DISCUSSION

Organophosphorus agents may block biological phenomena in at least three different ways:

(1) they may act as surface active agents. Such effects are characteristically rapid in onset, not critically dose dependent and little influenced by temperature.

(2) they may reversibly inhibit enzymes such as esterases and in this event their action disappears when they are washed off.

(3) they may irreversibly inhibit an OP sensitive esterase. This only occurs when the esterase is in its active form and not in an inactive precursor form.

Such inhibition is temperature, time and concentration dependent. The present experiments were undertaken to see if there was evidence for the involvement of OP sensitive esterases in cytotoxic killing. The problems posed by the OP fluoridates and the phosphonates will be discussed separately.

The fluoridates inhibited cytotoxic killing by immune spleen cells to a similar extent whether used as a pretreatment or as a concurrent treatment (i.e. while the cytotoxic effect was actually occurring). The inhibition of cytotoxicity was time, temperature and concentration dependent, and was abolished by hydrolysis of the inhibitors. Moreover, the fluoridates partially inhibited cytotoxic killing at concentrations which did not affect protein and RNA synthesis as measured by [^{14}C]leucine and uridine uptake. These considerations suggest that the fluoridates acted as irreversible inhibitors of an activated esterase, i.e. an esterase, present in the spleen cells in active form before contact with the target cells. The fact that the OP fluoridates were equally active when used concurrently or for pretreatment is some evidence against the importance of an activatable esterase. (See Introduction.)

In contrast to the fluoridates, the phosphonates had little effect when used as pretreatment. This may reflect their limited ability to inhibit the particular activated esterase involved in cytotoxic killing. The phosphonates, however, were moderately effective inhibitors of cytotoxic killing when used concurrently. They do not act by irreversibly inhibiting activated esterases, i.e. esterases already present in an active form before the cytotoxic reaction, as they have little effect when used as a pretreatment. It is also unlikely that they act by irreversibly inhibiting an activatable esterase which was only converted from the precursor to the active form during the cytotoxic reaction. The evidence for this is that the *p*-nitrophenyl phosphonates and the phenyl phosphonates both inhibited cytotoxic

killing to a similar degree when used concurrently, despite the fact that the *p*-nitrophenyl phosphonates are much more effective irreversible enzyme inhibitors than their phenyl analogues. The question remains open whether phosphonates act by reversibly inhibiting an activatable or activated esterase or whether they act in some other way.

Hydrolysis abolishes the ability of phenyl phosphonates to inhibit cytotoxic killing (concurrent treatment). However hydrolysis actually increased the ability of *p*-nitrophenyl phosphonates to inhibit cytotoxic killing in concurrent treatment. This was attributed to the liberation of *p*-nitrophenol and confirmed by studying the effects of shaking.

Cytochalasin B interferes with actomyosin and thereby limits cell movements (see Wessells, Spooner, Ash, Bradley, Luduena, Taylor, Wrenn and Yamada, 1971). The present results show that inhibition of cytotoxic killing produced by cytochalasin B and *p*-nitrophenol was partially reversed by shaking. This suggested that at least some of the action of cytochalasin B and *p*-nitrophenol is due to an inhibition of cell movements. The fact that the inhibition caused by fluoridates or phosphonates was not reversed by shaking suggests that these agents do not act primarily by inhibiting cell movements.

The reversal of the effect of cytochalasin B and *p*-nitrophenol by shaking has some theoretical interest. Both agents inhibit the migration of spleen cells from capillary tubes and it is reasonable to think that they act in part by reducing cell movements. One possibility is that a minimal area of contact is needed for cytotoxic killing in the present system and this is normally provided by chance contact followed by movement of the plasma membranes which leads to an increase in the area of contact. Shaking may compensate for this lack of movement by pushing the cells together.

The general conclusion is that pretreatment with fluoridates probably acts by irreversible inhibition of an activated OP sensitive esterase which is required for cytotoxic killing. If this interpretation is correct, cytotoxic killing by immune spleen cells resembles polymorph chemotaxis and phagocytosis which are inhibited by OP agents in a way which suggests the role of an activated esterase. Such enzyme inhibition might affect one of the two steps of this killing reaction—either the production of receptor antibody which enables the spleen cells to interact with the target cells, or the killing reaction itself. The finding that pretreatment with OP fluoridates inhibited cytotoxic killing by normal spleen cells when PHA was added or when antibody was passively provided suggests that the OP fluoridates interfere with the cytotoxic step and not with the production of receptor antibody (Mauel, Rudolf, Chapuis and Brunner, 1970). This is corroborated by the observation that the fluoridate pretreatment diminished cytotoxicity at concentrations which did not affect protein synthesis. The present data do not exclude the existence of an activatable esterase which becomes active when the spleen cell interacts with the target cell and the question remains open.

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