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TOXICITY OF ORGANOTIN COMPOUNDS FOR POLYMORPHONUCLEAR LEUKOCYTES: THE EFFECT ON PHAGOCYTOSIS AND EXOCYTOSIS

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Abstract—Phagocytosis and concomitant release of enzymes by rabbit polymorphonuclear leukocytes (PMNs) are inhibited by micromolar concentrations of triphenyltin and tributyltin; inhibition by triethyltin occurs at higher concentrations. Chemotactic peptide-induced exocytosis is inhibited at the same concentrations as phagocytosis. Tributyltin causes cell lysis at slightly higher concentrations as required for inhibition of phagocytosis and exocytosis. The organotin compounds have little effect on ATP level in PMNs, which makes an effect on metabolic energy providing processes unlikely. The increase of Ca^{2+}-permeability of the plasma membrane, induced by chemotactic peptide, is inhibited by the organotin compounds. Inhibition of exocytosis by triphenyltin can be counteracted by a number of sulfhydryl compounds. The results suggest that the organotin compounds interfere with PMN function in an early phase of cell activation, where all functions have a common pathway, and where vulnerable sulfhydryl groups play a pivotal role.

A number of organotin compounds have been used on an extensive scale because of their biocidal properties. Triphenyltin halide is widely used as an agricultural fungicide and agaricide. Tributyltin compounds have applications as an industrial biocide and are used as a molluscicide. Trialkyltin salts are very effective against several fungi. The toxicity of these compounds, especially of triethyltin, has in some cases led to intoxication because their use resulted in exposure of man to organotin residues [1–3]. There have been a number of reports in literature about the effects of organotin compounds on animals and on mammalian cells. Vos *et al.* [4] found that triphenyltin interfered with the immune system of the rat. Studying the effects of triorganotin compounds in male rats, Snoeij *et al.* [5] found that tributyltin and triphenyltin are primarily immunotoxic, whereas triethyltin is essentially neurotoxic. The alkyltin compounds with short chainlength of the alkyl group are potent neurotoxicants [1, 6]. Trialkyltin compounds and triphenyltin cause hemolysis of erythrocytes, though erythrocytes of different species differ in sensitivity [7].

A number of important enzymes and biochemical systems are sensitive to organotin compounds. In low concentrations trialkyltin compounds inhibit oxidative phosphorylation in mitochondria; triphenyltin inhibits ATPase activity of submitochondrial particles from beef heart mitochondria [8–11]. Triethyltin is a strong inhibitor of hexokinase and may therefore interfere with glycolysis [12, 13].

Polymorphonuclear leukocytes (PMNs) play an important role in the defense of the body against infection by microorganisms. By means of phagocytosis and subsequent killing of invaders they represent the primary defense line of the body against infection. Impairment of phagocytosis and related activities of the PMN may result in an enhanced susceptibility for infection [15, 16]. Recently it has been found that some PMN functions, such as chemotaxis and the production of superoxide radicals in the metabolic burst, were inhibited by triphenyltin [17–19].

In this study we investigated the effect of triphenyltin, tributyltin and triethyltin on phagocytosis and degranulation by rabbit PMNs. Degranulation occurs concomitant with phagocytosis, but may also be induced in the absence of phagocytosis by certain soluble activators, such as the chemotactic peptide formylmethionylleucylphenylalamine (fMLP) in the presence of cytochalasin B; in that case degranulation is measured as exocytosis of granule constituents.

MATERIALS AND METHODS

PMNs. Polymorphonuclear leukocytes were obtained from rabbits, injected intraperitoneally with 200 ml isotonic saline containing 1.5 mg/ml glycogen. After 4 hr the exudate was collected by flushing the peritoneal cavity with isotonic saline containing citrate (0.4%, pH 7.4). The cells were centrifuged and washed with medium. The medium used consisted of 140 mM NaCl, 5 mM KCl, 10 mM glucose and 20 mM Hepes pH 7.3. In the exocytosis experiment the medium was supplemented with 1 mM Ca^{2+}, in the phagocytosis experiments 1 mM Ca^{2+} and 1 mM Mg^{2+} was present. The final cell suspension during the experiments contained 3×10^6 PMNs per ml.

Phagocytosis. Uptake of opsonized zymosan was

taken as a measure of phagocytosis. Zymosan was opsonized by incubating 50 mg zymosan with 10 ml rabbit serum for 30 min at 37°, followed by extensive washing with medium to remove adherent serum. 3×10^6 PMNs per ml were preincubated with or without tin compound for 5 min at 37°. After preincubation opsonized zymosan (0.5 mg/ml, final concentration) was added, followed by incubation for 30 min at 37°. The mixture was centrifuged, and the release of enzymes in the supernatant was determined.

To the residue was added 5×10^{-3} M EDTA to block phagocytosis. Subsequently the zymosan particles taken up were counted using oil immersion microscopy. A magnification of 1000× enables a reasonable discrimination between uptake and adherence. In case of doubt the particle was considered as adherent and not as being phagocytized. Cells with two or more zymosan particles were counted as phagocytic.

Exocytosis. Exocytosis was measured as the release of the granule-associated enzyme lysozyme, under conditions that there was no significant release of the cytoplasmic marker enzyme lactate dehydrogenase (LDH). As a stimulus to induce exocytosis (in the absence of phagocytosis) the chemotactic peptide formylmethionylleucylphenylalanine (fMLP) (10^{-8}) in the presence of cytochalasin B (5×10^{-6} M) was used.

PMNs were preincubated with or without tin compound for 5 min at 37°, then fMLP + cytochalasin B were added, followed by incubation for 30 min at 37°. The mixture was centrifuged and enzyme release in the supernatant was determined.

Lysozyme release and LDH release were determined as described previously [20].

ATP. For the determination of ATP, all cellular processes were blocked by adding an equal volume of ice-cold trichloroacetic acid (10%). After 10 min the mixture was neutralized with sodium acetate (1.5 M). The ATP content was determined by the bioluminescence technique, using luciferin-luciferase from fire-fly tails, as described by Strehler [21]. In the experiments where the ATP level was measured, glucose was omitted from the medium.

[^{45}Ca] *uptake.* The association of [^{45}Ca] with the cell was measured as described by Korchak *et al.* [22] with minor modifications. Cells were preincubated with or without tin compounds for 5 min at 37°. Then, together with the activator fMLP + cytochalasin B, 1 μCi [^{45}Ca] was added to the cells to a final Ca^{2+} concentration of 1 mM. After incubation for 5 min at 37° the cells were rapidly filtered through Schleicher and Schuell BQ85 filters with a pore-width of 0.45 μm. The filters were washed and dried, and subsequently the radioactivity associated with the cells was measured.

In control experiments the effect of tin compounds on fMLP + cytochalasin B-induced exocytosis was measured after an incubation time of 5 min.

Materials. Triphenyltinchloride and triethyltinbromide were obtained from Ventron GmbH, Karlsruhe, F.R.G.; triethyltinchloride and other chemicals were obtained from Sigma Chemical Co, St. Louis, MO. From the tin compounds a concentrated solution was made in ethanol; immediately before use this was diluted in medium, and added to the mixture of reagents.

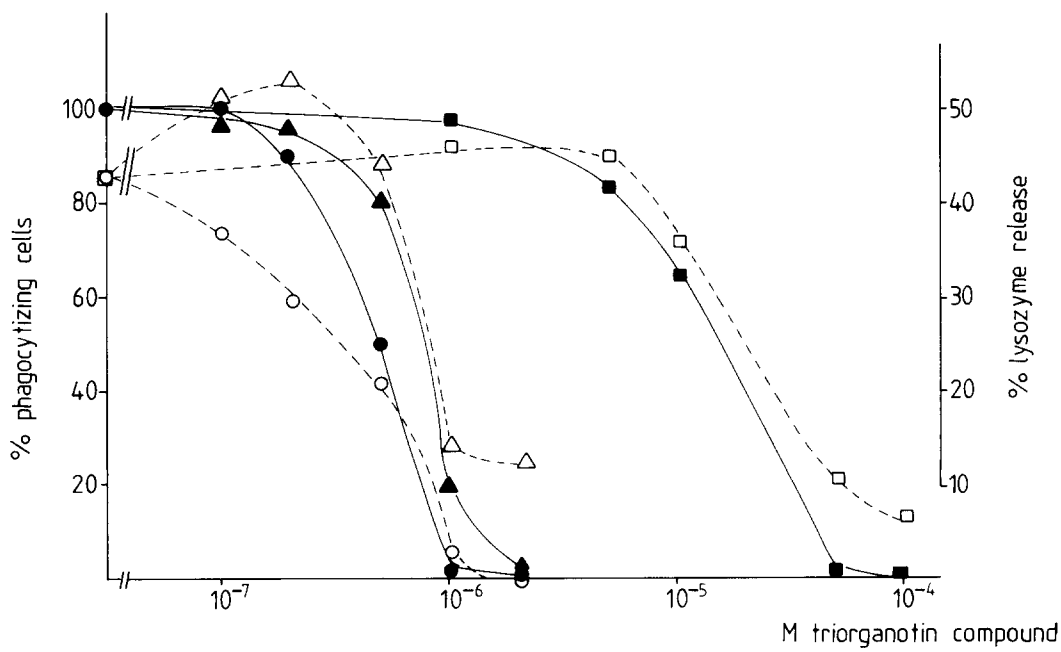

Fig. 1. Effect of triorganotin compounds on phagocytosis of opsonized zymosan, and on concomitant lysozyme release. The values given are the mean value of three experiments. —●— triphenyltin, phagocytosis; —○— triphenyltin, lysozyme release; —▲— tributyltin; phagocytosis; —△— tributyltin, lysozyme release; —■— triethyltin, phagocytosis; —□— triethyltin, lysozyme release.

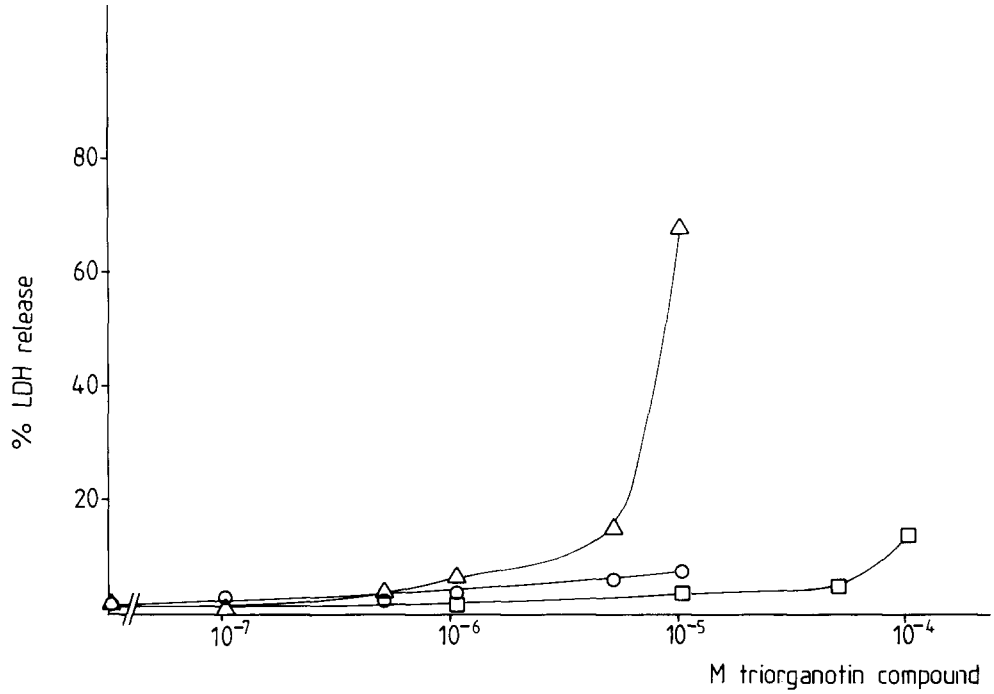

Fig. 2. Effect of triorganotin compounds on cell integrity: LDH release as a function of organotin concentration. Cells were incubated with the indicated amount of organotin compounds for 30 min at 37°, followed by centrifugation and analysis of the supernatant. —○— triphenyltin; —△— tributylin; —□— triethyltin. The values given are the mean values of three experiments.

RESULTS

The organotin compounds inhibit phagocytosis and concomitant enzyme release (Fig. 1). Inhibition by triphenyltin and tributyltin occurs in the micromolar concentration range, inhibition by triethyltin requires a 50-fold higher concentration. Inhibition of phagocytosis and concomitant enzyme release occurs at the same concentration.

Triphenyltin and triethyltin do not cause significant cytolysis at concentrations slightly higher than those used in our experiments. Tributyltin is cytolytic at a concentration (10 μM) which is slightly higher than the inhibitory concentration. This is demonstrated by the strong release of the cytoplasmic enzyme LDH at that concentration (Fig. 2).

Exocytosis, induced by the chemotactic peptide fMLP, in the presence of cytochalasin B, can be measured by determining lysozyme release in the range where no LDH release is observed. Exocytosis is inhibited by the tin compounds in the same way as phagocytosis: a strong inhibition by triphenyl and tributyltin in the micromolar concentration range, inhibition by triethyltin at higher concentrations (Fig. 3). The strong increase in lysozyme release at 10 μM tributyl tin coincides with LDH release (Fig. 2) and is apparently due to cytolysis.

The classical inhibitors of glycolysis, such as deoxyglucose, iodoacetate and fluoride cause a strong decrease of ATP level in resting PMNs. As compared with these compounds, the organotin compounds have little effect on ATP level, even in concentrations which are higher than those at which phagocytosis and degranulation are inhibited (Table 1).

In a number of cases a strong reduction of the inhibitory power is observed, when triphenyltin is preincubated with sulfhydryl compounds, followed by exposure to PMNs and induction of exocytosis. Reduction of inhibition strongly depends on the type of sulfhydryl compound. Inhibition is most strongly

Table 1. Effect of organotin compounds on ATP level of polymorphonuclear leukocytes

	nmol ATP/10^8 cells	% of control
—	52.9 ± 0.8	100
1 μM triphenyltin	50.0 ± 1.2	95
5 μM triphenyltin	49.7 ± 1.7	94
1 μM tributyltin	49.2 ± 1.9	93
5 μM tributyltin	44.8 ± 1.9	85
50 μM triethyltin	50.6 ± 1.8	96
200 μM triethyltin	48.5 ± 2.0	92

Cells (3×10^6/ml) were incubated for 20 min with the given concentration of organotin compound, after which the reaction was blocked by adding trichloroacetic acid. The values given are the mean values of three experiments ± S.D. The ATP content of cells which were exposed to 10 mM 2-deoxy-D-glucose, 1 mM iodoacetate or 20 mM sodium fluoride for 20 min, was reduced to 19, 20 and 6% of control, respectively.

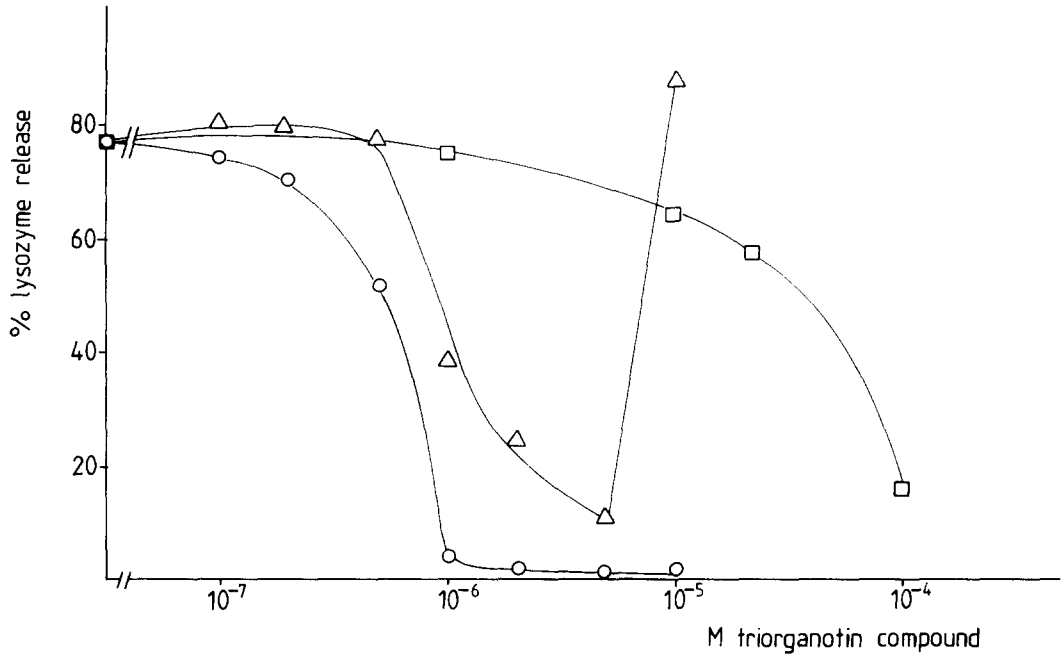

Fig. 3. Lysozyme release from fMLP ± cytochalasin B activated cells as a function of triorganotin compounds —○— triphenyltin; —△— tributyltin; —□— triethyltin. Values given are the mean value of four experiments.

reduced by dithiothreitol, sodium sulfide and dimercaptoethanol. Glutathione and mercaptoethanol are less effective, and cysteine has no effect on the inhibitory potency of triphenyl tin (Table 2).

During activation of the PMN by the chemotactic peptide fMLP a strong increase of [^{45}Ca] association with the cell can be observed, due to an increased permeability of the plasma membrane for Ca^{2+}. All tin compounds tested inhibit the fMLP (+ cytochalasin B)-induced increase of [^{45}Ca] association at concentrations which are inhibitory for phagocytosis and exocytosis (Fig. 4).

DISCUSSION

The toxic effect of organotin compounds on polymorphonuclear leukocytes is evident on two levels. Tributyltin has a cytotoxic effect; the release of the

Table 2. Effect of sulfhydryl compounds on triphenyltin inhibition of CB/fMLP induced exocytosis

	Lysozyme release (%)		
SH-compound	Without TPhT 100 μM SH compound	With 2 μM TPhT 10 μM	With 2 μM TPhT 100 μM SH compound
—	80 ± 5		2 ± 2
Dithiothreitol	86 ± 5	82 ± 5	87 ± 6
Glutathione	88 ± 4	2 ± 1	39 ± 4
Mercaptoethanol	90 ± 3	9 ± 3	18 ± 3
Cysteine	66 ± 10	4 ± 2	3 ± 2
BAL	67 ± 11	77 ± 6	65 ± 12
Sodium sulfide	86 ± 9	83 ± 8	81 ± 6

The sulfhydryl compounds in the concentrations indicated were exposed to 2 μM triphenyltin (TPhT) for 5 min at 37°. Then PMNs were added and after preincubation for another 5 min the activator CB/FMLP was added, followed by incubation for 30 min at 37°. To establish an effect of the sulfhydryl compounds on exocytosis, PMN's were exposed to 100 μM sulfhydryl compounds and after preincubation for 5 min. treated with activator CB/fMLP (left column). The results given are the mean value of four experiments ± SD.

CB: 5×10^{-6} M cytochalasin B; fMLP: 10^{-8} M formylmethionylleucylphenylalanine; TPhT: triphenyltinchloride.

BAL: British Anti Lewisite = 2,3-dimercapto-1-propanol.

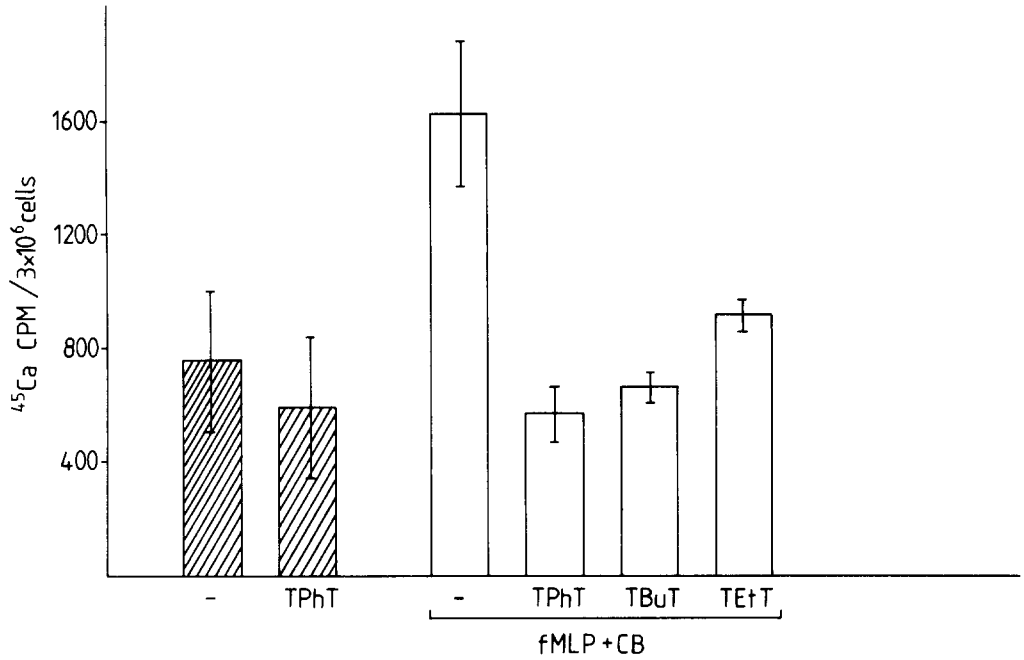

Fig. 4. $[^{45}Ca]^{2+}$ uptake by unstimulated cells, and by fMLP + cytochalasin B stimulated cells, and the effect of triorganotin compounds on $[^{45}Ca]^{2+}$ uptake. The values given are the mean value of four experiments ± SD. Shaded areas: unstimulated cells, without or with 2 μM triphenyltin (TPhT). Unshaded areas: fMLP + cytochalasin B (fMLP + CB)-stimulated cells, without or with 2 μM triphenyltin, 2 μM tributyltin (TBuT) or with 100 μM triethyltin (TEtT).

cytoplasmic enzyme LDH suggests a plasma membrane-damaging effect. This cytolytic effect of tributyltin occurs at a concentration of 5 μM, and is not observed with the other organotin compounds tested.

The other level of inhibition is more specific, occurs at very low concentrations—at least for triphenyltin and tributyltin—and refers to inhibition of particular PMN functions, such as phagocytosis and release of granule-associated enzymes by exocytosis. It appears that triphenyltin and tributyltin are much more toxic for the PMNs than triethyltin. Impairment of PMN function by triethyltin, which is extremely poisonous for mammals because of its neurotoxicity [1, 6], is only of a modest nature.

It has been suggested that organotin compounds may interfere with metabolism [9–12], and that such an interference with metabolic energy production might be the basis of an inhibiting effect. Inhibition of ATP production may be due to an interference with mitochondrial oxidative metabolism, or due to an interaction of organotin compounds with hexokinase with glycolysis [13, 14]. It seems unlikely, however, that inhibition of ATP generation is the basis of the toxicity of organotin compounds in PMNs. Glycolysis is considered to be the main source of metabolic energy in the PMN because this cell type has few or no mitochondria [23–25]. The PMN has large stores of glycogen which can be used in the absence of extracellular glucose, thus bypassing the enzyme hexokinase. The experimental observation that organotin compounds have little effect on ATP level in PMNs as compared with the large effects of the classical inhibitors of glycolysis, supports the idea that interference with ATP production is not the base of inhibition by organotin compounds in PMNs.

Ca^{2+} plays an important role in phagocytosis and in chemotactic peptide-induced exocytosis [15, 26, 27]. Activation by fMLP is accompanied by an increase of Ca^{2+}-permeability of the plasma membrane [28]. All organic tin compounds inhibit the enhanced influx of Ca^{2+} due to fMLP-induced change of Ca^{2+}-permeability. A change in permeability for Ca^{2+} may be a key step in the activation process and interference with this step might be explanation of inhibition by organotin compounds. There is, however, another explanation. PMN activation consists of a series of steps and if the organotin compounds inhibit one of the first steps then it may be that the subsequent steps—one of which could be the change in permeability for Ca^{2+}—are not taking place. Though phagocytosis and exocytosis require extracellular Ca^{2+} for optimal functioning, chemotaxis and the metabolic burst may be activated in the absence of extracellular Ca^{2+}. Nevertheless, these functions have been found to be equally sensitive to inhibition by triphenyltin; therefore the latter explanation, though still speculative, is certainly attractive.

Other investigators have shown that chemotaxis and the metabolic burst by PMNs are inhibited by triphenyltin at the same concentration as we found for inhibition of phagocytosis and exocytosis [17–19]. Phagocytosis, exocytosis, chemotaxis and the metabolic burst are complex PMN functions, which consist of a number of steps, eventually resulting in a given function. Some of these steps are common to all these functions, whereas others are specific for

one function. The fact that all PMN functions are inhibited at about 1–2 μM triphenyltin, suggests that this compound interferes with a step or biochemical entity that all these functions have in common.

In previous studies we have shown that PMN functions require a sulfhydryl group containing protein, probably located on the inner side of the plasma membrane, and that the functions are dramatically dependent on the intactness of the sulfhydryl group [29–32]. Sulfhydryl reagents inhibit all PMN functions and this occurs for all functions at about the same concentration of the reagent [29–32]. Organotin compounds are known to react with sulfhydryl groups [7, 14]. If triphenyltin is given the opportunity to react with a sulfhydryl group-containing compound, it consequently loses its ability to inhibit PMN functions. Therefore, it seems likely that triphenyltin exerts its inhibiting effect by reacting with sulfhydryl groups.

Taken together, the results suggest that organotin compounds exert their inhibiting effect on PMN function by interference with vulnerable sulfhydryl groups, and that these sulfhydryl groups are involved in an early step of activation, where all functions have a common pathway. Blockade of these sulfhydryl groups results in annihilation of all subsequent events which normally occur in PMN activation.

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