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Influences of a novel immunopotentiator Polyactin A on interleukin 1 production and responsiveness in mice

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ABSTRACT Effects of a novel immunopotentiator Polyactin A (PAA), developed in China, on production and responsiveness of murine interleukin 1(IL-1]) were investigated. The results demonstrated that: (1) PAA 0.01–100 $\mu g \cdot ml^{-1}$ directly induced IL-1 synthesis and secretion from murine peritoneal macrophages (PMØ) and markedly enhanced IL-1 production of the mouse PMØ stimulated by lipopolysaccharides (LPS) of *E coli*; (2) IL-1 release from the PMØ cultured in PAA 0.1 $\mu g \cdot ml^{-1}$ was detectable as early as 2 h after the incubation, peaked at 24 h, and then decreased gradually; (3) PAA stimulated and enhanced both IL-1 synthesis and release, but its effect on IL-1 release was stronger; (4) PMØ from the mice given po PAA 200 $mg \cdot kg^{-1} \cdot d^{-1}$ for 7 d produced a higher level of IL-1 than those from control group, and the increase in extracellular IL-1 was more significant than that in intracellular one; (5) *in vivo*, PAA had no effect on IL-1 receptor expression and IL-1 responsiveness of murine lymphocytes, but eliminated the suppressing effects of cyclophosphamide (Cyc) on IL-1 receptor expression and IL-1 responsiveness of mouse lymphocytes. The above findings provide new explanation for action of PAAand new basis for wider clinical applications of PAA.

KEY WORDS immunologic adjuvants; polyactin A; interleukin 1; inbred C57BL mice

The antibiotic polyactin A (PAA), newly developed in China, is a polymannopeptide isolated from fermenation broth of buccal α-hemolytic streptococci strain No 33. Experimental and clinical investigations revealed its antitumor and immunopotentiating effects[1-3], but the mechanisms of action are not yet elucidated. It is known that several immunopotentiators, such as lipopolysaccharides (LPS), muramyldipeptide[4] and interferons[5] can stimulate IL-1 production. IL-1 plays a crucial role in the initiation of an immune response[6]. However, there has been no information on PAA concerning IL-1 production. In order to understand the actions of PAA in immune responses, we examined its effects on IL-1 production and responsiveness in mice.

MATERIALS AND METHODS

Reagents PAA (No 85184A) was manufactured by Sichuan Industrial Institute of Antibiotics, Chengdu, China; LPS (*E coli* 055:B5) was obtained from Difco, USA; concanavalin A (Con A) from Sigma, USA; RPMI 1640 mediumfrom Gibco Lab, USA; human IL-1 from Genzyme, USA. All other chemicals were of the highest grade commercially available.

Mice Inbred mice C57BL/6j, aged 2–4 months (for IL-1 production) and 6–8 wk (for IL-1 assay) were obtained from Sichuan Industrial Institute of Antibiotics. For *in vivo* study, groups of the mice (8 in each group) were given po either PAA in 0.9% saline (200 $mg \cdot kg^{-1} \cdot d^{-1}$) or only 0.9% saline for 7 d. Cyc groups were given ip Cyc (100 $mg \cdot kg^{-1}$) in 0.9% saline on d 4. PMØ and splenocytes were obtained on d 8 from all groups.

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Preparation of PMØ Mouse peritoneal lavage with cold Hank's balanced salt solution (HBSS0 was obtained from the mice given ip 10% thioglycollate broth (1 ml / mouse) 4 d before harvest. The cells were washed with HBSS and resuspended in complete RPMI 1640 medium (CM) supplemented with 10% fetal calf serum (FCS), HEPES 2 mmol · L^{-1} and penicillin–streptomycin (100 U – 100 $\mu g \cdot ml^{-1}$). The cell suspension was then introduced into FCS–precoated plastic dishes (Falcon, USA), incubated at 37℃ in CO_2 incubator for 30 min. The medium containing non–adherent cells was decanted and the dishes were rinsed 5 times with CM to discard all the non–adherent cells. Having been removed from the dishes by incubation with PBS containing 0.2% ethylenediamine tetraacetate and 5% FCS at 4 ℃ for 1 h, the adherent cells were washed with HBSS and resuspended in CM. More than 95% cells were confirmed to be macrophages by both their morphology and phagocytosis of yeasts.

Preparation of splenic lymphocytes Single–cell suspensions were prepared in CM from mouse spleen, and allowed to settle for 5 min to remove debris. Having been washed with HBSS, the cells were resuspended in CM and treated 3 times in the way described above to remove adherent cells. The macrophage–depleted splenic lymphocytes thus obtained were more than 92% viable as determined with trypan blue exclusion and contained less than 0.5% macrophages as judged by the phagocytosis and esterase test.

IL–1 production PMØ were plated in 24–well plates (Linbro, USA) at 1×10^6 / well in 1 ml CM. IL–1 production was initiated by the addition of PAA at varying concentrations or / and LPS (10 $\mu g \cdot ml^{-1}$) at 37℃ in air of 5% CO_2 for 24 h. Control wells contained cells without PAA or LPS. At the end of incubation, PAA (0.1 $\mu g \cdot ml^{-1}$) and / or LPS (10 $\mu g \cdot ml^{-1}$) were added to the control wells. Then all the supernatants containing extracellular IL–1 were collected and clarified by centrifugation. The remaining adherent cells were covered with 1 ml fresh CM, frozen and thawed repeatedly in a methanol bath, and then sonicated. This supernatant containing intracellulaar IL–1 was obtained by centrifugation. Preliminary experiments determining IL–1 activity in this material indicated that the cell disruption was complete. All the supernatants obtained were dialysed at 4℃ for 24 h against PBS in a volume of 1 : 200 to remove low molecular weight substances which might affect thymocyte proliferation. Finally, the supernatants were passed through a millipore filter (0.22 μm) and stored at −70℃ until tested for IL–1 activity.

IL–1 measurement IL–1 activity was evaluated by thymocyte proliferation assay. Thymocytes (1.5×10^6 / well) isolated from C57BL / 6j mice were co–cultured, in triplicated wells of 96–well flat–bottomed microtiter plates (Costar, USA), with PMØ cultural supernatant in the presence of Con A at a final concentration of 0.5 $\mu g \cdot ml^{-1}$. The cultures were incubated CM for 72 h, pulsed with [^{3}H]thymidine ([^{3}H]TdR) 46.25 kBq / well during the final 16 h and harvested onto glass fiber strips with a microharvester. The [^{3}H]TdR uptake by the thymocytes was assayed with a liquid scintillation counter, and the results were given as dpm incorporation at a final 1 : 4 dilution of the sample supernatant.

IL–2 determination IL–2 activity was measured as the ability to support IL–2–dependent cytotoxic T–cell line (CTLL) proliferation as described by Bunges *et al*[7].

IL–1 absorption and responsiveness To study IL–1 absorption, 1×10^7 macrophage–depleted splenocytes were fixed with paraformaldehyde and incubated in 0.5 ml CM containing standard IL–1 at 37℃ for 90 min. Then residual IL–1 activity was quantitated in absorbed and incubated unabsorbed supernatants. To study IL–1 responsiveness of spleen cells, 1×10^6 macrophage–depleted splenocytes were cul–

Tab 1. Kinetics of IL-1 secretion from murine peritoneal macrophages induced by PAA 0.1 μg · ml^{-1} or / and LPS 10 μg · ml^{-1}. $n=3$, $\bar{x}\pm$ SD. $^{***}P<0.01$ *vs* medium. $^{+}P>0.05$, $^{+++}P<0.01$ *vs* LPS.

Incubation time (h)	IL-1 secretion from PMØ incubated in (dpm) Medium	PAA	LPS	PAA + LPS
0	1 143 ± 312	1 312 ± 548	1 677 ± 365	1 876 ± 635
2	812 ± 201	3 220 ± 566***	3 894 ± 608	6 550 ± 1 278^{+}
12	1 530 ± 566	4 841 ± 452***	5 381 ± 471	29 825 ± 2 302^{+++}
24	2 040 ± 836	7 868 ± 407***	6 516 ± 619	37 405 ± 3 272^{+++}
36	1 503 ± 357	7 254 ± 812***	5 005 ± 696	25 460 ± 2 709^{+++}
48	1 606 ± 659	6 452 ± 725***	3 913 ± 466	21 090 ± 1 519^{+++}
60	1 278 ± 230	4 685 ± 701***	3 153 ± 566	18 008 ± 1 876^{+++}

tured in the medium containing Con A 0.5 μg · ml^{-1} with or without 5 U standard IL-1 for 72 h ([^{3}H]TdR present during the final 18 h). [^{3}H]TdR incorporation was measured as described above.

Statistical analysis Differences between the test guoups and the controls were examined with t test. All data are given as $\bar{x}\pm$ SD of representative results from 2–4 determinations in triplicates.

RESULTS

Kinetics of IL-1 production from PMØ treated with PAA PMØ were cultured with PAA 0.1 μg · ml^{-1} or / and LPS 10 μg · ml^{-1}, and the supernatants were harvested at various intervals. The IL-1 activities in the supernatants are given in Tab 1. In each group, augmentation of IL-1 level was found as early as 2 h after starting incubation. In all of the 3 groups the IL-1 production began to increase at 2 h. The IL-1 level reached peaks at 24 h and then declined gradually.

IL-2 activity in supernatant of PMØ culture Since IL-2, a T-cell growth factor, could cause murine thymocytes to proliferate, it was possible that IL-2 in PMØ culture supernatants was involved in the enhancement of IL-1 activity in this system. To ascertain this possibility, the supernatants were examined for IL-2 activity against CTLL proliferation. In result, no IL-2 activity was seen (dpm values <515) in all the cultural supernatants of PMØ treated with PAA or / and LPS.

Effect of PAA on extracellular IL-1 production from PMØ *in vitro* Supernatants were generated by culturing the PMØ with incremental concentrations of PAA, or with CM alone, for 24 h. As assessed by the thymocyte proliferation assay, PAA stimulated extracellular IL-1 production from PMØ (Tab 2). There was no significant effect on thymocyte proliferation of the CM, which contained various concentrations of PAA or / and LPS 10 μg · ml^{-1}, during incubation

Tab 2. *In vitro* effect of PAA on release of IL-1 from murine peritoneal macrophages. Murine peritoneal macrophages 1×10^6 ml^{-1} were incubated with drug(s) for 24 h. $n=3$, $\bar{x}\pm$ SD. $^{**}P<0.05$, $^{***}P<0.01$ *vs* control.

PAA (μg · ml^{-1})	LPS (μg · ml^{-1})	[^{3}H]TdR incroporation (dpm) Experiment 1	Experiment 2
0	0	2 127 ± 295	1 696 ± 525
0.001	0	2 522 ± 673	1 362 ± 354
0.01	0	9 705 ± 993***	8 533 ± 641***
0.1	0	9 456 ± 949***	10 340 ± 1 235***
1	0	7 212 ± 783***	8 887 ± 771***
10	0	8 276 ± 1 156***	8 539 ± 1 135***
100	0	8 107 ± 546***	8 091 ± 884***
0	10	5 195 ± 985	7 644 ± 895
0.001	10	4 571 ± 1 017	10 276 ± 1 873
0.01	10	36 776 ± 4 998***	51 878 ± 4 163***
0.1	10	33 724 ± 2 034***	30 765 ± 5 564**
1	10	23 676 ± 2 490**	30 285 ± 2 544**
10	10	24 622 ± 2 800**	26 746 ± 3 298**
100	10	23 824 ± 1 817**	31 749 ± 3 671***

of 24 h and dialysis (data not shown).

Additive stimulation of IL-1 secretion by PAA and LPS Results shown in Tab 2 also indicated that PAA and LPS stimulated extracellular IL-1 production in an additive manner, suggesting that stimulation of extracellular IL-1 production by PAA and LPS occurs via independent mechanisms.

Effect of PAA on intracellular IL-1 production from PMØ IL-1 synthesis and secretion by macrophages may well be 2 distinct biological events[8]. The results in Tab 3 demonstrated that either in the presence or absence of LPS, PAA resulted in elevation of not only extracellular but also intracellular IL-1, and that in both cases, the stimulating effects of PAA on extracellular IL-1 were stronger than those on intracellular one.

Tab 3. ***In vitro*** **effects of PAA on production of extra- and intracellular IL-1 by murine macrophages.** $n=5$, $\bar{x}\pm$ SD. $^{**}P<0.05$ *vs* **intracellular IL-1.**

Stimulus added	IL-1 activity (dpm) Extracellular	Intracellular
None	2 024 ± 536	1 536 ± 323
PAA (0.1 μg · ml^{-1})	9 584 ± 1 387	5 376 ± 1 192
PAA / None ratio	4.74 ± 0.46	3.50 ± 0.29
LPS (10 μg · ml^{-1})	7 264 ± 1 144	4 048 ± 699
PAA + LPS	37 773 ± 2 739	15 947 ± 1 712
(PAA + LPS) / LPS ratio	5.2 ± 0.4**	3.94 ± 0.27**

In vivo **effects of PAA on IL-1 synthesis and secretion** The mice were given ip PAA and PMØ were then collected and assayed for extra- and intracellular IL-1 activities. As shown in Tab 4, *in vivo*, PAA not only induced extra- and intracellular IL-1 production, but also markedly enhanced IL-1 synthesis and secretion from the PMØ stimulated with LPS. Furthermore, in both cases, PAA showed stronger promoting effect on IL-1 secretion than that on IL-1 synthesis. These findings were quite similar to those observed in the *in vitro* experiments.

In vivo **effects of PAA on IL-1 absorption and responsiveness of splenocytes** *In vivo*, IL-1 effects on immune responses depend on not noly the IL-1 level but also the number of IL-1 receptors and IL-1 responsiveness of lymphocytes. Thus, in addition to IL-1 level, we also evaluated the *in vivo* effects of PAA on IL-1 absorption and responsiveness murine macrophage-depleted splenocytes. As shown in Tab 5 and 6, both the parameters were not significantly changed. But Cyc obviously suppressed the absorption of and responsiveness to IL-1 of the spleen cells, while PAA inhibited the suppressing effects of Cyc.

DISCUSSION

The above data demonstrated that the novel immunopotentiator PAA in a wide

Tab 4. ***In vivo*** **effects of PAA on extra- and intracellular IL-1 production by murine peritoneal macrophages.** $n=3$, $\bar{x}\pm$ SD. $^{***}P<0.01$ *vs* **intracellular IL-1.**

Macrophage donors	LPS added to PMØ culture (μg · ml^{-1})	IL-1 activity (dpm) Extracellular	Intracellular
Control mice	0	5 732 ± 508	3 841 ± 556
Mice receiving PAA	0	13 659 ± 1 028	7 631 ± 1 187
% of increase in IL-1		138.3 ± 0.88***	98.7 ± 0.13
Control mice	10	11 265 ± 944	7 606 ± 670
Mice receiving PAA	10	35 625 ± 4 354	19 169 ± 1 323
% of increase in IL-1		216.3 ± 1.83***	152.0 ± 1.38

Tab 5. *In vivo* **effect of PAA on IL−1 absorption by murine adherent cell−depleted splenocytes.** $n=3$. **The results are given as** $\bar{x}\pm SD$ **(% activity absorbed).** $^{**}P<0.05$ *vs* **control mice.**

IL−1 absorbed with spleen cells from	Residual IL−1 activity (dpm) Experiment 1	Experiment 2
None	44 505 ± 5 129	58 944 ± 5 598
Control mice	8 437 ± 874(81)	14 929 ± 1 316(75)
Mice receiving PAA	10 018 ± 929(77)	16 106 ± 1 482(73)
Mice receiving Cyc	19 886 ± 1 386(55)**	32 881 ± 3 227(44)**
Mice receiving Cyc + PAA	10 614 ± 1 548(76)	17 783 ± 2 227(70)

Tab 6. *In vivo* **effects of PAA on proliferative response of murine adherent cell−depleted splenocytes to IL−1. Adherent cell−depleted splenocytes from each mouse were incubated wityh 0.5** $\mu g\cdot ml^{-1}$ **of Con A and in the absence or presence of standard IL−1 for 72 h. Cell proliferation was determined by [^{3}H]TdR incorporation during the final 16 h.** $n=3$, $\bar{x}\pm SD$. $^{**}P<0.05$ *vs* **control mice.**

Splenocyte donors	[^{3}H]TdR uptake by splenocyte (dpm) −IL−1	+IL−1	Stimulation index
Control	25 434 ± 2 460	123 106 ± 12 939	4.84 ± 0.92
PAA	24 667 ± 2 586	112 727 ± 13 010	4.57 ± 1.18
Cyc	21 826 ± 2 207	57 402 ± 7 773	2.63 ± 0.79**
Cyc + PAA	25 439 ± 4 606	102 013 ± 10 475	4.01 ± 0.81

range of concentration augmented IL−1 synthesis and secretion from murine peritoneal macrophages both *in vitro* and *in vivo*, and was able to eliminate the suppressing effect of CY on immune response.

In the IL−1 assay PAA and LPS did not affect the IL−1 enhancement of Con A−induced proliferation of the thymocytes from the C57BL / 6j mice, because after incubation and dialysis the CM containing PAA or / and LPS did not enhance the suboptimal Con A−induced proliferative response of the thymocytes (data not shown). Thus our dialysis was effective and the residual PAA or LPS in the PMØ cultural supernatants was not responsible for the enhancement observed. In addition, IL−2 activity was also not present in the cultural supernatants of PMØ treated with PAA or LPS as demontrated by CTLL assay in this study.

Clinical investigations[3] have demonstrated the efficacy or adjuvant effect of PAA in treatment of the tumor patients. Recent studies have shown the involvement of IL−1 in the tumoricidal activity of macrophages[9] and generation of cytotoxic T−cells[7]. Thus, the present data suggest that the augmentation of IL−1 synthesis and secretion after stimulation with PAA might, in part, mediate the antitumor activity of PAA, and that the antagonism of PAA against the suppressing effects of the commonly−used antitumor agent Cyc on IL−1 receptor expression and responsiveness of lymphocytes is one of the mechanisms of PAA in adjuvant treatment of tumors.

In addition to the antitumor activity and the key role in the generation of an immune re-

sponse, IL-1 also mediates many other biological events, including stimulation of T-cell proliferation and IL-2 production[10], co-stimulation of B-lymphocytes[11], regulation of B-cell differentiation[12]. Thus the findings in this investigation also provide experimental basis for possible clinical application of this novel drug in a wider range.

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新型免疫促进剂多抗甲素对小鼠 IL-1 生成及其反应性的影响

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提要 本文观察多抗甲素对小鼠 IL-1 生成及其反应性的影响. 结果表明：多抗甲素能诱导小鼠腹腔巨噬细胞产生 IL-1，这一活性与 LPS 的作用有相加的作用；多抗甲素无论在体内或体外其诱导 IL-1 分泌的作用比诱导 IL-1 合成作用强；多抗甲素在体内尚能拮抗环磷酰胺对淋巴细胞表达 IL-1 受体及其对 IL-1 反应性的抑制作用. 上述结果提示多抗甲素可能有更广泛的临床治疗作用.

关键词 免疫佐剂类；多抗甲素；白细胞介素-1；小鼠