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Biphasic Protection Against Bacterial Infection in Mice Induced by Vaccination of *Propionibacterium acnes*

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A single intraperitoneal injection of the phenol-treated cells of *Propionibacterium acnes* into mice showed nonspecific resistance against subsequent lethal doses of an intraperitoneal challenge of *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Streptococcus pyogenes*. The protection showed a biphasic pattern. The maximum protection, designated as the early phase protection, was seen in mice injected with *P. acnes* vaccine 1 to 2 days before the challenge, whereas the late phase protection was seen in mice vaccinated 16 to 22 days before the challenge. The activity of the reticuloendothelial system in mice after vaccination also showed a biphasic pattern with the peak on days 4 and 12. The delayed activation of the reticuloendothelial system lasted up to 3 weeks and coincided with the period of the late phase protection. The early phase resistance was markedly impaired by the treatment with hydrocortisone and carrageenan, but not by the treatment with anti-thymocyte serum, actinomycin D, or cyclophosphamide. The number of peritoneal polymorphonuclear leukocytes in vaccinated mice increased on days 1 to 2. The number of macrophages also increased at 2 to 21 days after vaccination and reached its maximum on day 14. Total activities of acid phosphatase, Nitro Blue Tetrazolium reduction, and the phagocytic activities of peritoneal exudate cells were also enhanced on and after day 1 after the injection of *P. acnes* vaccine.

Since Halpern et al. reported that *Corynebacterium parvum* (*Propionibacterium acnes*) stimulated carbon-clearing activity in mice (9), anaerobic coryneforms have been proposed as an immunopotentiator, and many biological activities have been reported (8). The pretreatment of experimental animals with anaerobic coryneforms enhanced the host defense activity to planted tumor cells (11) and protozoal (14), bacterial (1, 6), and viral infections (7). In acute infection, an intense stimulation of the reticuloendothelial system (RES) (15) and activation of macrophages (4) by anaerobic coryneforms may be involved nonspecifically in part of a host resistance mechanism.

In the preceding paper of the series, chemical, biochemical, and antigenical studies were made on *P. acnes* isolated from human bone marrow and skin, and it was found that an insoluble middle layer including phenol-treated cells obtained by a hot phenol water extraction was the most effective means for the enhancement of phagocytic activity in mice, as well as for tumor protection and adjuvant action (20). The purpose of the present study is to clarify the characteristics and the mechanism of resistance exerted in mice by the phenol-treated cells of *P. acnes* against subsequent bacterial infections.

MATERIALS AND METHODS

Bacterial strains. *Klebsiella pneumoniae* K-34, *Streptococcus pyogenes* S-23, *Staphylococcus aureus* Smith, and *Escherichia coli* GN 2411 were used. These strains were stored at −70°C until use.

Animals. Male 5-week-old ICR-JCL mice weighing 23 to 27 g (CLEA Japan Inc., Tokyo, Japan) were used throughout these experiments. These mice were housed under a temperature-controlled environment and given solid feeds and water ad libitum.

Preparation of the phenol-treated cells of *P. acnes*. The phenol-treated cells of *P. acnes* C-7 were prepared by the method described in the preceding paper (20). The suspension containing 20 mg (dry weight) of the phenol-treated cells per ml of saline was usually used.

Immunosuppressive agents. Hydrocortisone acetate (J. P. Micronized, Diosynth, Holland), cyclophosphamide (Shionogi & Co., Ltd., Osaka, Japan), actinomycin D (P-L Biochemicals, Inc., Milwaukee, Wis.), and carrageenan (Sigma Chemical Co., St. Louis, Mo.) were obtained commercially. Rabbit anti-mouse thymocyte serum (ATS) was prepared by a subcutaneous injection of ICR mouse thymocytes (10^7 to 10^8 cells) mixed with Freund complete adjuvant or incomplete adjuvant (1:1) once weekly for 4 weeks. The ATS obtained was absorbed with mouse erythrocytes and stored at −70°C. By a dye exclusion test, 2^7 dilutions of the ATS completely killed mouse thymocytes. The treatment of mice with immunosuppres-

sive agents was carried out as described in the legend to Table 4.

Bacterial challenge. *K. pneumoniae*, *E. coli*, and *S. aureus* were cultured overnight in heart infusion broth (Difco Laboratories), and *S. pyogenes* was cultured overnight in Todd Hewitt broth (Difco Laboratories) at 37°C. In the challenge of *S. aureus* and *E. coli*, one volume of the diluted culture was mixed with an equal volume of 5% gastric mucin (1701W-type, Wilson Laboratories, Chicago, Ill.), and others were appropriately diluted with physiological saline. The mice challenged were observed daily for 1 week, and the 50% lethal dose was calculated by the method of Van der Waerden (23).

Measurement of acid phosphatase. Peritoneal exudate cells (PEC) were obtained by washing from mice with Eagle minimal essential medium (Nissui Seiyaku Co., Tokyo, Japan) on various days, after 1 mg *P. acnes* injection. Each PEC suspension pooled from five mice was sonicated with an ultrasonic apparatus (Ohtake Co., Tokyo, Japan) at 20 kc for 5 min. After centrifugation at 10,000 × *g* for 30 min, the supernatant fluid obtained was used as crude enzyme solution. To 1 ml of M/10 acetate buffer (pH 4.7), 0.5 ml of enzyme solution was added. After incubation at 37°C for 5 min, 1 ml of 0.2% *p*-nitrophenylphosphate was added to the reaction mixture, and the mixture was stopped by the addition of 4 ml of 1 N NaOH. The amount of *p*-nitrophenol released was measured photometrically at 430 nm and calculated from the calibration curve of standard solution.

NBT reduction test. The Nitro Blue Tetrazolium (NBT) reduction activity of PEC in four to six samples per experimental group was determined by the method of Baehner and Nathan (2). The reaction mixture was incubated at 37°C for 30 min.

Assay of phagocytic activity of RES. The phagocytic activity was tested by a clearance of colloidal carbon from mouse peripheral blood by the procedure described by Biozzi et al. (3).

Statistical analysis. A comparison of the final mortality of *P. acnes*-treated and untreated mice was made with the Fisher exact test. The difference between mean numbers of bacterial cells in *P. acnes*-treated mice and those in the untreated mice was compared by Student's *t* test. A *P* value <0.05 was considered to be significant.

RESULTS

Biphasic protection induced by an intraperitoneal injection of *P. acnes* vaccine. Several groups of mice were vaccinated intraperitoneally with 1 mg of the phenol-treated cells of *P. acnes* before the bacterial challenge and after that were challenged intraperitoneally with 0.4 ml of bacterial suspension. As shown in Table 1, an injection of *P. acnes* vaccine in mice showed the biphasic pattern in protection against the lethal doses of a challenge of *K. pneumoniae*, *S. aureus*, and *S. pyogenes*. Early phase protection was observed in mice injected with *P. acnes* vaccine 1 to 2 days before the challenge. Late phase protection was seen in mice vaccinated on 16 to 22 days before the challenge. However, no protection was observed on the group challenged with a minimum lethal dose of *E. coli*.

Optimum conditions for enhancing resistance. Table 2 shows the effective doses of *P. acnes* vaccine on early phase protection. Significant protection was found in mice treated with 0.07 mg of vaccine for *S. aureus*, with 0.13 mg for *S. pyogenes*, and with 0.5 mg for *K. pneumoniae*. With the range 0.07 to 1.0 mg, the protective effects were dependent upon the dosage of *P. acnes* vaccine. The effects of adminis-

TABLE 1. *Protective effects against experimental infection in mice treated with P. acnes various days before bacterial challenge*[a]

Time before treatment with 1 mg of *P. acnes*	Bacterial infection with:[b]							
	S. aureus		*S. pyogenes*		*K. pneumoniae*		*E. coli*	
	S/T[c]	P[d]	S/T	P	S/T	P	S/T	P
Untreated	0/10		0/10		0/10		0/10	
2 h	3/10	NS	0/10	NS	4/10	0.05	2/10	NS
1 day	7/10	0.01	9/10	0.001	10/10	0.001	3/10	NS
2 days	8/10	0.001	3/10	NS	7/10	0.01	2/10	NS
4 days	8/10	0.001	0/10	NS	7/10	0.01	1/10	NS
7 days	3/10	NS	0/10	NS	5/10	0.05	1/10	NS
11 days	1/10	NS	0/10	NS	1/10	NS	3/10	NS
16 days	4/10	0.05	0/10	NS	5/10	0.05	3/10	NS
22 days	8/10	0.001	2/10	NS	1/10	NS	1/10	NS

[a] Mice were intraperitoneally injected with 1 mg of *P. acnes*. Time intervals refer to the single untreated control group.

[b] *S. aureus* (2.2×10^5) and *E. coli* (1.0×10^2) were intraperitoneally challenged into mice mixed with 5% gastric mucin, and *S. pyogenes* (1.0×10^3) and *K. pneumoniae* (5.7×10^3) were intraperitoneally injected without mucin.

[c] Number of mice survived/number of mice tested.

[d] The Fisher exact test. NS, Not significant.

tration routes of *P. acnes* vaccine are shown in Table 3. The early phase protection was sufficiently obtained by the intraperitoneal injection, but the other three routes did not show any significant protective effects. However, the late phase protection was remarkably induced by the intravenous injection rather than by the intraperitoneal injection.

Effect of immunosuppressive agent. To see the effect of immunosuppressive agents on early phase and late phase protection, several immunosuppressive agents were administered to mice before or after the injection of *P. acnes* vaccine. As shown in Table 4, treatment with hydrocortisone, actinomycin D, and carrageenan significantly reduced the defense mechanisms of untreated control mice. The treatment with ATS, however, enhanced the resistance of mice to infection. The resistance induced by *P. acnes* vaccine was impaired by the treatment with hydrocortisone and carrageenan. The impairment seems to be greater in the early phase protection than in the late phase protection.

Activation of phagocytic cells in the peritoneal cavity. Table 5 summarizes the cell population and the enzyme activity of mouse PEC after the injection of *P. acnes* vaccine. In normal mice, macrophages were dominant, and polymorphonuclear leukocytes (PMN) were few or undetectable. However, in vaccine-treated mice, the number of PMN markedly increased on days 1 to 2 after the vaccination. The number

TABLE 2. *Effects of doses of P. acnes vaccine on the early phase protection*

Dose of *P. acnes* (mg)	Bacterial infection with:[a]					
	S. aureus		*S. pyogenes*		*K. pneumoniae*	
	S/T[b]	*P*[c]	S/T	*P*	S/T	*P*
Untreated	0/10		0/10		0/10	
0.07	4/10	0.05	2/10	NS	0/10	NS
0.13	7/10	0.01	4/10	0.05	1/10	NS
0.25	8/10	0.001	5/10	0.05	1/10	NS
0.5	8/10	0.001	6/10	0.01	4/10	0.05
1.0	8/10	0.001	8/10	0.001	6/10	0.01
2.0	3/10	NS	2/10	NS	10/10	0.001

[a] *S. aureus* (2.5×10^6 with 5% gastric mucin), *S. pyogenes* (2.8×10^2), and *K. pneumonia* (5.2×10^3) were intraperitoneally challenged into mice 1 day after intraperitoneal injection of *P. acnes*, respectively.
[b, c] See the footnotes to Table 1.

TABLE 3. *Effects of administration route of P. acnes vaccine on bacterial protection*[a]

Administration route	*P. acnes* (dose [mg] × time)	Treatment with *P. acnes* before:			
		1 day		14 days	
		S/T[b]	*P*[c]	S/T	*P*
Untreated		0/10		0/10	
Intraperitoneal	1, ×1	8/10	0.001	4/10	0.05
Intravenous	1, ×1	1/10	NS	8/10	0.001
Subcutaneous	1, ×1	2/10	NS	1/10	NS
Oral	1, ×1	0/10	NS	0/10	NS
	1, ×14	1/10	NS	NT[d]	

[a] *K. pneumoniae* (3.4×10^3) was challenged intraperitoneally into mice 1 day and 14 days after treatment with *P. acnes*, respectively.
[b, c] See the footnotes to Table 1.
[d] Not tested.

TABLE 4. *Effects of immunosuppressive agents on Klebsiella protection in P. acnes-treated and untreated mice*

Bacterial challenge[a]	Treatment (i.p.)	Log_{10} LD_{50}[b] cells/mouse (confidence limit)			
		Vaccination (−)	Ratio	Vaccination (+)[c]	Ratio
Expt 1, 1 day after *P. acnes*	Untreated	1.56 (1.16–1.95)	1.00	3.16 (2.67–3.64)	1.00
	Hydrocortisone[d]	0.26	0.17	0.36 (0–0.84)	0.11
	Cyclophosphamide[e]	1.36 (0.88–1.84)	0.87	3.95 (3.32–4.57)	1.25
Expt 2, 1 day after *P. acnes*	Untreated	1.93	1.00	4.73 (4.05–5.41)	1.00
	Actinomycin D[f]	0.93	0.48	3.73 (3.34–4.13)	0.79
	Carrageenan[g]	0.43	0.22	0.93 (0.25–1.61)	0.20
	ATS[h]	2.73	1.41	5.13 (4.51–5.75)	1.08
Expt 3, 14 days after *P. acnes*	Untreated	1.94 (1.55–2.34)	1.00	2.94 (2.26–3.62)	1.00
	Hydrocortisone			1.14 (0.46–1.82)	0.39
	Carrageenan			1.54 (0.92–2.16)	0.52

[a] Mice were challenged with *K. pneumoniae* 1 or 14 days after *P. acnes* vaccination.
[b] LD_{50}, 50% Lethal dose.
[c] Mice were given 1 mg of *P. acnes* intraperitoneally (i.p.).
[d] Mice were treated with 5 mg of hydrocortisone 1 and 2 days before *P. acnes* vaccination.
[e] Six milligrams of cyclophosphamide was injected 4 days before.
[f] Ten milligrams of actinomycin D was injected 1 h before.
[g] Three milligrams of carrageenan was injected 1 day before and 1 day after *P. acnes* vaccination.
[h] Mice were treated with 0.3 ml of ATS 1 and 4 days before.

TABLE 5. *Changes in cell population and enzyme activities of PEC in mice after P. acnes injection*

Days after *P. acnes* injection[a]	No. of PEC × 10^6/mouse (%)				Acid phosphatase[c] ($\mu g/10^6$)	NBT reduction[d] ($OD/10^6$)
	Total[b]	Macrophages	PMN	Lymphocytes		
Untreated	3.8 ± 0.5	2.4 (63)	0 (0)	1.4 (37)	7.4 ± 0.6	0.037 ± 0.002
1	8.4 ± 1.2	2.4 (29)	5.1 (61)	0.8 (10)	7.8 ± 1.1	0.114 ± 0.006
2	11.1 ± 1.0	6.2 (56)	4.8 (44)	0 (0)	16.1 ± 2.5	0.083 ± 0.005
4	9.4 ± 0.9	7.4 (79)	1.0 (11)	0.9 (10)	13.7 ± 1.5	0.086 ± 0.006
7	9.0 ± 1.1	5.0 (55)	3.2 (35)	0.9 (10)	14.7 ± 2.4	0.103 ± 0.003
14	18.0 ± 0.4	11.0 (61)	4.5 (25)	2.5 (14)	9.0 ± 0.7	0.086 ± 0.011
21	11.0 ± 3.0	7.4 (67)	0 (0)	3.6 (33)	10.6 ± 0.8	0.046 ± 0.005

[a] Mice were given 1 mg of *P. acnes* intraperitoneally.
[b] Mean ± standard error of 15 to 30 mice.
[c] The enzyme activity was expressed as the mean ± standard error of amount of *p*-nitrophenol released in three to six samples.
[d] The activity was expressed as the mean ± standard error of the optical density (OD) at 515 nm in four to six samples.

of macrophages also increased on days 2 to 21, and the maximum was seen on day 14. The number of lymphocytes also increased at the late phase. The total activity of acid phosphatase of mouse PEC was enhanced on and after day 1 due to the increased cell number, although the activity of the lyzosomal enzyme per 10^6 cells of PEC was enhanced on and after day 2. The NBT reduction of PEC was also enhanced on and after day 1, indicating an increase of digestive activity and the bactericidal activity in peritoneal phagocytic cells.

Activation of mouse RES. The changes in the activity of mouse RES after the vaccination are shown in Fig. 1. The phagocytic index (*K* value) in normal mice was 0.017. The phagocytic activity was gradually enhanced on and after day 1 by the vaccination and showed a peak on day 4. A second peak followed a transient decline and was observed on day 12. The second activation of RES lasted up to 3 weeks after the vaccination and coincided with the period of the late phase protection.

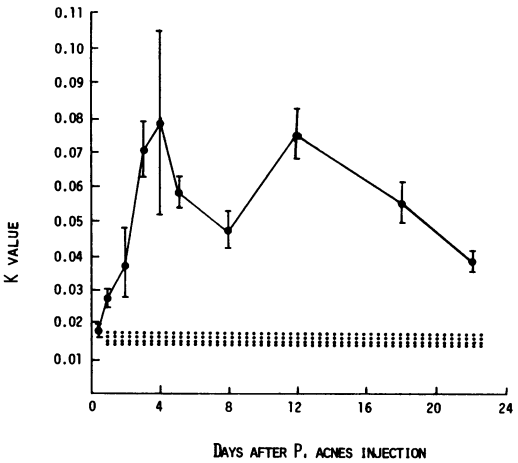

FIG. 1. *Changes in the activity of mouse RES after P. acnes injection. Each point represents the mean ± standard error of five to seven mice. Stippled area indicates normal range of K values in control mice.*

DISCUSSION

An intraperitoneal injection of *P. acnes* vaccine elicited two distinct activities. One is a short-term and local stimulation in the peritoneal cavity, and the other is a long-term and systemic stimulation on the reticuloendothelium. The results are also supported by the study of the tissue distribution of ^{14}C-labeled *P. acnes* in mice after an intraperitoneal injection, which showed short-term distribution with a large amount in the peritoneal cavity and long-term distribution with a moderate amount in spleen, pancreas, liver, bone marrow, and lymphonodus of thoracis duct (unpublished data).

That the early phase protection appeared at 1 to 2 days after the vaccination coincided with the enhanced number of PMN in mice and the enhanced activities of NBT reduction of PEC, but it did not always coincide with the activities of RES and the number of macrophages. Furthermore, the early phase resistance was easily impaired by the treatment of animals with hydrocortisone and carrageenan, which have been known to impair the function of phagocytic cells (21, 24). Administration of cyclophosphamide, which has been known to impair a B-cell function (22), did not reduce the induced resistance of mice. Therefore, the participation of B-cells in the early phase resistance induced by the vaccination of *P. acnes* was not found, whereas the requirements for B-cells were reported in endotoxin-induced resistance (17) and in the resistance of mice to *Listeria* infection (5). On the other hand, the treatment with ATS, which

mainly impairs a T-cell function (12), enhanced the resistance, supporting the results reported by Parent et al. (17) and Campbell et al. (5). Since the enhancement of phagocytic activity and of resistance to bacterial infection was observed in T-cell-deprived mice (25) and in nude mice (18), our result indicates that at least, even under the conditions of the impaired T-cell function, the early phase protection is enhanced.

A lipid content of anaerobic coryneforms has been reported to be an important factor for the promotion of the early phase activation of macrophage (16) and of its chemotaxis (19). However, the lipid content of the phenol-treated cells of *P. acnes* used in this experiment was 0.6% and was less (4.2%) than that of whole cells (unpublished data). Consequently, we should have consideration for a factor(s) other than lipid content on the activation of macrophage.

The late phase protection of mice appeared to be induced by the long-term activation of RES mediated by the systemic increased number of macrophage and the remarkable multiplication of reticuloendothelial cells (13). Furthermore, the participation of T-cells and B-cells in the late phase protection may be considered, because the increased number of lymphocytes was observed at the late phase of vaccination. The period of maximum protection in our study did not agree with that of other reports (1, 6). The details of this discrepancy are unclear, but the following causes are considered, i.e., the different pathogenesis due to the difference of bacterial species challenged, the distinct sensitivity of peritoneal macrophages to *P. acnes* owing to the difference of mouse strain, and the difference of stimulating activity for animals due to strain differences of anaerobic coryneforms used.

P. acnes has been known to be a potent activator of complement (10), which plays an important role in host defense mechanism. To clarify the mechanism of the activation of phagocytic cells by *P. acnes* injection, the participation of complement for the activation of PEC is being studied.

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