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High-Level Induction of Gamma Interferon with Various Mitogens in Mice Pretreated with *Propionibacterium acnes*

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Various T-cell mitogens induced high levels of circulating gamma interferon (IFN-γ) in mice that had been pretreated with *Propionibacterium acnes*. Administration of lipopolysaccharide, a B-cell mitogen, to these mice also caused pronounced production of IFN-γ in addition to IFN-α and IFN-β. The enhanced induction was most marked at about 1 week after the pretreatment.

Immune or gamma interferon (IFN-γ) is produced in animals upon challenge with specific antigens (14) or in lymphocyte-macrophage cultures by stimulation with polyclonal T-cell mitogens (17). However, production of IFN-γ is very low or undetectable in animals upon administration of T-cell mitogens which are active IFN-γ inducers in vitro. *Propionibacterium acnes* (formerly designated *Corynebacterium parvum*) has been known to have various immunomodulating and antitumor effects (13) and also to enhance IFN production both in vivo (23) and in vitro (19), besides being an IFN inducer in cultured lymphocytes (6). Here we report that various polyclonal mitogens induce high levels of IFN-γ in the circulation of mice pretreated with *P. acnes*. Notably, administration of lipopolysaccharide (LPS), a B-cell mitogen to these mice also effectively caused pronounced production of IFN-γ and relatively low levels of IFN-α and IFN-β, in contrast to the generally held notion that LPS is an inducer of IFN-α or IFN-β.

MATERIALS AND METHODS

Mice. SPF mice of various strains 8 to 10 weeks old, were from Shizuoka Laboratory Animals Center.

Bacteria and reagents. *P. acnes* (ATCC 11827) was grown in brain heart infusion medium supplemented with L-cysteine (0.03%) and Tween 80 (0.03%), and the harvested cells, washed by centrifugation with phosphate-buffered saline, were killed by exposure to 60°C for 1 h. Staphylococcal enterotoxin A (SEA) was purified to electrophoretic homogeneity by the method described by Shinagawa et al. (15). LPS (*Escherichia coli*) and phytohemagglutinin (PHA) were purchased from Difco Laboratories. Concanavalin A (ConA) and galactose oxidase were obtained from Pharmacia Fine Chemicals Inc. and Worthington Biochemicals Corp., respectively.

Treatment of mice. Mice were injected intraperitoneally with large doses of heat-killed *P. acnes* (10 mg [wet weight] per mouse), and at appropriate time intervals they were challenged intravenously with various substances. Blood was taken from the heart at different times after challenge, and the serum IFN levels were assayed.

IFN assay. IFN assays were carried out by measuring the capacity to inhibit the cytopathic effect of vesicular stomatitis virus on L-cells. Titers were expressed as international reference units, using National Institutes of Health reference IFN (no. G002-905-511).

Antisera and neutralization test. Anti-mouse IFN-γ sera from rabbits immunized with IFN from lymphocytes induced by PHA or SEA were kindly given by E. Falcoff (Institut Curie, Paris) (16) and by J. A. Georgiades (University of Texas, Galveston) (12). Anti-IFN-α and anti-IFN-β, formerly designated as anti-IFN-F and anti-IFN-S, respectively, were prepared as described before (9, 22). Anti-L-cell IFN (anti-IFN-α,β) supplied by the Antiviral Substances Program, National Institute of Allergy and Infectious Diseases, Bethesda, Md., was also used. The specificity of these antibodies was ascertained by using IFN-α and IFN-β from L-cells (22) and IFN-γ from SEA-induced mouse spleen cells.

Neutralization tests were carried out by determining the IFN titration endpoint (50% cytopathic effect) in the presence of a fixed concentration of one of the antisera or their mixtures (8). The antibody concentrations used were such that IFN-α and IFN-β were neutralized to a few percent of the control IFN without antibody, and IFN-γ was neutralized to 10%. If a substantial level of IFN remained unneutralized in the presence of, for instance, anti-IFN-α,β, the observed titer was taken to represent IFN-γ, after confirming that it was further reduced by anti-IFN-γ added together with anti-IFN-α,β. In this way, the α, β, and γ compositions of various IFN samples were semiquantitatively estimated by appropriate use of various antisera. The sum of the titers of the individual IFNs thus estimated was about equal to or marginally lower than the titer of control IFN without added antibodies. That is, the phenomenon of potentiation (3, 4), in which IFN-α,β and IFN-γ when mixed exhibit greater antiviral activities than the sum of the individual activities, was not readily observable in our IFN assay system.

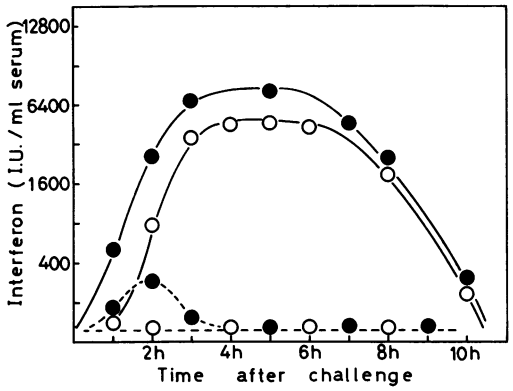

FIG. 1. In vivo production of IFN triggered by SEA or LPS in *P. acnes*-treated mice. C57BL/6 mice which had been intraperitoneally injected with 10 mg (wet weight) of heat-killed *P. acnes* 7 days before were challenged intravenously with 1 μg of SEA or 1 μg of LPS. Blood was taken at the indicated times, and the serum samples were assayed for IFN activity. Each value represents the average of five samples. *P. acnes*-treated mice were challenged with SEA (○—○) or with LPS (●—●). Untreated mice were injected with SEA (○---○) or with LPS (●---●).

TABLE 1. In vivo production of IFN triggered by various substances in *P. acnes*-treated mice[a]

Challenge with:	Amt per mouse	IFN activity in serum
SEA	1 μg	5,198 ± 1,380
LPS	1 μg	6,411 ± 2,695
Galactose oxidase	10 U	6,033 ± 2,399
PHA	40 μg	135 ± 86
ConA	20 μg	26 ± 18
ConA	100 μg	3,560 ± 1,272
Protein A	250 μg	83 ± 61
Neuraminidase	10 U	46 ± 22
Heated galactose oxidase	20 U	80 ± 68
Heated LPS	2 μg	4,056 ± 1,483

[a] C57BL/6 mice that were treated with *P. acnes* 1 week earlier were intravenously challenged with various substances, and serum samples were harvested 6 h after challenge. For ConA, blood was taken at 4 h postchallenge. Heating of galactose oxidase and LPS was performed at 100°C for 30 min. Average values and standard deviations of six samples are shown.

RESULTS

IFN induction in *P. acnes*-treated mice. Figure 1 shows the induction kinetics of in vivo production of IFN induced with SEA and LPS in C57BL/6 mice which were pretreated with *P. acnes* 7 days before. IFN activity appeared in the blood at around 1.5 h after challenge with SEA and reached a maximum (4×10^3 to 10^4 U/ml) at about 3 h. The peak level was maintained for 3 to 4 h thereafter. The time course was similar to that observed when *Mycobacterium bovis* BCG-infected mice were challenged by purified protein derivative (20), but the IFN levels seemed higher in the present case. Mice not pretreated with *P. acnes* failed to produce IFN until 22 h after challenge with SEA. Production of IFN by LPS was somewhat different. Injection of LPS to untreated mice also induced low levels of IFN which peaked at about 2 h postinjection as described by other workers (18). This type of IFN was reported to have a common antigenicity to L-cell IFN (21). Mice treated with *P. acnes* showed a greatly enhanced and prolonged production upon challenge with LPS. The lag period of induction was shorter than in the case of SEA, and the maximum activity observed was even higher.

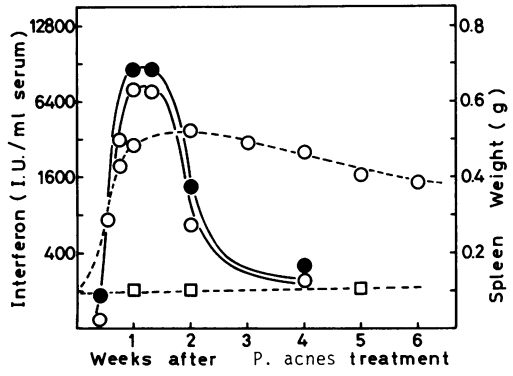

FIG. 2. Changes with time after *P. acnes* treatment of serum IFN levels induced by mitogens and of spleen weight. Mice were challenged with SEA (○) or LPS (●) on different days after treatment with *P. acnes*. All of the serum samples for IFN assay were taken 6 h after the mitogen challenge. Spleens were weighed from treated mice on the indicated days after injection of *P. acnes* (○---○) and from untreated mice (□---□). The values are the averages of six samples.

The enhanced production of IFN by SEA or LPS was observable starting from 4 to 5 days after treatment with *P. acnes* and could hardly be observed after 3 weeks (Fig. 2), the optimal time being around 7 days posttreatment. During this period, a huge hepatosplenomegaly was observed. As shown in Fig. 2, the spleen weight reached a maximum at about 7 days after injection of *P. acnes* and diminished only slightly during the following several weeks.

Other substances tested for in vivo induction of IFN are listed in Table 1. Galactose oxidase, which is a T-cell mitogen and can induce IFN-γ in vitro (1, 2) could induce production of IFN in *P. acnes*-treated mice. Heating of galactose oxidase (100°C for 30 min) largely eliminated its inducing ability, whereas heating of LPS did not. This shows that the inducing ability of galactose oxidase was not due to possible contamination by LPS. ConA, a well known T-cell mitogen,

TABLE 2. Acid stability and antigenicity of IFN harvested from the circulation of *P. acnes*-treated mice after challenge with SEA or LPS

Treatment	IFN titer (IU/ml) at time (h) after challenge:			
	SEA		LPS	
	2	6	2	6
None	800	5,700	2,240	10,400
pH 2	80	200	2,200	320
Anti-IFN-α, β	760	5,160	100	9,600
Anti-IFN-γ	160	980		2,400

induced little IFN at a low dose (20 μg per mouse), but at a high dose (100 μg per mouse), almost the same level of IFN activity as was obtained with SEA appeared in the circulation of *P. acnes*-treated mice. However, most of the mice, both normal and *P. acnes* treated, died by 4 to 5 h after administration of ConA at this dose. Mice which were not pretreated with *P. acnes* failed to produce IFN by injection of galactose oxidase or ConA. PHA, which is a good inducer of IFN-γ in vitro, did not induce IFN production at the dose employed. Higher dose of PHA could not be employed because of its toxicity. Protein A and neuraminidase were poor inducers.

Molecular types of IFN produced. These IFNs produced as described above were characterized as to their molecular types by acid stability and neutralization tests. SEA-induced IFNs harvested at different times consisted of the γ type, since they were inactivated after exposure to pH 2 (dialysis against 0.02 M glycine-hydrochloride buffer for 20 h at 4°C) and neutralized by anti-IFN-γ antiserum, but not by anti-IFN-α,β antiserum. The data on 2- and 6-h samples are shown in Table 2. On the other hand, LPS-induced IFNs showed variations in the molecular composition depending on the time of harvest. IFN that appeared 2 h after LPS challenge was resistant to pH 2 treatment and was neutralized by anti-IFN-α,β antiserum. However, IFN activity that appeared at 6 h postchallenge was unstable at pH 2 and neutralized by anti-IFN-γ antiserum but not by anti-IFN-α,β antiserum.

By using the specific anti-IFN-α, anti-IFN-β, and anti-IFN-γ antisera, LPS-induced IFNs were further analyzed for their molecular compositions as described above. The results are shown in Fig. 3. IFN at 1 h postchallenge was mostly β-type; that at 2 h was a mixture of α and β types; and those at 4 h and later were predominantly γ type. In harmony with this finding, the 1- and 2-h samples were acid stable, whereas the later ones were unstable. Thus, LPS appeared to trigger IFN-γ production in a manner similar to that of SEA in *P. acnes*-treated mice but, in addition, it induced early-appearing IFN-α and IFN-β. IFNs induced by ConA and galactose oxidase had mostly the properties of IFN-γ.

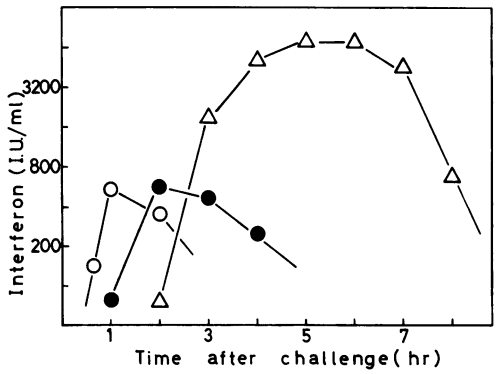

FIG. 3. Variation with time after LPS challenge in the molecular composition of IFN in the circulation of *P. acnes*-treated mice. ICR mice pretreated with *P. acnes* were challenged with LPS, and the blood was taken at appropriate times. Determination of the IFN types was performed by the acid stability test and neutralization test, using the anti-IFN-α, anti-IFN-β, or anti-IFN-γ antiserum and their combinations as described in the text. Symbols: IFN-α,●; IFN-β, ○; IFN-γ, △.

DISCUSSION

Our experiments thus indicated that administration to mice of various T-cell mitogens and a B-cell mitogen, LPS, which do not appreciably induce IFN-γ in vivo, results in production of high levels of IFN-γ if the mice have been pretreated with *P. acnes*. Live *M. bovis* BCG instead of *P. acnes* also produced similar effects in mice (unpublished data). The mechanism of enhanced production of IFN-γ remains to be clarified. One could suspect that the administration of bacteria might have sensitized the mice immunologically against the inducers given later. This, however, seems unlikely, because SEA or LPS did not cause any delayed hypersensitivity in *P. acnes*-treated mice when examined by footpad reactions (data not shown). Also, the relatively rapid decline of enhanced production of IFN (Fig. 1) may not be consistent with immunological sensitization. Bacterial organisms such as *P. acnes* and *M. bovis* BCG have been reported to activate the reticuloendothelial system (5, 10), and, in our mice, a pronounced splenomegaly was observed (Fig. 2). It may therefore be possible that activation of regulatory cells such as macrophages is involved in the enhanced IFN-γ production. Or, since suppressor cells which inhibit IFN-γ production have been reported to be generated in mitogen-injected mice (7), it could be surmised that their

generation is suppressed in bacteria-treated mice.

Production of high levels of IFN-γ upon stimulation with LPS was unexpected but observed in at least three strains of mice examined (C57BL/6 and outbred ICR and ddY). This casts doubt on the current belief that LPS induces only type I IFN (α or β), although it remains to be seen whether the actual inducing stimulus was LPS or *P. acnes*. Evans and Johnson (3) recently reported that *P. acnes* induced IFN-γ as well as IFN-α,β in mouse spleen cells. Thus, it may be possible that the role of LPS is to stimulate some accessory cells which in turn help the IFN-γ-producing cells responding to an inducer other than LPS. That the lag period of IFN-γ production is longer than that of IFN-α,β may be consistent with this idea (Fig. 1 and 3).

Induction of IFN-γ production in *M. bovis* BCG-sensitized mice was reported to be under genetic control (11). This seems to be also true for the IFN-γ induction by polyclonal mitogens studied here since mice of different strains differed markedly in their responses after *P. acnes* treatment, with C57BL/6, B.10, ICR, and ddY being high responders and C3H/He, A/J, and BALB/c being low responders (manuscript in preparation). This suggests that the genetic control may operate on levels other than antigen recognition.

The present experiments demonstrated a novel mode of IFN-γ production in vivo. Its possible relationship to antitumor and immunomodulating effects of *P. acnes* will be worth exploring.

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