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Original Article

Pulmonary granulomas caused experimentally in mice by a recombinant trigger-factor protein of *Propionibacterium acnes*

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Etiology of sarcoidosis remains unknown. A trigger factor from *Propionibacterium acnes* causes a cellular immune response in some sarcoid patients but not in nonsarcoid subjects. We examined whether experimentally induced hypersensitivity to the trigger factor gives rise to granulomas. Female C57BL/6 mice primed intravenously with *P. acnes* or not were sensitized with recombinant-protein RP35, a fragment of *P. acnes* trigger factor, and complete Freund's adjuvant. In controls, RP35 was replaced with *P. acnes* or one of two control proteins. In primed and unprimed mice, pulmonary granulomas were found in some of the mice sensitized with RP35 or *P. acnes* but in no control-protein-sensitized mice. Detection of pulmonary granulomas (25-57%) did not differ significantly between mice sensitized with RP35 or *P. acnes*, primed or not. No difference in popliteal lymph-node-cell reactivity and serum antibodies to these two antigens was found between mice with and without pulmonary granulomas. *P. acnes* was cultured from the lungs of 8 (33%) of 24 untreated mice. The recombinant trigger-factor protein of *P. acnes* caused pulmonary granulomas in primed and unprimed mice sensitized with the protein and adjuvant. Sarcoid granulomas may form during hypersensitivity to antigens of *P. acnes* indigenous to the affected organ.

Key words: *Propionibacterium acnes*, Sarcoidosis, Experimental model, Pulmonary granulomas, Trigger factor

Introduction

Sarcoidosis, of unknown etiology, may result from exposure of a genetically susceptible subject to a specific environmental agent(s)[1], possibly an infectious one, although none has been identified. *Propionibacterium acnes* is so far the only bacterium to be isolated from sarcoid lesions.[2] Many genomes of *P. acnes* have been detected in sarcoid lymph nodes by the quantitative polymerase chain reaction.[3,4] By hybridization *in situ*, *P. acnes* DNA was found in sarcoid lymph nodes in and around sarcoid granulomas.[5] These results point to an etiological link between *P. acnes* and some cases of sarcoidosis. However, *P. acnes* is indigenous to the skin of healthy humans; in addition, it has been isolated in culture from tissue samples from some patients with diseases other than sarcoidosis[2], and a few genomes of *P. acnes* have been detected in some lymph nodes from patients without

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sarcoidosis.[3,4] Host factors may be more critical than agent factors in the etiology of sarcoidosis, as already suggested from the phenomenon of the Kveim test, in which a suspension of sarcoid tissues injected intracutaneously causes sarcoid granulomas in patients with sarcoidosis but not in healthy people or patients with other diseases.[6] The inflammatory response in sarcoidosis involves many activated T cells and macrophages[7], with a pattern of cytokine production in the lungs consistent with a helper T cell type 1 (Th1) immune response triggered by a still undefined antigen(s).[8] If *P. acnes* caused sarcoidosis, it is likely that an antigen arising from the bacterium gave rise to a Th1 immune response in the subject. Ebe and colleagues[9] recently reported that a recombinant trigger-factor protein, RP35, from *P. acnes* causes a cellular immune response in some patients with sarcoidosis, but not in subjects without sarcoidosis. In that study, this antigenic protein was recovered from a λgt11 genomic DNA library of *P. acnes* by immunoscreening with sera from patients with sarcoidosis, but it is unknown whether this antigen is responsible for formation or maintenance of granulomas in patients with sarcoidosis.

In experimental animals, granulomatous lesions can be induced by *P. acnes*. A single intravenous injection of *P. acnes* into mice causes many granulomas in the liver[10-12], but in a preliminary study we found no granulomas in the lungs after such treatment. Pulmonary granulomas can be induced by an intravenous injection of *P. acnes* into sensitized rats[13] and rabbits[14]. In these two studies of experimental pulmonary granulomas, heat-killed *P. acnes* was used as a sensitizer and then as a challenge, intended to lead to granuloma formation. What antigen(s) from *P. acnes* is involved is not known. Here, we designed experiments with mice to examine the hypothesis that pulmonary granulomas in patients with sarcoidosis may have developed because of hypersensitivity to *P. acnes* that was indigenous or that had proliferated ectopically in the lungs. An intravenous injection of *P. acnes* preceded the sensitization with an adjuvant or was omitted from the experiment. We used *P. acnes* trigger factor as one of the antigens to evaluate the ability of this antigen to cause granuloma formation. The aim of the study was to link sarcoidosis and *P. acnes* through experimental induction of pulmonary granulomas of mice with *P. acnes* trigger factor as the sensitizer.

Materials and Methods

Preparation of RP35 recombinant protein

DNA of *P. acnes* prepared as described elsewhere[4] was subcloned into an expression vector, pGEX-6P-1 (Amersham Biosciences, Uppsala, Sweden). This plasmid, and the same plasmid without the insert DNA, were used to transform *Escherichia coli* JM109 cells. Recombinant protein RP35 fused with glutathione S-transferase (GST) and GST alone were obtained from these cells with and without the insert DNA as described by Amersham. The fusion protein was cleaved with PreScission Protease (Amersham). The sequence of the RP35 insert DNA was confirmed by sequencing of the recombinant clone with a pGEX sequencing primer. The insert encoded a fragment 256 amino acid residues long.

Preparation of *P. acnes* antigen

A clinical isolate from a patient with sarcoidosis was used. The strain was isolated in our laboratory from a subcutaneous lesion of a 25-year-old woman with sarcoidosis. No difference was found between the clinical isolate and a type strain (ATCC 6919) by a bacteriologic method or by the polymerase chain reaction with 16S rRNA. *P. acnes* was grown in Gifu-anaerobic-medium broth (Nissui Pharmaceutical Co., Ltd., Tokyo) at 37 °C. After growth to the late exponential phase, cells were harvested by centrifugation (10,000 × *g*). Bacteria were killed by incubation at 60 °C for 30 min and sonicated for 25 min with an ultrasonic disruptor (Tomy Seiko Co., Ltd., Tokyo). The sonicate was suspended in phosphate-buffered saline (PBS) and used for immunization. The soluble fraction of the sonicated bacteria was obtained by centrifugation (550,000 × *g*) for 20 min and used for lymphoproliferative and antibody assays.

P. acnes-induced pulmonary granulomatosis

Female C57BL/6 (H-2b) mice 5-6 weeks old were obtained from Clea Japan Inc. and maintained under specific-pathogen-free conditions. The animal experimental protocol used in this study was approved by the Institutional Review Board of the Tokyo Medical and Dental University and was performed in accordance with the guidelines of the above review board. Mice were sensitized with RP35 in one of two protocols: with and without being primed first with a single intravenous injection of *P. acnes* (Fig. 1). In the first experiment, mice were primed with a single intravenous injection of 1 mg of *P. acnes* suspended in 0.2 ml of

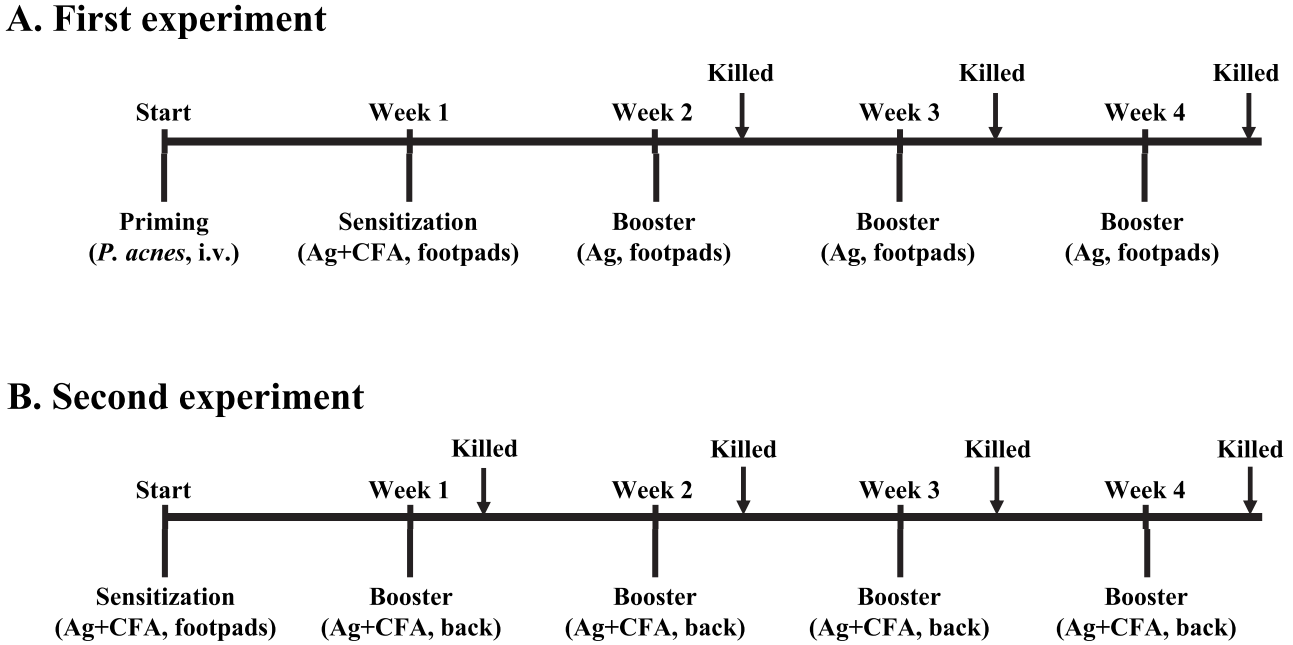

Fig. 1. Immunization protocols. Priming was done by a single intravenous injection of heat-killed *P. acnes*. Sensitization was done by subcutaneous injections of one of the antigens (Ag): RP35, *P. acnes*, ovalbumin, or GST, emulsified with complete Freund's adjuvant (CFA), into the hind footpads. Boosters were subcutaneous injections of the same antigen in PBS into the hind footpads in the first experiment (A), and subcutaneous injections of the antigen emulsified with CFA into the animal's back in the second experiment (B). Mice were killed three days after the last booster as shown by the arrows.

PBS. One week later, the mice were sensitized by subcutaneous injections of a total of 50 μg of RP35 emulsified with complete Freund's adjuvant (CFA; BD Biosciences, Franklin Lakes, NJ) into the hind footpads. Booster doses of subcutaneous injections into the hind footpads of 50 μg of RP35 in PBS but without CFA were repeated weekly. Mice were examined three days after the last booster, done 2, 3, or 4 weeks after the priming. In the second experiment, without being primed, mice were sensitized by subcutaneous injections of a total of 50 μg of RP35 emulsified with CFA into the hind footpads. Then subcutaneous injections of 50 μg of RP35 with CFA were made into the animals' backs weekly, but not into areas inflamed because of earlier injections. (Multiple CFA injections at the same site may cause venous embolization of the adjuvant owing to capillarization of the inflamed area.) Mice were examined three days after the last booster, done 1, 2, 3, or 4 weeks after the sensitization. In both experiments, for the control treatment, RP35 was replaced with 1 mg of *P. acnes*, 50 μg of ovalbumin (Sigma, St. Louis, MO), or 50 μg of GST.

Histologic examination

After being anesthetized and bled, the mice were killed by cervical dislocation. The lungs and liver were removed and fixed in 10% neutral buffered formalin. Before the fixation, the lungs were filled with formalin through the trachea, which was then sutured closed. Lungs and liver were embedded in paraffin, sectioned, and stained with hematoxylin and eosin. For evaluation of whether granulomas were present, three histologic sections 4 μm thick were prepared from lungs and liver at 50-μm intervals. Mice were considered to have pulmonary granulomas when one or more granulomas were found in each of the three sections from the lungs. In the groups of mice sensitized with RP35, other organs (brain, eyes, heart, esophagus, stomach, duodenum, colon, spleen, pancreas, kidneys, skeletal muscle, and mesenteric lymph nodes) were examined as well.

Antibody assay

Serum was obtained from the blood samples mentioned above. Flat-bottomed microplates (Nalge Nunc International, Roskilde, Denmark) were coated with 100 ng of RP35 or with 30 μg of *P. acnes* in carbonate-bicarbonate buffer (pH 9.6) and left for 90 min at 37 °C. As negative controls, the plates were treated with this buffer without any antigen. The plates were

washed with Tris-buffered saline containing 0.05% Tween 20 (T-PBS), and treated with blocking solution diluted 1:4 (Block Ace, Dainippon Pharmaceutical Co., Ltd., Osaka, Japan) overnight at 4 °C. After the plates were washed with T-PBS and coated with 25 μl of blocking solution diluted 1:5, 25 μl of serum diluted 1:100 was added to each well, and the plates were incubated for 2 h at room temperature. After plates were washed as before, they were incubated with goat anti-mouse IgG, IgA, or IgM (all labeled with biotin; Zymed Laboratories Inc., San Francisco, CA) for 30 min, and then with europium-labeled streptavidin (PerkinElmer Life Sciences, Boston, MA) for 30 min, both at room temperature. After plates were washed again, wells were treated with 'enhancement solution' (PerkinElmer), which causes a fluorescent chelate of europium to form. The fluorescence was measured with a time-resolved fluorometer. The amount of antibodies in a sample is expressed as counts per second of fluorescence from a well coated with RP35 or *P. acnes* after subtraction of the background fluorescence from an uncoated well (negative control).

Lymphoproliferative assay

Cells for the stimulation assay were obtained from popliteal lymph nodes of mice, and suspended in RPMI 1640 medium (Gibco, Rockville, MD) containing 10% fetal calf serum, 10 mM Hepes buffer, 100 units/ml penicillin, and 100 μg/ml streptomycin sulfate. Cells (5×10^5/well) were cultured in 96-well round-bottomed microtiter plates (Corning Costar Corp., Acton, MA) for 3 days at 37 °C in humidified air with 5% CO_2. Triplicate cultures were done for each condition, with the medium only, 10 μg/ml RP35, or 10 μg/ml *P. acnes*; then [*methyl*-^{3}H]thymidine (6.7 Ci/mmol, PerkinElmer) was added (1 μCi per well) and the cells were harvested 24 h later. For cultures stimulated with RP35 or *P. acnes*, the stimulation index was calculated as the mean count per minute of stimulated cultures divided by the mean count per minute of unstimulated cultures.

Bacterial culture

Normal untreated C57BL/6 female mice 6 or 12 weeks of age were used in the culture for *P. acnes*. The lungs, liver, and mesenteric lymph nodes were removed under sterile conditions. Specimens were stored in seed tubes for transport under anaerobic conditions (Eiken Chemical Co., Ltd., Tokyo) at 4 °C until the culture procedure was started. After the organs were weighed, they were homogenized in four volumes per weight of reduced Gifu-anaerobic-medium broth. Fifty microliters of the homogenate was used to inoculate Anaero Columbia agar with rabbit blood (Nippon Becton Dickinson Co., Ltd., Tokyo) and the agar was incubated for 5 days at 35 °C under anaerobic conditions. At the same time, enrichment culture was done for 7 days at 35 °C under anaerobic conditions with brucella broth (BD Biosciences) with hemin and menadione or this broth with 16% sucrose. After incubation, colonies resembling *P. acnes* in appearance were counted. For colony identification, Gram staining was done of each kind of colony different in appearance from the others, and then the VITEK system (bioMérieux SA, Marcy-l'Etoile, France) was used for further identification by standard methods.

Statistical analysis

The chi-square test for proportions with Yates' correction was used for evaluation of differences in frequency between pairs of groups. The Mann-Whitney *U*-test was used to compare the lymphoproliferative responses and also the serum levels of antibodies in mice treated by the same immunization procedure and grouped by whether pulmonary granulomas were found or not. In both tests, differences with p values of less than 0.05 were considered to be statistically significant. The Kruskal-Wallis test was used to search for differences among all groups. When a significant difference was found, the Mann-Whitney *U*-test with Bonferroni's correction was used to compare the values between pairs of groups. Here, the difference with a p value of less than 0.0018 was considered to be statistically significant. StatView software (version 5.0; SAS Institute, Cary, NC) was used.

Results

Granulomatous inflammation was found only in the liver and lungs of some mice, and not in any of the other organs examined. Hepatic granulomas were found in all primed mice (first given a single intravenous injection of *P. acnes*) whether later sensitized or not, but in no unprimed mice. When mice were primed in this way and later sensitized with RP35 or control proteins, there were many hepatic granulomas at 2 weeks after the priming, fewer at 3 weeks, and almost none at 4 weeks, leaving occasional foci of lymphocytic infiltration. No differences in these changes were found from the mice primed but not later sensitized. In livers of the primed mice later sensitized with *P. acnes*, there were

more granulomas at 3 weeks than at 2 weeks after the priming, and still more at 4 weeks.

Whether there was priming or not, pulmonary granulomas were found in some of the mice sensitized with RP35 or *P. acnes*, but not in any of the mice sensitized with ovalbumin or GST (Table 1). The proportion of mice with pulmonary granulomas was from 33% to 57% and 25% to 50% in the RP35 groups with and without priming, respectively. In the *P. acnes* groups with and without priming, the proportions were from 25% to 30% and 25% to 38%, respectively. No difference in the frequency was found when the RP35 and *P. acnes* groups were compared, whether primed or not. Histologic findings of pulmonary granulomas were similar in mice sensitized with RP35 or *P. acnes*, whether mice were primed or not. Granulomas were scattered throughout the lungs, especially in subpleural areas (Fig. 2A). Granulomas were composed of a core of epithelioid cell intermingled with a few mononuclear cells and surrounded by many such cells (Fig. 2B and D). Immature granulomas were sometimes found in localized areas of alveolitis (Fig. 2C). Vascular granulomas were sometimes found (Fig. 2E), and perivascular granulomas were frequent (Fig. 2F).

P. acnes was cultured from the lungs of 8 (33%) of 24 normal mice, with no difference depending on age (Table 2). The frequency of success in culture was higher with the lungs than with the livers or mesenteric lymph nodes. *P. acnes* was cultured from these three organs of one mouse, and from the lungs and liver of another mouse. The number of *P. acnes* in the cultures ranged from 100 to 800 cfu/g of tissue. Results of the lymphoproliferative and antibody assays are shown in Table 3, in which mice treated by a single protocol and then grouped depending on whether pulmonary gran-

Table 1. Detection frequency of pulmonary granulomas in primed and unprimed mice sensitized with various antigens

	Sensitization		Numbers (%) with pulmonary granulomas of mice/total at:			
Priming	**Sensitizer**	**Booster**	**1 week**	**2 weeks**	**3 weeks**	**4 weeks**
Done	None	None	0/6 (0)	0/6 (0)	0/6 (0)	0/6 (0)
Done	RP35 + CFA	RP35	N.E.*	4/12 (33)[a]	13/23 (57)[b]	6/12 (50)[c]
Done	*P. acnes* + CFA	*P. acnes*	N.E.	3/12 (25)[a]	4/15 (27)[b]	3/10 (30)[c]
Done	OVA + CFA	OVA	N.E.	0/6 (0)	0/6 (0)	0/6 (0)
Done	GST + CFA	GST	N.E.	0/6 (0)	0/6 (0)	0/6 (0)
Not done	RP35 + CFA	RP35 + CFA	0/8 (0)	2/8 (25)	8/16 (50)[d]	10/20 (50)[e]
Not done	*P. acnes* + CFA	*P. acnes* + CFA	0/8 (0)	2/8 (25)	6/16 (38)[d]	3/12 (25)[e]
Not done	OVA + CFA	OVA + CFA	0/6 (0)	0/6 (0)	0/6 (0)	0/6 (0)
Not done	GST + CFA	GST + CFA	0/6 (0)	0/6 (0)	0/6 (0)	0/6 (0)

Priming was by a single intravenous injection of *P. acnes*. Sensitizing and booster doses were injected at weekly intervals.

* Not examined.

[a]$p = 0.65$, [b]$p = 0.070$, [c]$p = 0.34$, [d]$p = 0.48$, [e]$p = 0.16$, chi-square test of proportions with Yates' correction, pairwise.

Table 2. Results of *P. acnes* culture from lungs, liver, and mesenteric lymph nodes of normal untreated mice

	Number (%) of successes in culture [cfu/g] with normal mice		
Organ	**6 weeks old (n = 12)**	**12 weeks old (n = 12)**	**Total (n = 24)**
Lung	4 (33) [500[a], 300, 800, 100[b]]	4 (33) [100, 100, 100, 100]	8 (33)*[†]
Liver	2 (17) [100[a], 100[b]]	0 (0)	2 (8)*
Lymph node	1 (8) [100[a]]	0 (0)	1 (4)[†]

[a, b] Organs with the same superscripts were taken from the same mice. *$p = 0.033$ and [†]$p = 0.0096$, chi-square test of proportions with Yates' correction, pairwise.

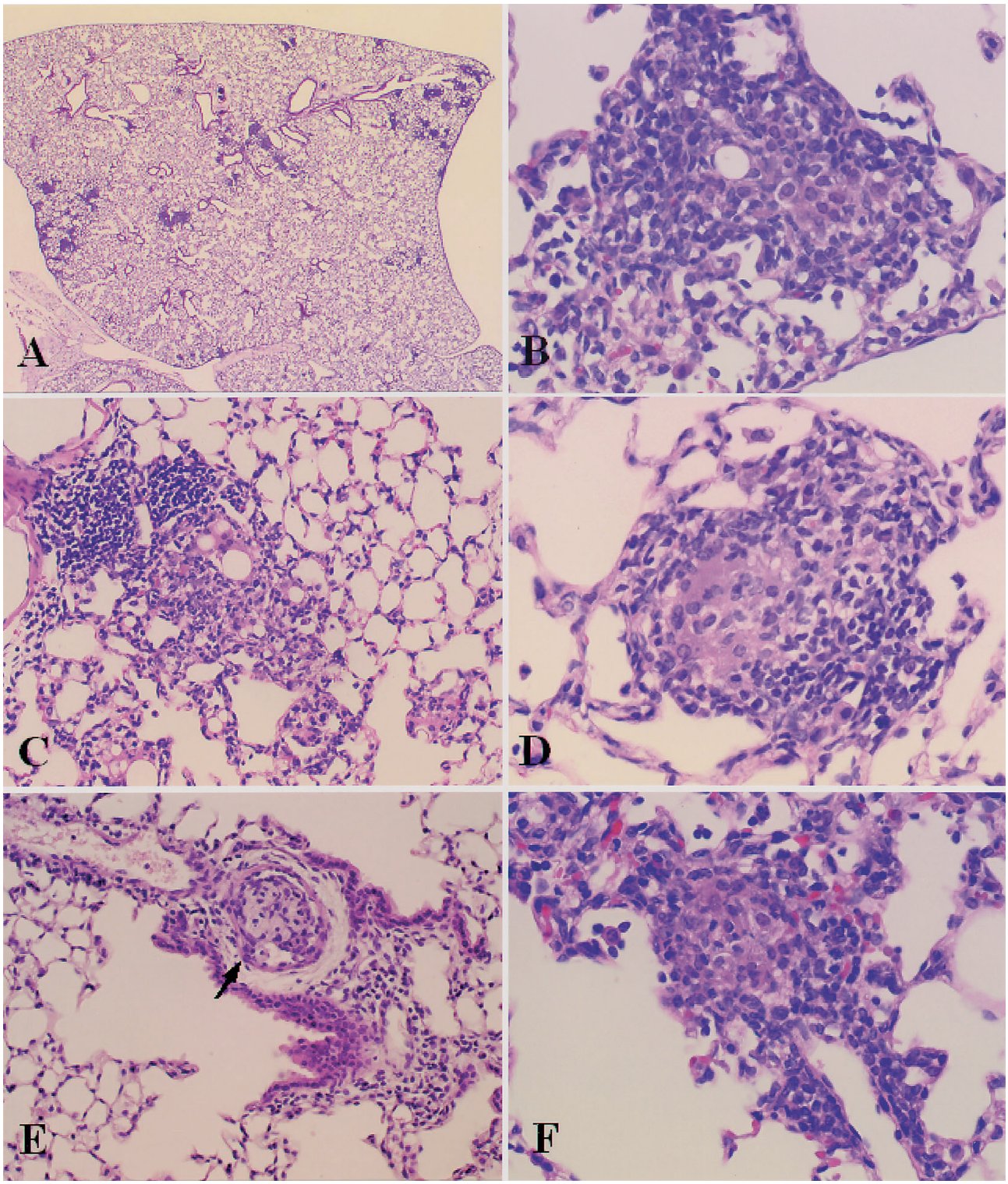

Fig. 2. Pulmonary granulomas of primed and unprimed mice sensitized with RP35 or *P. acnes*, examined 3 weeks after the first treatment. A-C, from unprimed mice sensitized with RP35; D and E, from primed mice sensitized with RP35; F, from unprimed mice sensitized with *P. acnes*. Note the many granulomas scattered throughout the lung (A, ×22), a granuloma with many lymphocytes around a core of epithelioid cells with rich eosinophilic cytoplasm (B and D, ×600), focal alveolitis with granulomas developing (C, ×300), a granuloma of the arterial wall (arrow) (E, ×300), and a granuloma with lymphocytes in the perivascular lymphatic space (F, ×600).

Table 3. Lymphoproliferative response and serum levels of IgG, IgA, and IgM to RP35 and *P. acnes* in primed and unprimed mice sensitized with RP35 and *P. acnes*, examined 3 weeks after the first treatment

					Response (SI) to:		IgG antibodies to:		IgA antibodies to:		IgM antibodies to:	
Priming	**Antigen**	**Gra.***		***n***	**RP35**	***P. acnes***	**RP35**	***P. acnes***	**RP35**	***P. acnes***	**RP35**	***P. acnes***
Done	RP35	+		13	48.4[a]	7.5[a]	177[r]	145	1	11	26[b]	132[b]
					(41.8, 74.5)	(4.0, 9.0)	(123, 466)	(119, 173)	(0, 2)	(11, 14)	(15, 39)	(118, 151)
Done	RP35	-		10	44.6[c]	7.3[c]	418[s]	103	< 1	9	29[d]	137[d]
					(34.3, 48.4)	(5.7, 9.4)	(233, 514)	(97, 159)		(9, 16)	(14, 41)	(102, 157)
Done	*P. acnes*		+	4	5.3	9.4	3[e, r]	259[e]	< 1	12	4[f]	165[f]
					(3.4, 6.0)	(8.8, 10.1)	(1,4)	(250, 288)		(9, 15)	(1, 11)	(117, 174)
Done	*P. acnes*		-	11	8.3	10.7	< 1[g,s]	246[g]	< 1	9	5[h]	115[h]
					(6.0, 9.7)	(9.8, 11.5)		(241, 354)		(9, 12)	(2, 6)	(95, 153)
Not done	RP35	+		8	86.1[i]	9.6[i]	312[j, t]	17[j]	2	8	20	21
					(76.5, 97.2)	(6.0, 11.6)	(276, 388)	(10, 37)	(2, 2)	(7, 8)	(12, 30)	(19, 32)
Not done	RP35	-		8	74.6[k]	9.0[k]	337[l, u]	13[l]	2	6	7	19
					(38.6, 92.1)	(4.3, 21.2)	(313, 382)	(9, 18)	(1, 4)	(4, 8)	(5, 11)	(12,34)
Not done	*P. acnes*		+	6	3.5	6.0	< 1[m,t]	123 [m]	< 1	9	< 1[o]	88 [o]
					(2.8, 4.9)	(3.9, 7.2)		(97, 152)		(8, 9)		(51, 121)
Not done	*P. acnes*		-	10	9.0	11.1	< 1[p, u]	168 [p]	< 1	10	2[q]	86[q]
					(8.2, 9.8)	(8.3, 13.1)		(118, 193)		(7, 11)	(0, 4)	(66, 104)

* Granuloma formation detected in the lungs. The stimulation index (SI) and levels of antibodies (counts per second) are expressed as medians, with 25th and 75th percentiles in parentheses. Values for the levels of antibodies are to be multiplied by 10^3. The p-values of *a* to *u* were less than 0.001. Kruskal-Wallis test for overall differences; Mann-Whitney *U*-test with Bonferroni's correction used to evaluate any significant differences found (here, $p < 0.0018$ was taken to be significant).

ulomas were detected or not are compared. In both kinds of assays, no difference was found between mice with and without pulmonary granulomas (Mann-Whitney *U*-test). In the mice sensitized with RP35 with and without priming, the lymphoproliferative response to RP35 was greater than that to *P. acnes* ($p < 0.001$). In mice sensitized with *P. acnes*, whether primed or not, the lymphoproliferative response to *P. acnes* did not differ from that to RP35. In all mice, antibody levels to the antigen used for sensitization and priming (if done) were at detectable levels, and significant differences in the extent of increase between RP35 and *P. acnes* were found, depending on the sensitizer used.

Discussion

RP35, a recombinant protein of 256 amino acid residues with the calculated molecular mass of 28,133 Da, is the C-terminal region of *P. acnes* trigger factor, which has 529 amino acid residues and the calculated molecular mass of 57,614 Da.[9] The C-terminal sequence (Asp-463 to Lys-529) seems to be unique to *P. acnes*, with no similarity to sequences of other bacterial proteins deposited in the Swiss-Prot database. The region of Ser-491 to Lys-529 at the C terminus has been found in conformational analysis to be highly antigenic.

RP35 caused sarcoidosis-specific proliferation of peripheral blood mononuclear cells from 9 (18%) of 50 patients with sarcoidosis.[9] The same study established that serum levels of IgG and IgA antibodies to RP35 are high for patients with sarcoidosis and other lung diseases. In bronchoalveolar lavage, levels of IgG and IgA antibodies were high in 7 (18%) and 15 (39%), respectively, of 38 patients with sarcoidosis, and in 2 (3%) and 2 (3%), respectively, of 63 patients with other lung diseases. The results of that study suggested an etiological link between sarcoidosis and *P. acnes*, but it is not known whether hypersensitivity to this antigenic protein from *P. acnes* is involved in the pathogenesis of this disease.

In this study, we designed the first experiment to examine whether experimentally induced hypersensitivity to the trigger factor gives rise to granulomas. Because *P. acnes* seems to be indigenous to humans but not to mice, a single intravenous injection was given to expose the animals to *P. acnes* before the sensitization. We avoided transbronchial administration of *P. acnes* for either priming or challenge on the basis of results from a previous report[13] that the intravenous injection of *P. acnes* into sensitized mice causes sarcoid-like interstitial granulomas, but that the transbronchial administration of *P. acnes* causes intra-alveolar granulomas similar to those found in hypersensitivity pneumonitis rather than sarcoidosis. For

sensitization of mice with RP35, we used a single subcutaneous injection of this protein emulsified in CFA, followed with repeated subcutaneous injections of RP35 in PBS, because we intended to induce most antigen-specific immune responses possible. Results of the first experiment suggested that priming of mice by an intravenous injection of *P. acnes* before sensitization induced hepatic granulomas and seemed to have no effect on the induction of pulmonary granulomas, and led us to design the second experiment without a priming step. In the second experiment, we used repeated injections of RP35 emulsified in CFA because the results from the first experiment suggested that a single use of CFA may be not enough for sensitization giving pulmonary granulomas in all mice treated by identical immunization protocols. Repeated injections with one week intervening was done so that the protocol would match that of the first experiment as closely as possible. In fact, variations in the immunization protocol with CFA, such as injections every two weeks, did not change the results (data not shown).

Sensitization of mice with RP35 in CFA caused a strong immune response to RP35 and resulted in pulmonary granulomas. Experimental models of allergic diseases such as encephalomyelitis[15], thyroiditis[16], and orchitis[17] have been produced by the immunization of animals with self antigens (myelin basic protein, thyroglobulin, and testicular homogenate, respectively) emulsified in CFA, which is essential for the experiment. Autoimmune inflammatory lesions are induced in this way only in the organs from which the self antigens used for the sensitization originated. In our study, sensitization of mice with RP35 or *P. acnes* in CFA induced granulomatous inflammation confined to the lungs. This finding suggests that such antigens from *P. acnes* exist in the lungs of mice even before the experiment. *P. acnes* antigens may have cross-reacted with self antigens of the mouse lung, but this is unlikely because more than half of the mice undergoing identical immunizations were free from such inflammation, although no differences were found in cellular and humoral immunity between the mice with and without pulmonary lesions. Another possibility is that this bacterium normally resides in mouse lungs. Bacterial culture of the lungs suggests this explanation to be more likely. *P. acnes* was cultured from the lungs, liver, and lymph nodes from some untreated normal mice, and culture was most often successful with the lungs. Culture was not done with the mice used in the experiments to avoid possible contamination during sampling. However, there was unexpected concordance in the rate of culture from normal lungs and the frequency of detection of pulmonary granulomas in mice sensitized with RP35 or *P. acnes*. The concordance suggests that mice without granulomas may have been free from *P. acnes* in the normal indigenous flora of their lungs before and during the experiment. Infection of *P. acnes* occurring during the experiments is unlikely because no difference was found in the culture rates between mice 6 and 12 weeks of age. An eradication experiment with antibiotic treatment of mice to eliminate *P. acnes* and an infection experiment with direct pulmonary exposure of mice to *P. acnes* could be used to examine further our hypothesis that *P. acnes* endogenous to mouse lungs is the focus for granuloma formation. The design of such experiments, including preliminary experiments, must take into consideration the findings that the commensalism of *P. acnes* in the lungs seems to be different from colonization by bronchial microflora or the usual kinds of pulmonary infection.

Pulmonary granulomas being induced by the sensitization of mice with RP35 used instead of *P. acnes* suggests that *P. acnes* trigger factor is one of the antigens responsible for *P. acnes*-induced pulmonary granulomas. It is unlikely that substances such as endotoxins still contaminating RP35 after its purification from *E. coli* contributed to granuloma formation, because GST purified in the same way from *E. coli* did not induce granulomas when used as the sensitizer in the same protocol. The involvement of antigens from *M. tuberculosis* of CFA in the pulmonary granulomas is unlikely; results with the control groups rule out this possibility.

P. acnes was used as a positive control in this study, with an emulsion of *P. acnes* with CFA being injected in the same way as for RP35. In an earlier study of pulmonary granulomas caused by *P. acnes* in rabbits[14], an emulsion of *P. acnes* with incomplete Freund's adjuvant was injected subcutaneously for sensitization, and granulomas developed after a single intravenous injection of *P. acnes* into the sensitized rabbits. The researchers may have decided to avoid using a mixture that contained *M. tuberculosis* (in CFA) as well as *P. acnes*, and might have expected an adjuvant effect from *P. acnes* itself.[18] The mechanism of granuloma formation when only sensitization with *P. acnes* was done in our experiment is not known, but our protocol may give a more satisfactory model of sarcoidosis than those reported previously[13,14] in the following ways. First, hypersensitivity to *P. acnes* trigger factor, experimentally induced here, has already been found in some patients with sarcoidosis. Second, situ-

ations resembling an intravenous challenge with *P. acnes* are rare in humans, and sarcoidosis can start in asymptomatic persons without evidence of septicemia. Third, priming was not an essential part of our protocol.

Hepatic granulomas were found in all mice primed with a single intravenous injection of *P. acnes*, similar to results reported earlier.[10-12] In our study, hepatic granulomas were numerous at 4 weeks in the mice sensitized with *P. acnes*, but when sensitization was with RP35, almost all such granulomas had disappeared by that time. This discrepancy between the groups means that hypersensitivity to RP35 did not contribute to the formation or maintenance of hepatic granulomas. *P. acnes*-induced granulomas in the lungs and livers may be caused by different mechanisms; maintenance of the number (or increases in the number) of hepatic granulomas in primed mice later sensitized with *P. acnes* may not be owing to hypersensitivity to *P. acnes* but simply to the additional amount of insoluble components of *P. acnes* entering the liver after the booster injections. When we used soluble components of *P. acnes*, instead of the *P. acnes* of booster doses, the hepatic granulomas changed with time in the same way as when PR35 was used (results not shown). Kupffer cells of the liver may have trapped the insoluble components of *P. acnes* in the PBS without CFA given as booster doses.

The trigger factor is an abundant protein of about 58 kDa found in all eubacteria. Little is known about it. The factor can cross-link to a variety of nascent secretory and cytoplasmic proteins[19] and has ATP-independent chaperone-like activity.[20] Unlike typical molecular chaperones like some heat-shock proteins, however, the trigger factor does not seem to recognize exposed hydrophobic surfaces.[21] Heat-shock proteins in different bacteria have sequence similarity, and sometimes immunologic cross-reactivity occurs.[22] The trigger factor in different bacteria has little sequence similarity (usually less than 30%), and the sequence most similar to that of *P. acnes* trigger factor is that of *M. tuberculosis*.[9] The mechanism by which some patients with sarcoidosis have hypersensitivity to *P. acnes* trigger factor has not been identified. Sarcoidosis may arise from a Th1 immune response to one or more antigens of *P. acnes* indigenous to or proliferating in the affected organ in an individual with a hereditary or acquired abnormality of the immune system.

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