ENHANCEMENT OF MOUSE IMMUNE SYSTEM BY PYRROLOMYCIN B

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Pyrrolomycin B enhanced both humoral immune response and delayed-hypersensitivity against sheep red blood cells in mice. In spleen cell culture it was a weak inhibitor of mitogenesis. However, in combination with concanavalin A, there was stimulation of mitogenesis in spleen cell culture. Pyrrolomycin B also enhanced phagocytosis of yeasts by peritoneal macrophages after *in vivo* administration to mice. Thus, pyrrolomycin B, formerly isolated as an antibiotic agent, is an immunopotentiator possibly acting on the membrane of lymphocytes or macrophages.

Pyrrolomycin B is an antibacterial and antifungal antibiotic[1] isolated from the culture broth of *Actinosporangium vitaminophilum* SF-2080.

We have also isolated pyrrolomycin B from the culture filtrate of *Streptomyces fragilis* MG303-fF8 as a potent stimulator of haemolysis induced by melittin[2]. It was also found to enhance cellular arachidonic acid release induced by melittin or tumor promoters such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA) or teleocidin B. This observation suggested that pyrrolomycin B might enhance tumor promotion induced by TPA[2].

TPA is known to enhance mitogenesis of cultured lymphocytes synergistically with concanavalin A[3] and to induce inflammation in mouse skin[4]. Therefore, a mechanistic relationship has been suggested between tumor promotion and immune reactions.

We studied modification by pyrrolomycin B of humoral and cell-mediated immune responses against sheep red blood cells (SRBC) in mice.

Materials and Methods

Pyrrolomycin B in crystalline state (LD_{50} 100 mg/kg intraperitoneally in JCL-ICR mice)[1] was kindly supplied by the Central Research Laboratories, Meiji Seika Kaisha, Ltd. Female CDF_1 mice were obtained from Shizuoka Experimental Animals Farm. Groups of five mice were used for each assay.

For antibody formation assay, 10^8 SRBC were intravenously (iv) injected into 8-week-old female CDF_1 mice. Pyrrolomycin B was injected intraperitoneally (ip) at the same time. After 4 days the spleen was removed and a cell suspension from it was subjected to JERNE's plaque forming assay with SRBC and Guinea pig complement[5].

Delayed-hypersensitivity was assayed as described by LAGRANGE *et al.*[6] Female CDF_1 mice were injected subcutaneously (sc) with 10^8 SRBC first in the right hind footpad and again four days later in the left hind footpad. The thickness of left hind footpad was measured 24 hours after the second injection. Pyrrolomycin B was injected (ip) at the time of immunization.

For mitogenesis assay the spleen cell suspension was also obtained from female CDF_1 mice. Spleen cells (10^6) were incubated in 1 ml of the RPMI-1640 medium supplemented with 1% foetal bovine serum for 72 hours, with or without pyrrolomycin B and/or concanavalin A. Tritiated thymidine (1 μg/ml, 1 μCi/ml) was added to the incubation mixture in the last 18 hours. After incubation, the cells were collected on a Whatman 3 MM filter. The filters were soaked in 5% trichloroacetic acid for 1 hour twice, in ethanol for 30 minutes, and then dried. The amount of tritiated thymidine incorporated into the acid insoluble fraction was determined by liquid scintillation spectrometry. The results reported are means for duplicate samples.

Macrophage phagocytosis assay was carried out essentially as described in the study of bactobolin[7]. CDF_1 mice were injected (ip) with 1 ml of thioglycollate broth and four days later peritoneal macrophages were collected by washing the peritoneal cavity with 5 ml of Dulbecco phosphate buffered saline (DPBS). Pyrrolomycin B was injected (ip) 1 day before the harvest of the cells. The peritoneal cells were washed and suspended in DPBS at 5×10^5 cells/ml. One ml aliquots of the cell suspension were placed in 35 mm plastic dishes (Falcon 3001) and incubated at 37°C for 2 hours. After the incubation, the dishes were washed with DPBS to remove non-adherent cells. Then, the cells were washed with DPBS thoroughly, and 1 ml of DPBS containing 0.2 ml of heat-inactivated *Saccharomyces cerevisiae* at 3.75×10^7 cells/ml was added to the dish and incubated at 37°C for 45 minutes. The cells were again washed with DPBS and stained with May-Grünwald and Giemsa solution. Quantitative evaluation of phagocytic cells which ingested yeasts was performed by counting 200 macrophages microscopically (400×).

Results

Pyrrolomycin enhanced antibody formation against SRBC when intraperitoneally injected in mice at the time of immunization, as shown in Table 1. It enhanced antibody formation about 40% at 16 μg/mouse. However, at doses of 250 μg/mouse or higher, pyrrolomycin B was inhibitory to the antibody formation.

Table 1. Enhancement of humoral immune response in mice by pyrrolomycin B.

Pyrrolomycin (μg/mouse)	PFC/spleen	
None	175,100±28,400	
1	182,500±22,500	
4	201,200±32,000	
16	249,300±37,300	$P<0.05$
62.5	218,600±22,000	
250	133,100±35,300	
1,000	53,800±10,800	$P<0.01$

Table 2. Enhancement of delayed-hypersensitivity in mice by pyrrolomycin B.

Pyrrolomycin (μg/mouse)	Increase of footpad thickness (0.1 mm)	
	ip	sc
None	7.4±2.0	6.3±1.8
0.5	11.3±1.0*	—
2	11.7±2.8	10.2±1.6*
8	13.6±2.0**	—
31	12.0±1.4**	12.1±1.5**
125	12.1±2.9*	—
500	11.5±2.1	11.1±1.3**

* $P<0.05$, ** $P<0.01$.

Pyrrolomycin B also enhanced delayed-hypersensitivity against SRBC in mice when administered at the time of immunization, as shown in Table 2. It was effective either by

Table 3. Effect of pyrrolomycin on phagocytosis of yeasts by peritoneal macrophages.

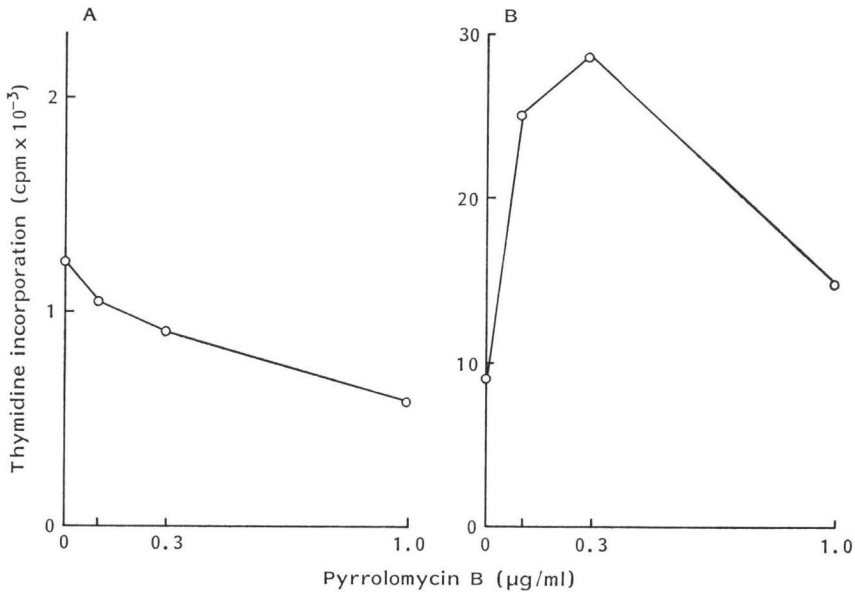
A

Pyrrolomycin (μg/mouse, ip)	No. of phagocytes (mean±S.D.)	Relative %
None	64.9± 8.5	100
0.1	53.3± 6.5	82 N.S.
1	81.0± 4.2	125 $P<0.01$
10	96.7±11.4	149 $P<0.01$
100	91.0± 5.2	140 $P<0.01$

B

Days of injection of pyrrolomycin (1 μg)	No. of phagocytes (mean±S.D.)	Relative %
None	104.8±12.0	100
~5 days	91.5± 9.2	87
~3 〃	95.5±15.9	91
~1 〃	148.0±17.5	141 $P<0.01$

Fig. 1. Enhancement of spleen cell mitogenesis by pyrrolomycin B.
A) Mouse spleen cells were incubated with pyrrolomycin B and labelled thymidine.
B) Concanavalin A (0.1 μg/ml) was added to the incubation mixture.



intraperitoneal or subcutaneous injection. It enhanced delayed-hypersensitivity about 80% at 8 μg/mouse (ip). Higher doses of pyrrolomycin B also stimulated delayed-hypersensitivity.

In mouse spleen cell culture pyrrolomycin B was slightly inhibitory to thymidine incorporation at 0.1 ~ 1.0 μg/ml, as shown in Fig. 1A. But when it was added with a suboptimal dose (0.1 μg/ml) of concanavalin A, it synergistically increased thymidine incorporation by the spleen cells, as shown in Fig. 1B.

Pyrrolomycin enhanced phagocytosis by peritoneal macrophages in mice, as shown in Table 3A. It enhanced yeast phagocytosis about 50% at 10 μg/mouse. Pyrrolomycin B was active when it was given one day before the harvest of macrophages, but not when it was given 3 or 5 days before (Table 3B).

Discussion

Bestatin was first isolated as an inhibitor of aminopeptidase B and leucine aminopeptidases, enzymes which are located on the cell membrane. Later it was found to enhance immunity in experimental animals[8] and in humans[9]. Forphenicine[10] which inhibited chicken intestinal alkaline phosphatase (also located in the cell membrane) and its derivative, forphenicinol[11] were also found to potentiate immune responses. Therefore, compounds which selectively affect cell membrane enzymes are likely candidates among which to search for modifiers of immune reactions.

Pyrrolomycin B enhances mellitin-induced haemolysis of horse erythrocytes and TPA-induced arachidonic acid release, both of which effects are on the cell membrane[2]. Therefore, it is most likely that pyrrolomycin B also acts on the cell membrane. Its receptor is not known but it must be different from that of TPA, since it does not inhibit phorbol ester binding[2].

Enhancement of mouse immune responses by pyrrolomycin B may be mediated by activation of macrophages, although the possibility of direct activation by pyrrolomycin B of lymphocytes can not be excluded.

SD-170 (3′,5′-dichloro-2,4′-dihydroxybenzanilide) which inhibited delayed-hypersensitivity in mice[12] was also found to inhibit tumor promotion[13]. Therefore, this is another example in which the mechanistic relation between immune reactions and tumor promotion is suggested.

Since pyrrolomycin B alone does not induce arachidonic acid release in cell culture and since it does not bind to the phorbol ester receptor, it is unlikely to be a tumor promoter. Non-toxic structural analogues of pyrrolomycin B may become clinically useful immunopotentiators.

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