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Adjuvant Effect of *Photobacterium phosphoreum* PJ-1 on Humoral Immune Response of ddY Mice to Sheep Erythrocytes

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The effects of *Photobacterium phosphoreum* PJ-1 from a luminous marine fish on the immune response of ddY mice to sheep erythrocytes (SRBC) were examined.

Simultaneous intravenous injection of *P.phosphoreum* PJ-1 and SRBC markedly enhanced the formations of the splenic direct and indirect plaque-forming cells (PFC) against SRBC on day 7 after the immunization. The anti-SRBC hemolysin titers in sera were also markedly enhanced by the simultaneous administration of *P.phosphoreum* PJ-1 and SRBC, and this effect of the organism on hemolytic response to SRBC was still observed on day 34 after the immunization.

The injection of *P.phosphoreum* PJ-1 one day after and 14 days before the immunization also significantly enhanced the splenic direct and indirect PFC responses to SRBC. In addition, the administration of *P.phosphoreum* PJ-1 one day before the immunization significantly enhanced only the splenic direct PFC response to SRBC.

These results show that the marine bacterium *P.phosphoreum* PJ-1 is available as an adjuvant for studies on the immune responses to SRBC in ddY mice.

Keywords——marine bacterium; luminous bacterium; symbiotic bacterium; adjuvant; immune response; *Photobacterium phosphoreum*

Introduction

It is well known that various microorganisms and their products are useful immunopotentiators and provide useful tools for investigation of the mechanisms of immune responses in animals, *e.g.*, *Mycobacteria*,[1] *Corynebacteria*,[2] *Hemophilus pertussis*[3] and bacterial endotoxin.[4]

A number of luminous bacteria have been already isolated from seawater and marine animals, and widely employed as experimental tools to study the phenomenon of bioluminescence.[5-7] However, there are few reports dealing with their effects on immune responses of animals. Therefore, we investigated the immunological effect of *Photobacterium phosphoreum* PJ-1 on the immune response of mice to sheep erythrocytes (SRBC). In this paper, we report that *P. phosphoreum* PJ-1 acts as an immunopotentiator of the immune response of ddY mice to SRBC.

Materials and Methods

Organism——A symbiotic luminous marine bacterium was isolated from the luminous organ of the luminous marine fish, *Physiculus japonicus* Hilgendorf. Fukasawa,[8] our co-worker, reported that this bacterium consists of a straight rod and a flagellum, and emits a blue-green luminescence (λ_{max}: 490 nm). More recently, we examined the biochemical characteristics of this bacterium according to *Bergey's Manual* (8th edition).[9] From the results obtained and those of other investigators,[5,10] we classified this bacterium as a gram-negative bacterium, *Photobacterium phosphoreum*, and designated it as "*Photobacterium phosphoreum* PJ-1". It was used exclusively in this work.

Preparation of Heat-killed Cells——*P.phosphoreum* PJ-1 was cultivated with shaking at 20°C for 22 h in a medium containing 30 g of NaCl, 0.2 g of $MgSO_4 \cdot 7H_2O$, 5.0 g of Bacto Peptone (Difco), 3.0 g of Yeast Extract (Difco), 5.0 g of glycerol and 2 ml of 1 M phosphate buffer (pH 7.2) in 1000 ml of distilled water (pH 7.2). The organisms were harvested by centrifugation at $2000 \times g$ for 15 min, washed twice with 3%

NaCl and three times with physiological saline, suspended in saline, and then killed by heating at 60°C for 30 min. The organisms were adjusted to a concentration of 10^9 cells/ml in sterile saline and used for the experiments. All procedures were performed aseptically.

Mice and Immunization——Male ddY mice were purchased from Shizuoka Experimental Animal Farm Co. (Shizuoka, Japan). Fresh sheep erythrocytes (SRBC) were purchased commercially. SRBC were washed three times, and then adjusted to a concentration of 10^9 cells/ml in the sterile saline. Six- to 8-week-old ddY mice were intravenously (*i.v.*) immunized with 0.1 ml of the SRBC suspension.

Estimation of Hemolytic Plaque-forming Cells——Mice injected with both SRBC and *P. phosphoreum* PJ-1 or SRBC alone were bled according to the schedule. The spleens were removed and placed in ice-cold Eagle's minimum essential medium (MEM). Spleen cell suspension was prepared by squeezing the organ with forceps and the number of nucleated cells was counted in a hemocytometer. Direct and indirect plaque-forming cells (PFC) were assayed by the methods of Cunningham *et al.*[11] and Dresser *et al.*,[12] respectively. The data obtained for *P.phosphoreum* PJ-1 treated and nontreated mice were analyzed by means of Student's *t*-test.

Titration of Anti-SRBC Antibody——Sera of the immunized mice were obtained by orbital sinus puncture at various times and kept at −20°C until titration. Titration of hemolytic activity was carried out by the microplate method; 25 μl aliquots of 1% SRBC and of guinea pig serum as a source of complement were added to 25 μl of anti-SRBC sera serially diluted with 10 mM gelatin veronal buffer (GVB; pH 7.5). Each mixture was incubated at 37°C for 90 min, and then the hemolysin titer was determined. For the assay of 2-mercaptoethanol(2ME)-resistant antibody, the sera were preincubated with an equal volume of 0.2 M 2ME at 37°C for 60 min before serial dilution. The hemolysin titer was expressed as $\log_2$ of the highest dilution of the sera giving positive hemolytic reaction.

Results

Enhancement of PFC Response to SRBC by *P. phosphoreum* PJ-1

The kinetics of the primary direct and indirect PFC responses in the spleens of mice injected *i.v.* with SRBC or SRBC plus *P. phosphoreum* PJ-1 are shown in Fig. 1. In control mice treated with SRBC alone, the number of splenic direct PFC increased rapidly up to 4 days after the antigen injection, decreased rapidly from 4 to 7 days and thereafter decreased gradually. In mice treated with *P. phosphoreum* PJ-1 and SRBC, direct PFC response was enhanced significantly on days 2, 7 and 11 but not on day 4 when the maximum number of direct PFC was observed. However, no adjuvant effect of *P. phosphoreum* PJ-1 on direct PFC response to SRBC was recognized except at 2 days after the immunization when the results were expressed in terms of PFC per 10^6 cells. This is a result of the increase of spleen cells in mice treated with the organism, as described the next section. Indirect PFC response of mice treated with *P. phosphoreum* PJ-1 and SRBC was also enhanced significantly on days 7, 11 and 14. Maximum adjuvant activity of *P. phosphoreum* PJ-1 on PFC response to SRBC was seen on day 7, when the numbers of splenic direct and indirect PFC in control mice were approximately equal. Therefore, 7 days after the immunization appears to be a good time to examine the relationship of the adjuvant activity of *P. phosphoreum* PJ-1 on anti-SRBC IgM and IgG PFC response of ddY mice to SRBC, and this timing was adopted in the present work.

Effects on Spleen Weight and the Number of Splenic Nucleated Cells

The injection of *P. phosphoreum* PJ-1 significantly increased the weight of the spleen and the number of splenic nucleated cells in the treated mice. These results are shown in Fig. 2. However, these effects of the organism on the spleen of mice were no longer observed from 11 and 14 days after the administration, respectively.

Relationship between Dose of SRBC and Adjuvant Effect of *P. phosphoreum* PJ-1 on PFC Response to SRBC

The relationship between dose of SRBC and adjuvant effect of *P. phosphoreum* PJ-1 was studied in terms of the PFC response to SRBC, and the results are shown in Table I. In mice injected simultaneously with SRBC and *P. phosphoreum* PJ-1, the formations of direct and indirect PFC were enhanced significantly as compared with that in control mice given SRBC

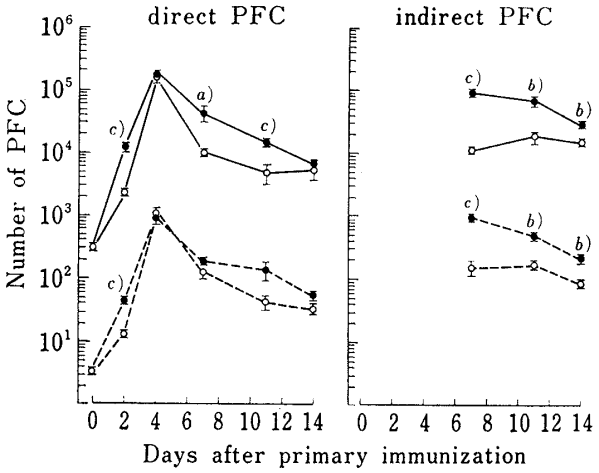

Fig. 1. Adjuvant Effect of *P. phosphoreum* PJ-1 on the Primary PFC Response of Mice to SRBC

Each point represents the mean ± standard error for 5 mice. (○) 10^8 SRBC (*i.v.*) alone; (●) 10^8 SRBC plus 10^8 *P. phosphoreum* PJ-1 (*i.v.*); (—) PFC response/spleen; (---) PFC response/10^6 cells.
a) $p<0.05$, *b*) $p<0.01$, *c*) $p<0.001$.

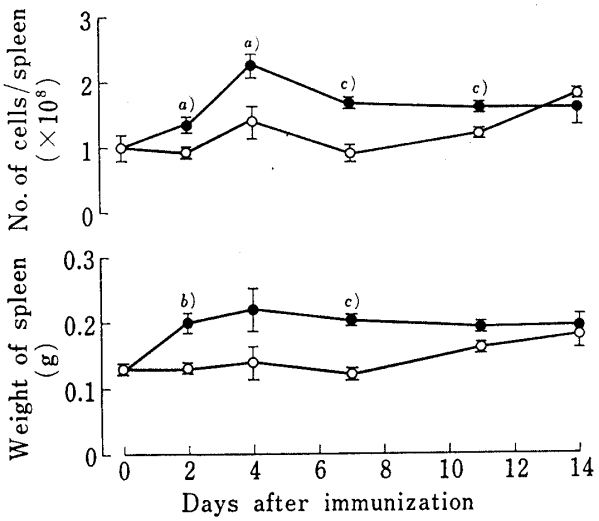

Fig. 2. Changes in the Number of Splenic Nucleated Cells and the Spleen Weight in Mice after the Injection (*i.v.*) of 10^8 SRBC with and without 10^8 *P. phosphoreum* PJ-1

Each point represents the mean ± standard error for 5 mice. (○) SRBC alone; (●) SRBC plus *P. phosphoreum* PJ-1.
a) $p<0.05$, *b*) $p<0.01$, *c*) $p<0.001$.

alone. Moreover, *P. phosphoreum* PJ-1 gave the highest adjuvant activity in the group of mice treated with 10^7 SRBC among the groups studied. In order to examine the effect of *P. phosphoreum* PJ-1 on the relationship between IgM and IgG PFC response to SRBC, *e.g.*, suppression or potentiation, the full PFC responses to antigen are required. Therefore, 10^8 SRBC were used as an antigen in this experiment. The number of nonspecific background PFC increased slightly in mice which received 10^8 *P. phosphoreum* PJ-1 alone.

Table I. Relationship between Dose of SRBC and Adjuvant Effect of *P. phosphoreum* PJ-1 on PFC Response to SRBC

Dose of SRBC injection (cells/mouse)	Dose of PJ-1[a] injection (cells/mouse)	Number of PFC/spleen on day 7			
		Direct PFC	Ratio[b]	Indirect PFC	Ratio[b]
1×10^6	None	7007± 1648	1.0	1849± 456	1.0
	1×10^7	10563± 1909	1.5	7992± 2365	4.3
	1×10^8	10500± 1202	1.5	4579± 1934	2.5
1×10^7	None	2115± 349	1.0	2295± 700	1.0
	1×10^7	8978± 636[f]	4.2	9068± 2985	4.0
	1×10^8	26775± 5953[e]	12.7	49590± 6691[f]	21.6
1×10^8	None	9800± 863	1.0	11450± 1690	1.0
	1×10^7	32797± 8823[d]	3.3	68266±14977[e]	6.0
	1×10^8	43063±12369[d]	4.4	96125± 6319[f]	8.4
None	None	300± 27	1.0	N.D.[c]	
	Saline	219± 32	0.7	N.D.	
	1×10^8	521± 87[d]	1.7	N.D.	

Mice were simultaneously injected *i.v.* with different doses of SRBC and of *P. phosphoreum* PJ-1, and the number of splenic direct and indirect PFC were estimated on day 7 after the injection. Each value represents the mean ± standard error for 5 mice.
a) *P. phosphoreum* PJ-1.
b) Ratio of PFC response in *P. phosphoreum* PJ-1 treated mice to that in control mice.

(Fig. 3). Thus, there is a difference between the PFC responses and the hemolytic responses as regards the adjuvant effects of *P. phosphoreum* PJ-1 on the immune responses of ddY mice to SRBC.

It is known that pretreatment with *Propionibacterium acnes*[13] or *Corynebacterium parvum*[15] suppresses immune response to SRBC. However, such immunosuppression was not shown by *P. phosphoreum* PJ-1 under our experimental conditions. In addition, *P. phosphoreum* PJ-1 induces an increase of splenic nucleated cells, increases spleen weight and enhances the background direct PFC response to SRBC in the spleen 7 days after the treatment (Table I and Fig. 2). These results suggest that *P. phosphoreum* PJ-1 has a polyclonal effect. Moreover, *P. phosphoreum* PJ-1 can be injected *i.v.* and exhibits a potent adjuvant effect on humoral antibody responses to SRBC even after a single shot (Fig. 3). The findings indicate that *P. phosphoreum* PJ-1 may be preferable to Freund's complete adjuvant for *i.v.* injection in some cases. Recently, we isolated lipopolysaccharide (LPS) from *P. phosphoreum* PJ-1 and this LPS was characterized as containing a trace (0.2%, w/w) of 2-keto-3-deoxyoctonate (KDO).[9] Thus, *P. phosphoreum* PJ-1 is a useful and interesting adjuvant material for studies on the immune response of ddY mouse to SRBC.

It has been reported that marine bacteria resemble morphologically terrestrial gram-negative bacteria which have straight or curved rods, flagella and cell walls.[16,17] Thus, the adjuvant effects of marine bacteria may occur by a mechanism similar to that by which terrestrial gram-negative bacteria act. We are now attempting to obtain more detailed information on the mechanism of immunopotentiation by *P. phosphoreum* PJ-1.

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