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Immune Response Modulation by Colonization of Germfree Rats with *Propionibacterium acnes*

CAROL L. WELLS† AND EDWARD BALISH*

Departments of Surgery and Medical Microbiology, University of Wisconsin Center for Health Sciences, Madison, Wisconsin 53706

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Propionibacterium acnes (oral and/or parenteral administration) had a modulating effect on antibody- and cell-mediated immune responses of germfree (GF) and monoassociated (MA) rats. In conventionally reared rodents, parenteral injection of killed *P. acnes* stimulated the splenic plaque-forming cell response to sheep erythrocytes. However, in GF rats and in rats monoassociated with viable *P. acnes*, parenteral injection of killed *P. acnes* antigen inhibited the plaque-forming cell response to sheep erythrocytes. When compared with the responses of GF control rats, splenocytes from GF rats parenterally injected with *P. acnes* antigen had a decreased in vitro blastogenic response to phytohemagglutinin and concanavalin A and an increased blastogenic response to homologous bacterial antigens. Conversely, a parenteral injection of *P. acnes* antigen into *P. acnes* MA rats resulted in an increased splenocyte blastogenic response to phytohemagglutinin and concanavalin A but not to homologous (*P. acnes*) antigens. Thus the presence or absence of intestinal antigenic stimuli (in MA and GF rats) had a modulating effect on the immune response to a parenteral injection of *P. acnes* antigen.

The ability to alter the immune response of the host by antigen feeding is a relatively untapped area of medical research. In studying the immune responses of germfree (GF) rats that had been monoassociated (MA) (orally) with viable bacteria, we noted different immune responses in GF, MA, and conventionally reared (CONV) animals (C. L. Wells, E. Balish, and C. E. Yale, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, B19, p. 18). Other investigators have shown that oral administration of various antigens can stimulate the immune response with substantial specificity (6, 15, 16) and little immunological memory (1, 9, 16). These findings indicated that the population of viable intestinal bacteria might be manipulated to the immunological advantage or disadvantage of the host. Available information indicates that antigen feeding can result in either positive stimulation of the immune response (7, 9, 13, 24) or in a state of hyporesponsiveness to the ingested antigen (1, 3, 6, 15, 16, 31, 33). Speculations on the causes of this hyporesponsiveness include the formation of secretory immunogloblulins at mucosal surfaces which interfere with antigen uptake (33), the formation of serum antigen-antibody complexes which inhibit the activity of B-lymphocytes (1), or the production of T- or B-suppressor cells, or both, which block cellular responses (3, 16, 31).

Although monoassociation of GF animals is an artificial situation, the model can be used to study the effect of a population of intestinal bacteria on the immune responses of the host. We chose *Propionibacterium acnes* as a test organism in this system for the following reasons: (i) This bacterium is a member of the normal flora of the human skin and gastrointestinal tract (8) and it can colonize the intestinal tract of GF rats. (ii) Parenteral injection of killed *P. acnes* has been widely used as an immunological adjuvant (5) and in cancer immunotherapy (20, 26, 30), and it would be important to know if intestinal bacteria can play a modulating role in the immune response to this parenteral *P. acnes* antigen.

MATERIALS AND METHODS

Animals. Six-week-old female HAIR/c Swiss white mice and 60- to 90-day-old Sprague-Dawley rats were purchased from Sprague-Dawley (Madison, Wis.). The GF and MA (60- to 90-day-old) Sprague-Dawley rats (males and females) were maintained in plastic isolators at the Gnotobiotic Laboratory, University of Wisconsin, Madison, Wis. Weekly fecal cultures from live rats and fecal cultures at the time of sacrifice yielded no extraneous microbial contamination (aerobic and anaerobic) for the duration of the study.

Organism and monoassociation of GF rats. *P. acnes* RC 248-2 was isolated as a laboratory contami-

† Present address: Anaerobe Laboratory, Virginia Polytechnic Institute, Blacksburg, VA 24060.

nant, identified according to recommended criteria (12), and maintained at the Wisconsin State Laboratory of Hygiene, Madison, Wis. (To avoid confusion, the term *P. acnes* [14, 22] will be used throughout this paper in referring to other work, even if the cited author(s) used the term *Corynebacterium parvum*.) Germfree rats were inoculated by swabbing the oral cavity with a 48-h peptone-yeast-glucose broth (12) culture that had been grown in an anaerobic glove box (2). Animal colonization with a pure culture was verified by weekly fecal cultures and by the recovery of 10^{10} viable organisms per g from the cecum and colon of the MA rats at sacrifice. Cultures of internal tissues from GF and MA rats (spleen, liver, lung, kidney, and heart) were consistently negative for microbial growth.

Antigens. A 72-h *P. acnes* broth culture was heat killed (60°C, 1 h), washed with sterile saline, and frozen in 2-ml portions at −40°C. The dry weight of *P. acnes* antigen was determined as milligrams per milliliter of suspension. Sterile sheep erythrocytes (SRBC; GIBCO, Madison, Wis.) were washed and suspended in sterile saline.

Antigens for in vitro lymphocyte blastogenesis included heat-killed cells (as previously described) and also soluble and particulate cell fractions that were prepared by passing whole cells through a modified French pressure cell (19), followed by centrifugation at 5,000 × *g* for 30 min. The soluble fraction was sterilized by filtration and the particulate fraction was sterilized with heat (60°C, 1 h). Antigen protein concentrations were determined by the Lowry method (17).

Enumeration of PFC. The plaque-forming cells (PFC) were enumerated by using a slide modification of the Jerne plaque assay (21). Complement for this assay was derived from pooled guinea pig serum that had been absorbed extensively with SRBC, frozen in 10-ml portions at −40°C, thawed, and then diluted 1:18 immediately before use. Rat immunoglobulin G (IgG) was prepared from the serum of CONV rats by an Na_2SO_4 precipitation (11), and this preparation was injected periodically into a rabbit. The rabbit anti-rat IgG (the facilitating antiserum for indirect plaques) was extensively absorbed with SRBC and with rat erythrocytes, had a 1:32 titer of contaminating antibody, and was used at a predetermined optimal dilution of 1:1,000.

Inoculation of CONV rodents. The adjuvant potential of our *P. acnes* isolate was determined by an accepted method (34). Specifically, groups of CONV rodents were inoculated intravenously on day 0 with either saline (0.2 ml) or *P. acnes* antigen (0.7 mg in 0.2 ml) and on day 7 with an intravenous injection of either saline (0.1 ml) or SRBC (10^8 in 0.1 ml). Direct PFC were enumerated on day 12 (5 days after SRBC injection).

Inoculation of GF and MA rats. GF rats and rats that had been monoassociated for 5 weeks with viable *P. acnes* were inoculated intraperitoneally on day 0 with either saline (0.4 ml) or *P. acnes* antigen (4 mg in 0.4 ml) and on day 8 with an intraperitoneal injection of either saline (0.5 ml) or SRBC (0.5 ml of a 10% suspension). Direct and indirect PFC from GF rats were enumerated on day 13 (5 days after SRBC injection); direct and indirect PFC from MA rats were enumerated on days 13 and 23 (5 and 15 days postinjection of SRBC).

In vitro lymphocyte blastogenesis. Splenic lymphocytes from GF and MA rats were analyzed for their blastogenic responses to polyclonal mitogens and to homologous (i.e., colonizing) bacterial antigens. The mitogen concentrations used in each assay, predetermined by previous experiments, included phytohemagglutinin-M (PHA; Difco, Detroit, Mich.), 50 and 25 μg; concanavalin A (ConA; Calbiochem, San Diego, Calif.), 2.5 and 1.25 μg; pokeweed (PWM; GIBCO Laboratories, Grand Island, N.Y.), 50 and 5 μg; and lipopolysaccharide (Difco; *Escherichia coli* 0111:B4 or 055:B5), 50 and 5 μg. The amount and kind of homologous *P. acnes* antigens used included heat-killed whole cells, 35, 3.5, and 0.35 μg; a soluble (cytoplasm) fraction, 10, 1, and 0.1 μg; and a particulate (cell wall and membrane) fraction, 385, 38.5, and 3.85 μg. Each mitogen and antigen concentration was assayed in either triplicate or quadruplicate.

Rat spleen cells were teased through 60-gauge wire mesh screens into phosphate-buffered saline. Leukocytes were separated on a Ficoll-Hypaque gradient, washed twice with phosphate-buffered saline and resuspended in RPMI 1640 medium that had been supplemented with 2 mM sodium pyruvate, 4 mM L-glutamine, 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer, 5,000 IU of penicillin, and 5 mg of streptomycin per 100 ml (all of the latter solutions and chemicals were obtained from Flow Laboratories, Rockville, Md.). Splenocyte viability, determined by dye exclusion, was usually 85 to 95%. Spleen cells (2×10^5 viable cells/well) were placed in flatbottomed microtiter wells (Falcon Plastics, Oxnard, Calif.) with 5% heat-inactivated, autologous serum and one of the concentrations of antigen or mitogen mentioned above. Cultures were incubated for 4 days (a previously determined optimum incubation time) at 37°C in 5% CO_2, pulsed with 1 μCi of [^{3}H]thymidine (specific activity, 2 Ci/mmol, New England Nuclear Corp., Boston, Mass.), harvested on a cell harvester (Otto Hiller Co., Madison, Wis.), precipitated onto glass fiber filter paper (Microbiological Associates, Bethesda, Md.), and air dried, a scintillation cocktail was added, and the counts per minute of incorporated [^{3}H]thymidine were determined in a model 3375 liquid scintillation counter (Packard Instrument Co., Inc., Downer's Grove, Ill.). The counts per minute for the antigen or mitogen concentration giving the highest average counts per minute are reported in the tables, and these cpm were converted to $\log_{10}$ values for statistical analysis.

Statistical analysis. All data were analyzed by a one-way analysis of variance followed by Fisher's test for least significant difference (30).

RESULTS

Effect of parenteral *P. acnes* on the direct PFC response to SRBC in the spleens of CONV rodents. An injection of *P. acnes* into mice, 7 days before an injection of SRBC, caused an eightfold increase in the number (per spleen) of direct PFC (Table 1). The data presented in Table 1 were the results of one of two similar

experiments. Splenomegaly (approximately fivefold) was observed in all *P. acnes* injected mice.

Comparable increases in PFC were obtained in similar experiments performed with CONV rats (data not presented, see Discussion). Splenomegaly was not observed in *P. acnes*-injected CONV rats.

Effect of parenteral *P. acnes* on the direct and indirect PFC response to SRBC in the spleens of GF rats. Germfree rats had a negligible background PFC response to SRBC that was not significantly altered by a parenteral *P. acnes* injection (Table 2). The substantial increase in the numbers of splenic PFC indicated that germfree rats could respond to an injection of SRBC (Table 2). An i.p. injection of GF rats with *P. acnes* 8 days before the injection of SRBC caused about a 50% reduction in the direct and indirect PFC enumerated on day 13, i.e., 5 days postinoculation with SRBC. Since assays were performed 5 days after SRBC injection, we would not expect to detect significant numbers of IgG-forming cells (23). The decreased response in indirect compared with direct PFC (Table 2) was interpreted to reflect both the absence of an IgG response and the presence of some anti-IgM activity in the facilitating antiserum. This anti-IgM activity resulted in a 9 and 28% inhibition of PFC in groups 3 and 4, respectively.

Effect of parenteral *P. acnes* on the direct and indirect PFC response to SRBC in the spleens of rats monoassociated with viable *P. acnes*. Neither monoassociation of GF rats with viable *P. acnes* nor a parenteral challenge of these MA rats with dead *P. acnes* caused a noticeable increase in the background PFC response to SRBC (Table 3). Rats monoassociated with viable *P. acnes* had a substantial PFC response to SRBC 5 days after injection of SRBC. Injection of *P. acnes* antigen 8 days before the injection of SRBC caused about a fourfold reduction in this PFC response (Table 3). Five days after SRBC injection slightly more direct than indirect plaques were detected, which was again interpreted to mean that the early PFC response of MA rats was primarily IgM-forming cells. On day 23 of the experiment (15 days after SRBC injection), the direct PFC response to SRBC was reduced (Table 3), reflecting a decline in IgM response. The decrease in the indirect PFC response on day 23 was somewhat difficult to interpret since our facilitating antiserum most probably also contained anti-IgM activity. Again this anti-IgM activity was 10 and 27% in groups 3 and 4 (Table 3), respectively. Thus, assuming that the anti-IgM activity was less than 30% in group 5 (Table 3), only a slight, if any, IgG response was present on day 23 (15 days after SRBC injection).

Effect of parenteral *P. acnes* on the in vitro lymphocyte blastogenic response of spleen cells from GF rats and from rats monoassociated with viable *P. acnes*. The effect of parenteral *P. acnes* on the blastogenic responses of spleen cells from GF and MA rats

TABLE 1. *Effect of parenteral P. acnes on the direct PFC response to SRBC in the spleens of CONV mice*

Group no.	Inoculation		No. of mice	Avg direct PFC/SE[a] on day 12	
	Day 0	Day 7		Per 10^6 cells	Per spleen
1	Saline	Saline	3	1 ± 1	50 ± 10
2	*P. acnes*	Saline	3	2 ± 1	1,100 ± 500
3	Saline	SRBC	5	500 ± 60	68,000 ± 3,300
4	*P. acnes*	SRBC	6	800 ± 230	544,800 ± 147,300[b]

[a] SE, Standard error of the mean.
[b] Increased over group 3, $P < 0.01$.

TABLE 2. *Effect of parenteral P. acnes on the direct and indirect PFC response to SRBC in the spleens of GF rats*

Group no.	Inoculation		No. of rats	Avg PFC ± SE[a] on day 13			
				Direct		Indirect	
	Day 0	Day 8		Per 10^6 cells	Per spleen	Per 10^6 cells	Per spleen
1	Saline	Saline	3	>1	40 ± 30	0	0
2	*P. acnes*	Saline	3	>1	70 ± 70	>1	50 ± 50
3	Saline	SRBC	2	530 ± 140	330,000 ± 45,000	480 ± 140	301,000 ± 49,900
4	*P. acnes*	SRBC	5	280 ± 30[b]	187,200 ± 47,400	210 ± 40	135,300 ± 29,900[b]

[a] SE, Standard error of the mean.
[b] Decreased from group 3, $P < 0.05$.

TABLE 3. *Effect of parenteral P. acnes on the direct and indirect PFC response to SRBC in the spleens of rats monoassociated with viable P. acnes*

Group no.	Inoculation		No. of rats	Avg PFC ± SE[a] on day 13			
				Direct		Indirect	
	Day 0	Day 8		Per 10^6 cells	Per spleen	Per 10^6 cells	Per spleen
1	Saline	Saline	3	>1	20 ± 20	>1	40 ± 20
2	*P. acnes*	Saline	3	>1	90 ± 50	>1	10 ± 10
3	Saline	SRBC	2	1,200 ± 140	650,000 ± 230,000	1,100 ± 20	582,800 ± 151,600
4	*P. acnes*	SRBC	3	300 ± 120[b]	183,800 ± 55,600[b]	240 ± 70[b]	134,600 ± 28,500[b]
5[c]	*P. acnes*	SRBC	3	98 ± 30	82,500 ± 38,400[d]	369 ± 260	84,600 ± 18,600[d]

[a] SE, Standard error of the mean.
[b] Decreased from group 3, $P < 0.01$.
[c] This group was assayed on day 23.
[d] Decreased from group 4, $P < 0.01$.

(Table 4) may be summarized as follows. (i) Lipopolysaccharide did not stimulate rat spleen cell blastogenesis in any of the groups tested. (ii) Monoassociation with *P. acnes* suppressed splenocyte responses to polyclonal mitogens. (iii) Injection of GF rats with *P. acnes* antigen decreased the PHA and ConA blastogenic responses, but not the PWM response. (iv) The MA rat splenocyte responses to PHA and ConA were increased after injection of *P. acnes* antigen. (v) The highest blastogenic responses to *P. acnes* antigen preparations were obtained with splenocytes from GF rats that had been injected with *P. acnes*; monoassociation with *P. acnes* interfered with this blastogenic response to *P. acnes* antigens.

As seen from the data in Table 4, *P. acnes* antigenation (parenterally) of GF rats decreased their splenocyte blastogenic response to PHA and ConA. Conversely, the same injection of *P. acnes* MA rats increased their blastogenic response to ConA and PHA. Injection of *P. acnes* antigen resulted in the formation of specifically sensitized lymphocytes in germfree rats but not in gnotobiotic rats that were colonized with *P. acnes*.

DISCUSSION

Our results with GF rats indicated that experience with a conventional microbial flora was not prerequisite for the formation of splenic PFC in response to SRBC injection, but this experience was necessary to achieve the immunostimulatory effects of *P. acnes*; i.e., parenteral *P. acnes* caused a twofold decrease in PFC response of GF rats to SRBC.

The PFC assays with *P. acnes* MA rats also indicated that a complex microflora was not necessary for the rats to respond to an SRBC injection. In *P. acnes* MA rats, however, parenteral *P. acnes* injection caused a fourfold decrease in the splenic PFC response to SRBC. This decrease was even greater than the decrease observed under similar conditions with GF counterparts. This observation does not agree with speculations, based on work in CONV animals (27, 29, 32), stating that the immunopotentiating effects of *P. acnes* may depend on prior exposure to cross-reacting bacterial antigens that have been shown to exist in animals and humans (35). The lack of antigenic stimulation and suppressive effects in GF mice may be due to a deficiency in populations of activated macrophages. However, MA rats were heavily colonized with a recognized macrophage activator (*P. acnes*), and the suppression of PFC response in MA rats was even greater than that observed in GF rats. The lack of substantial numbers of indirect PFC 15 days after SRBC injection demonstrated that MA rats did not possess IgG PFC at a time when one would expect these PFC to be present (23), indicating that perhaps the *P. acnes* MA rats were incapable of normal B-cell ontogeny (18).

At this time, we wish to call attention to the fact that our PFC assays utilized gnotobiotic rats and CONV mice. We originally used CONV rats for PFC assays and observed that a parenteral injection of CONV rats with *P. acnes* had an adjuvant effect on the number of PFC formed in response to a subsequent SRBC injection. However, spleen cells from CONV rats formed numerous mottled, hazy, background plaques that made exact quantitation extremely difficult, if not impossible. However, we believe we can accurately estimate the number of PFC in conventional rat spleen cells and we can state that parenteral injection of *P. acnes* had a comparable adjuvant effect on both mouse and rat spleen cells. It was interesting that spleen cells from GF or MA rats did not have this confusing background of hazy plaques, and PFC response to SRBC antigenation could be enumerated as easily as with the mouse model. Perhaps GF and

TABLE 4. *Effect of parenteral P. acnes on the in vitro lymphocyte blastogenic response of spleen cells from germfree rats and rats monoassociated with viable P. acnes*

No. of rats	Microbial status	Intraperitoneal inoculation	Blastogenic response							
			Polyclonal mitogen					Homologous antigen		
			None	PHA	ConA	LPS	PWM	Heat killed	Soluble fraction	Particulate fraction
4	GF	Saline	2.15[a] 174	4.10 13,344	4.55 36,600	2.19 170	3.39 3,301	2.47[b] 327	2.37[b] 270	2.44[b] 346
6	GF	*P. acnes*	2.60 430	3.84[c] 7,852	4.27[c] 20,860	2.46 307	3.57 5,179	3.02 1,708	2.78 681	2.93 1,115
3	MA	Saline	2.26 193	3.47[b, c, d] 3,817	3.58[b, c, d] 4,233	2.17 174	2.44[b, c] 295	2.29[b] 201	2.40[b] 265	2.12[b, d] 141
3	MA	*P. acnes*	2.48 321	3.86[c] 7,486	3.82[b, c] 7,324	2.42 311	2.74[b, c] 586	2.52[b] 393	2.54[b] 364	2.53[b] 589

[a] The top number is the average $\log_{10}$ value; the number below is the average counts per minute of [^{3}H]thymidine incorporated.

[b] Decreased from GF injected with *P. acnes*, $P < 0.005$.

[c] Decreased from GF, $P < 0.005$.

[d] Decreased from MA injected with *P. acnes*, $P < 0.005$.

MA rats have populations of suppressor cells that inhibit the formation of the hazy plaques, or perhaps spleen cells from CONV rats produce antibodies that cross-react with SRBC.

A study of the effects of parenterally injected *P. acnes* on the in vitro lymphocyte blastogenic responses of rat spleen cells also yielded some surprising results. In GF rats, parenteral *P. acnes* decreased the blastogenic response to PHA and ConA and increased the blastogenic response to *P. acnes* antigen preparations. The former observation supported the work of Scott (25) and Bash (4), who noted that parenteral *P. acnes* decreased the PHA responsiveness of spleen cells from CONV mice and rats. The decreased PHA response of lymphocytes from *P. acnes*-injected GF rats, coupled with a similar observation on splenocytes from CONV rats (4), is also consistent with the work of Scott, MacDonald, and Carter (28), who noted that parenteral *P. acnes* induced a suppressive effect on a delayed-type hypersensitivity response (footpad swelling) in GF and CONV mice. Thus, as seen with GF rats, experience with the normal microflora was not necessary for parenteral *P. acnes* to inhibit the in vitro spleen cell response to PHA or to stimulate a population of lymphocytes to divide upon subsequent in vitro exposure to the homologous (*P. acnes*) antigen.

The finding that *P. acnes*, when injected into GF rats, decreased the splenocyte response to PHA and ConA but not to PWM could be explained by the observations that *P. acnes* has an immunostimulatory effect on B-lymphocytes and an inhibitory effect on T-lymphocytes (5). Since PHA and ConA are primarily T-cell mitogens, whereas PWM is a mixed B- and T-cell mitogen (10), parenteral injection with *P. acnes* could decrease the PHA and ConA responses and have no effect on the PWM response. An example of the different immune responses that can be obtained by parenteral injection of killed organisms or by oral colonization was the observation that splenocytes from MA rats (viable *P. acnes*) had a decreased response to PWM, whereas GF rats that were parenterally injected with *P. acnes* (dead antigen) had an enhanced response to PWM.

Compared to the GF rat splenocytes, oral colonization with viable *P. acnes* decreased the splenocyte response to polyclonal mitogens. However, in contrast to our work in GF rats and to the work of Bash (4) in CONV rats, parenteral *P. acnes* stimulated the PHA and ConA responses of MA rats. Also, colonization of rats with *P. acnes* prevented the positive blastogenic responses to homologous antigen preparations that were observed after a parenteral injection of this antigen into GF rats; i.e., MA rats were hyporesponsive to homologous antigen preparation. Thus, in MA rats, parenteral *P. acnes* had an adjuvant effect on T-lymphocytes without a concurrent increase in their cellular responses to homologous *P. acnes* antigen. Bash (4) suggested that dissociation of the immunopotentiating properties from the T-cell-inhibiting properties might increase the efficacy of *P. acnes* in antitumor therapy. Perhaps oral feeding of viable *P. acnes* could block some of the T-cell-inhibiting properties of parenteral *P. acnes* and enhance the antitumor effect.

This work demonstrated that oral colonization with a pure culture of *P. acnes* can modulate the immune response of the host.

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