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**PROTODYNE:  
AN IMMUNOSTIMULATORY PROTEIN COMPONENT,  
PREPARED FROM GRAM-POSITIVE *BACILLUS SUBTILIS***

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**ABSTRACT**

A protein component derived from bacterial protoplasm, called Protodyne, increases the non-specific resistance to infections by bacteria and viruses. Here we show that Protodyne can be prepared not only from Gram-negative bacteria, but also from Gram-positive bacilli. Several preparations of Protodyne, prepared from *Bacillus subtilis* by phenol extraction or by ammonium sulfate precipitation, were evaluated for immunomodulatory activities in a variety of assays. Protodyne had a marked mitogenic activity on mouse spleen cells; it was a potent inducer of tumor necrosis factor (TNF) and stimulated production of interleukin-1 (IL-1) in human peripheral blood mononuclear cells; it increased the capacity of activated macrophages to undergo a respiratory burst, to produce intracellular killing of leishmanial parasite and extracellular lysis of mastocytoma cells; it also stimulated phagocytosis of latex particles, and prolonged survival of immunosuppressed mice infected with *Pseudomonas aeruginosa*. These activities were not inhibited by polymyxin B, indicating that the activity of Protodyne is not the result of contamination with exogenous lipopolysaccharide. It appears that Protodyne exerts its many immunomodulatory actions by inducing the release of soluble mediators, including TNF and IL-1.

**INTRODUCTION**

Protodyne, a protein component derived from bacterial protoplasm, was originally prepared from Gram-negative bacteria (e.g. *Escherichia coli*) by extraction with hot phenol and subsequent precipitation of the phenol phase with cold methanol (2). This isolation procedure distinguished Protodyne from endotoxin (lipopolysaccharide - LPS) derived from bacterial cell walls also by phenol extrac-

tion, but which was located in the aqueous phase (15). However, the fact that Protodyne exerted activities similar to LPS raised a question of possible contamination of Protodyne with LPS. Chemical composition and several other differences distinguished both preparations: in contrast to LPS, Protodyne was predominantly a protein and contained less than 1% of carbohydrates and lipids, had low toxicity, no pyrogenicity, did not contain 2-keto-3-deoxyoctonate, and was non-reactive in *Limulus* amoebocyte lysate assay (2, 3, 7, 12). Either preparation, Protodyne or LPS, prepared from Gram-negative bacteria, increased survival of animals infected with lethal doses of various micro-organisms (1, 2, 3), but the actual mechanisms by which Protodyne leads to the increased resistance, were not studied in detail.

Here we show that Protodyne can be prepared not only from Gram-negative bacteria, but also from Gram-positive bacilli considered not to contain LPS. Several batches of Protodyne were prepared from *Bacillus subtilis*, either by the original extraction with hot phenol and subsequent precipitation with cold methanol (A-41886) or by precipitation with ammonium sulphate at 75% saturation (AS). The samples of Protodyne were distributed to collaborating institutions for the determination of biological activities. Polymyxin B (PMXB), an agent known to inhibit the biological activities of LPS (9) was used in most of the experiments.

#### METHODS

The methods used are described in References.

#### RESULTS

##### ***Mitogenic activity***

The proliferation of murine spleen cells, measured by <sup>3</sup>H-thymidine uptake, was stimulated by Protodyne in a dose-dependent fashion as shown in Figure 1-A (full line). This activity was not inhibited by the addition of PMBX (dotted line).

##### ***Tumor necrosis factor (TNF)***

Protodyne also stimulated TNF production by human peripheral blood monocytes, measured by a sensitive TNF bio-assay (6) using human rhabdomyosarcoma A-673/6 cells, as shown in Figure 1-B (full lines = 3, 6 and 24 hours incubation). PMXB did not abrogate TNF induction (dotted line).

##### ***Interleukin-1-beta (IL-1-beta)***

The production of IL-1-beta by human peripheral blood monocytes, as measured by radio-immunoassay (5, 13), was stimulated by Protodyne and this activity was not reduced by PMXB (Table I).

##### ***Respiratory burst***

Respiratory burst, measured by hexose-monophosphate shunt assay (4), using mouse peritoneal macrophages, was only slightly stimulated by Protodyne in non-activated cells (Fig. 2-A, lower part, full line). However, a high stimulation was

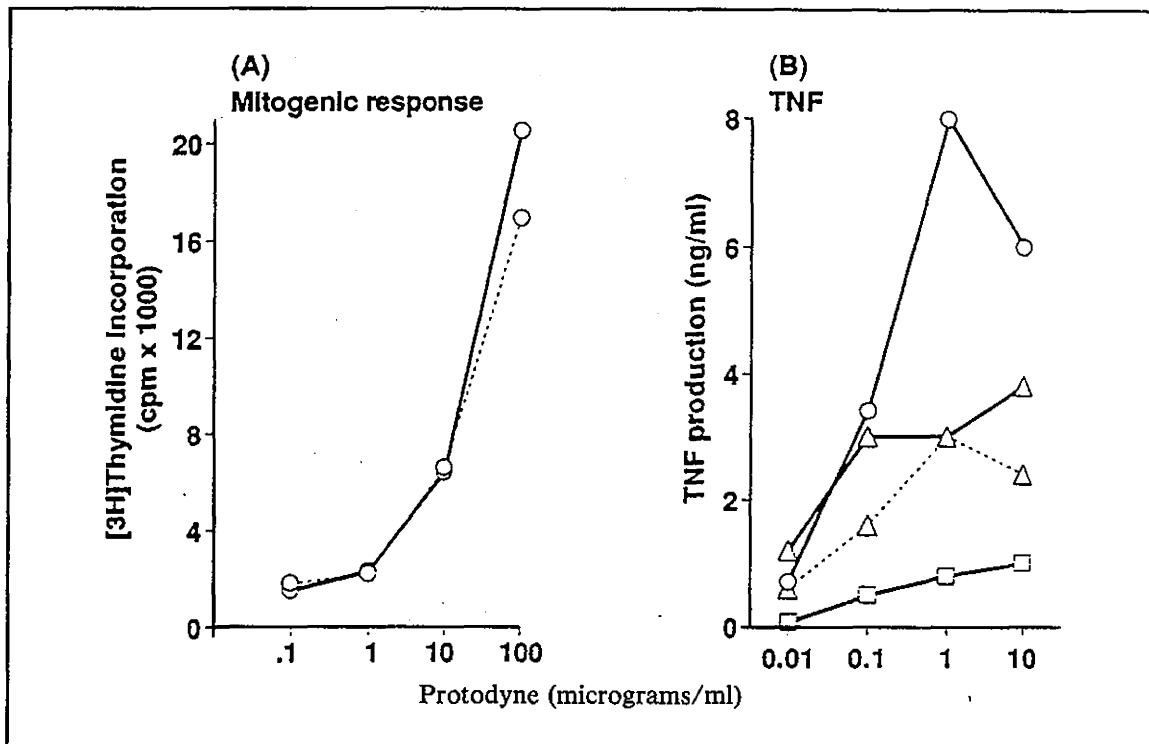


Fig. 1. Stimulation of mitogenic responses and TNF production by Protodyne A-41886 alone (full lines) and with 20  $\mu\text{g}/\text{ml}$  of polymyxin B (dotted lines). — A. Stimulation of proliferation of murine spleen cells by different doses of Protodyne measured by  $^3\text{H}$ -thymidine uptake. B. Stimulation of TNF production from human peripheral blood monocytes by Protodyne at 3 hrs (○), 6 hrs (△) and 20 hrs (□) incubation.

Table I. Stimulation of Interleukin-1 production by human peripheral blood monocytes *in vitro*.

Test samples	PMXB	Donor I		Donor II	
		-	+	-	+
Controls (PBS) .....		<0.03*	<0.03	<0.03	<0.03
LPS (1 $\mu\text{g}/\text{ml}$ ) .....		8.50	<0.03	9.75	0.03
Protodyne ( $\mu\text{g}/\text{ml}$ )					
1.0 .....		<0.03	<0.03	0.037	0.037
10.0 .....		11.25	12.00	12.50	11.70
100.0 .....		9.25	9.75	13.20	13.25

\* Total IL-1 (ng/ml), means of triplicates.

Human peripheral blood mononuclear cells from two donors were stimulated in culture by Protodyne or LPS for 24 hours in the absence (PMXB-) or presence (PMXB+) of polymyxin B (5  $\mu\text{g}/\text{ml}$ ) added together with test compounds. Total IL-1 was determined by RIA as described in Methods. The numbers represent the means of triplicate estimations.

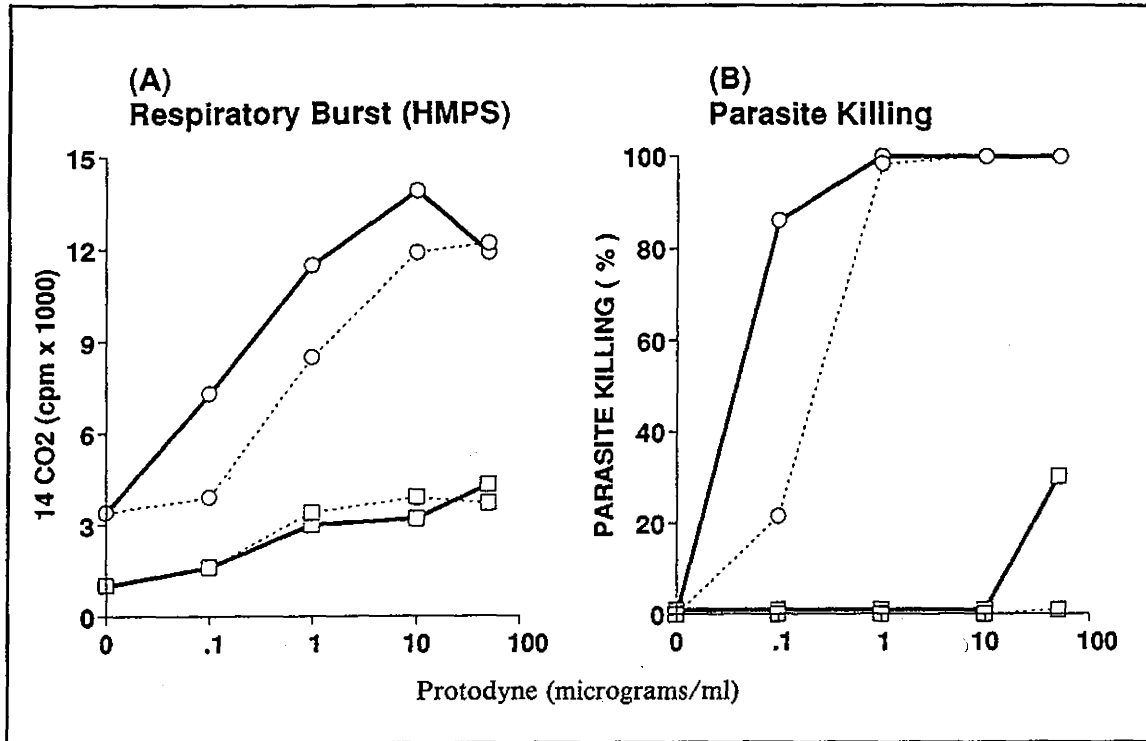


Fig. 2. Respiratory burst (HMPS) and intracellular parasite killing by peritoneal murine macrophages in the presence (○) or absence (□) of MAF. Stimulation by Protodyne A-41886 without (full lines) or with (dotted lines) polymyxin B (1 μg/ml). — A. BALB/c peritoneal macrophages were incubated with test samples for 24 hrs at 37°C; after washing with glucose-free HBSS, the cells were reincubated with [ $^{14}\text{C}$ ] glucose for 120 min, at which time the amount of  $^{14}\text{CO}_2$ (cpm) evolved by the cells was measured. — B. *L. enriettii* parasitized BALB/c peritoneal macrophages were incubated with test samples for 24 hrs at which time intracellular killing was determined after SDS lysis of macrophages, by the uptake of  $^3\text{H}$ -thymidine by the surviving parasites.

observed in macrophages activated by macrophage activating factors (MAF) as shown in the upper part. PMXB did not markedly inhibit this activity (dotted lines).

#### Intracellular killing of parasites (8)

Murine macrophages, exposed to Protodyne in the absence of MAF, did not induce intracellular killing of protozoan parasites (*Leishmania enriettii*) as shown in Figure 2-B (lower part). However, a substantial killing of the parasites was induced by macrophages activated by MAF, even at low concentrations of Protodyne (Fig. 2-B, upper part, full line). PMXB had little effect on this activity (dotted lines).

#### Cytotoxicity

The extracellular cytolytic capacity of macrophages activated by MAF, measured by specific release of  $^{51}\text{Cr}$  from prelabelled mastocytoma cells (11), was stimulated by Protodyne in a dose-response manner as shown in Figure 3-left part. Again, this activity was not influenced by added PMXB.

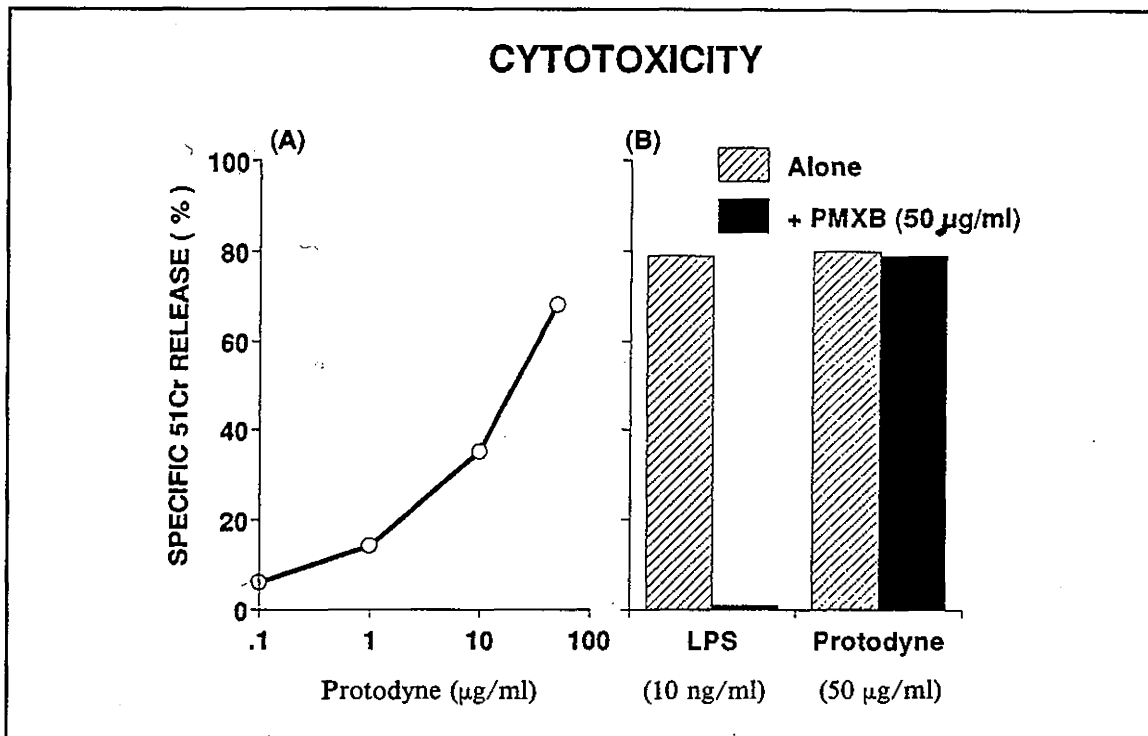


Fig. 3. Dose-response effect of Protodyne A-41886 to stimulate the extracellular capacity of macrophages, activated by MAF, to lyse mastocytoma cells prelabelled with  $^{51}\text{Cr}$ . Specific release of  $^{51}\text{Cr}$  was calculated and expressed as a percentage (A). The effect of polymyxin B (50  $\mu\text{g}/\text{ml}$ ) on the activity of Protodyne in comparison with LPS (B) is a representative result of 15 different fractions of Protodyne tested.

### Phagocytosis (10)

Protodyne stimulated *in vitro* phagocytosis of latex particles by mouse peritoneal macrophages (Tables II) but not by peripheral blood leukocytes. However, intra-peritoneal injections of Protodyne to animals 1 to 24 hours before bleeding increased the phagocytic activity of peripheral blood leukocytes *in vitro* in a dose-dependent fashion as shown in Figure 4.

### Survival of mice

Table III shows the effect of Protodyne on the survival of mice, severely immunosuppressed by cyclophosphamide and infected with lethal doses of *Pseudomonas aeruginosa* (14). Protodyne, injected 24 hours before infection, significantly increased the survival of mice, as compared with controls injected with saline.

The biological activities of Protodynes prepared by different procedures are compared in Table IV. There was no substantial difference in the activities of the preparations indicating that ammonium sulphate precipitation, which provided a water soluble product, might be a useful step for further purification to homogeneity and eventual identification.

Table II. Phagocytosis of latex beads by mouse adherent peritoneal exudate cells (macrophages) *in vitro*.

Test samples	% of phagocytic cells	No of beads in 100 cells	PI*
Saline . . . . .	40**	67	2.7
LPS (10 µg/ml) . . . . .	49	111	5.4
Protodyne (µg/ml)			
10.0 . . . . .	44	97	4.3
20.0 . . . . .	57	100	5.7
100.0 . . . . .	64	120	7.7

Mouse (C-58) adherent peritoneal exudate cells were incubated in microslide chambers with latex beads (labelled with fluorescein) and the test samples for 1.5 hrs at 37°C in 5% CO<sub>2</sub> and examined under the UV-microscope. The number of cells in 100 containing beads (% phagocytic cells) and the number of beads in those 100 cells were recorded for calculation of Phagocytic Index (PI):

$$*PI = \frac{\% \text{phagocytic cells} \times \text{No. beads}/100 \text{ cells}}{1,000}$$

\*\* The numbers represent the means of at least three experiments.

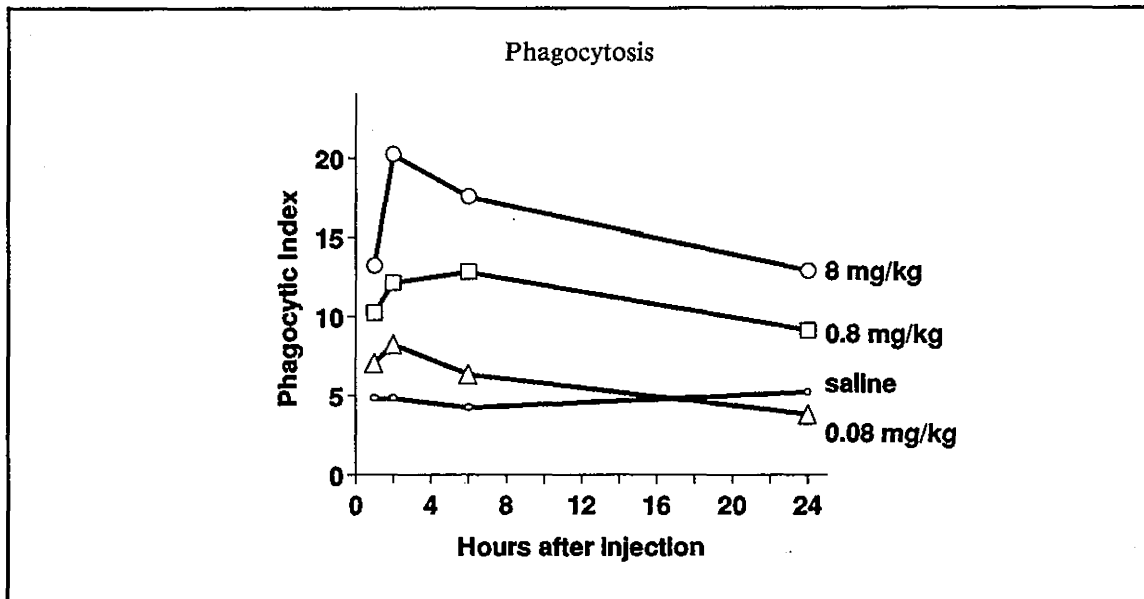


Fig. 4. Kinetics of phagocytosis stimulation by Protodyne A-41886. — Mice BALB/c and C<sub>3</sub>H/HeJ were injected intraperitoneally with different doses of Protodyne (as indicated) and with saline. Heparinized blood samples from mice, sacrificed at different time intervals, were mixed with Fluoresbrite (Latex) particles and incubated for 1 hr at 37°C. The smears were prepared on slides, stained by Giemsa and examined under the UV-microscope. Phagocytosis Index (PI) was calculated as described in Methods.

Table III. Effect of Protodyne treatment on the survival of immunosuppressed mice infected with *P. aeruginosa*.

Hours after infection	Saline (controls)	Protodyne (mg/kg)			Interleukin-1 ( $\mu\text{g}/\text{kg}$ )	
		0.1	1.0	10.0	3.0	30.0
24	17/24 (71%)**	23/23 (100%)	22/24 (92%)	24/24 (100%)	20/20 (100%)	24/24 (100%)
30	0/24 (0%)	7/23 (30%)	13/24 (54%)	20/24 (83%)	14/20 (70%)	20/24 (83%)
36	0/24 (0%)	2/23 (9%)	4/24 (17%)	9/24 (38%)	9/20 (45%)	7/24 (29%)
48	0/24 (0%)	0/23 (0%)	0/24 (0%)	1/24 (4%)	1/20 (5%)	0/24 (0%)
Significance versus controls: $\chi^2 =$ $p =$		3.72 >0.05	6.29 <0.05	15.39 <0.001	9.66 <0.01	15.28 <0.001

\* Number of survived/total number of animals.

\*\* % of survivors.

Mice, immunosuppressed by cyclophosphamide (150 mg/kg-4 days and 100 mg/kg-one day before infection), were infected with *Ps. aeruginosa*. Test compounds (Protodyne, interleukin-1 and saline) were given as a single i.p. injection 24 hours before infection. 20 to 24 mice were used in each group. Survival of mice was recorded at intervals of 24, 30, 36 and 48 hours.

Table IV. Comparison of activities of Protodyne (*B. subtilis*) prepared by different procedures.

Protodyne		Mitogenic activity (c.p.m.)*	Tumor necrosis factor (pg/ml)	Cytotoxicity (specific $^{51}\text{Cr}$ release) (%)	Parasite killing (%)	Phagocytosis PMNs <i>in vivo</i> (specific PI)
Method of preparation	Concentration	10 $\mu\text{g}/\text{ml}$	10 $\mu\text{g}/\text{ml}$	50 $\mu\text{g}/\text{ml}$	10 $\mu\text{g}/\text{ml}$	8 mg/kg
Phenol extraction		5,980	6,000	79	100	15.39
Precipitation by ammonium sulphate		7,490	6,500	81	Not done	11.17
Sephacryl S-400 1st (pooled) fraction		10,600	4,000	87	100	16.36

\* c.p.m. of background deducted.

## CONCLUSION

The data presented clearly indicate that the activity of Protodyne is not the result of contamination with exogenous lipopolysaccharide. It appears that Protodyne exerts its many immunomodulatory actions by inducing the release of soluble mediators, including TNF and IL-1.



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