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Induction of Alpha and Beta Interferons During the Hyporeactive State of Gamma Interferon by *Mycobacterium bovis* BCG Cell Wall Fraction in *Mycobacterium bovis* BCG-Sensitized Mice

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Characterization of interferon (IFN) induced by a second challenge with specific antigen was investigated during the development of hyporeactivity established after challenge of *Mycobacterium bovis* BCG-sensitized mice with BCG cell wall fraction (BCG-CW). When BCG-sensitized mice were challenged with BCG-CW, IFN-γ was detected in the circulation 4 h later and rapidly disappeared. After IFN-γ disappeared from their blood, mice became completely hyporeactive to the second challenge with BCG-CW for 1 day, and thereafter they recovered from this hyporeactive state. However, we always observed that IFN induced at first by the second challenge with BCG-CW during the hyporeactive state was type I IFN-α/β, but thereafter was entirely IFN-γ. Induction of IFN-α/β by the specific antigen during restoration from the hyporeactive state in BCG-sensitized mice is discussed.

Youngner and Salvin (12) showed that gamma interferon (IFN-γ) was produced in *Mycobacterium bovis* BCG-sensitized mice after challenge with old tuberculin, and thereafter these mice became hyporeactive to the homologous antigen (13). Minagawa and Ho (5) found that hyporeactivity factor was produced in the circulation of BCG-sensitized mice that were challenged with BCG, as reported by Stringfellow et al. (8–10), and suggested that there were two types of hyporeactivity factors, namely, IFN-γ itself and a substance antigenically associated with IFN-α/β, which were detected in the hyporeactive state. Alternatively, IFN-α/β as well as IFN-γ may be induced by challenge with BCG cell wall fraction (BCG-CW) in BCG-sensitized mice, although BCG-CW does not induce any IFN without sensitization with BCG in vivo. In fact, some inducers can induce both types of IFN. Recently, we found the alternative induction of IFN-α/β or IFN-γ by *Listeria monocytogenes* in vivo and in vitro (6; A. Nakane and T. Minagawa, manuscript in preparation). Therefore, we investigated the level and type of IFN detected in the circulation after challenge of BCG-sensitized mice with BCG-CW. We report here that IFN-α/β can be induced by a second challenge with BCG-CW during restoration of these mice from the hyporeactive state, and that recovery of IFN-γ production follows the disappearance of IFN-α/β production.

MATERIALS AND METHODS

Mice. Female ddY outbred mice, 5 to 10 weeks old (Hokkaido Experimental Animal Center, Sapporo), were used in all experiments.

Preparation of BCG organisms and BCG-CW. BCG cells (Japan strain, Japan BCG Co., Tokyo) were grown in Dubos broth medium (Difco Laboratories, Detroit, Mich.) supplemented with bovine albumin (0.6%, fraction V; Armour Pharmaceuticals Co., Phoenix, Ariz.), glucose (0.75%), and glycerol (5%) for 10 to 12 days; the cells were then prepared and maintained as previously reported (5). BCG-CW was prepared according to the method of Azuma et al. (2). Briefly, the cell wall fraction was differentiated from sonically disrupted bacterial suspensions by centrifugation. The cell wall fraction isolated was washed three times with sterilized double-distilled water. The lyophilized BCG-CW preparation was stocked at 4°C until use.

Sensitization with BCG. Mice were injected in the tail vein with 0.2 ml of 8×10^6 colony-forming units of live BCG in 0.01 M phosphate-buffered saline (PBS, pH 7.4) containing 0.02% Tween 80. Three weeks after sensitization, the mice were inoculated intravenously with BCG-CW reconstituted with PBS.

Viruses. Vesicular stomatitis virus, Indiana strain, was prepared in primary mouse embryo fibroblasts and had a titer of 10^7 PFU per 0.1 ml in human amnion FL cells.

Inducers of IFN-α/β. IFN-α/β was induced by lipopolysaccharide (LPS) from *Salmonella typhimurium* (Difco), purified by the method of Westphal and Jann (11), and polyriboinosinic-polyribocytidylic acid sodium salts [poly(rI-rC)] Yamasa Shoyu Co., Choshi,

Chiba). These inducers were suspended in PBS and injected intravenously into mice.

Cell culture and media. Mouse L929 cells were purchased from Flow Laboratories, Rockville, Md., and RPMI 1640 medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with penicillin G (100 U/ml), streptomycin (100 μg/ml), and fetal bovine serum (5%) was used in all cell cultures and for certain dilutions.

IFN assays. IFN assays were done by the dye-binding assay (1) by using a continuous line of mouse L929 cells and vesicular stomatitis virus as the challenge virus. The titer represented the dilution which inhibited 50% cytopathology of the virus. One unit of IFN in these assays was the equivalent of 1 U of a reference mouse standard IFN (catalog no. G-002-904-511) provided by the Antiviral Substances Program of the National Institute of Allergy and Infectious Diseases.

Stability of IFN at pH 2.0. Sera diluted with RPMI 1640 medium containing 5% fetal bovine serum were dialyzed against 0.2 M KCl-HCl buffer, pH 2.0, for 48 h at 4°C and then redialyzed against Eagle minimal essential medium (GIBCO) for 24 h at 4°C. These samples were assayed immediately for antiviral activities. Control samples were obtained by dialyzing against 0.01 M PBS (pH 7.4) instead of pH 2.0 buffer.

Neutralization with anti-mouse IFN-α/β serum. Anti-L-cell IFN-α/β serum (catalog no. G-024-501-568) was obtained from the Antiviral Substances Program, National Institute of Allergy and Infectious Diseases. Samples of sera serially diluted twofold were mixed with equal volumes of rabbit anti-mouse IFN-α/β serum diluted 1:200. This diluted antiserum completely neutralizes 50 IU of mouse IFN-α/β induced by Newcastle disease virus (NDV) in L cells. The mixtures were incubated for 1 h at 37°C before being assayed for IFN activities.

RESULTS

IFN responses by BCG-CW, LPS, or poly(rI-rC) after challenge with BCG-CW in BCG-sensitized mice. Figure 1 shows the schedule of experiments. Pooled sera of specimens were obtained from five mice. Figure 2A shows IFN responses of BCG-sensitized mice by the second challenge with BCG-CW on each day after the first administration of the same antigen. On day 1 after the challenge with BCG-CW, reinduction of IFN by the same antigen was completely abolished, and thereafter the hyporeactive state was substantially restored. On the other hand, when LPS or poly(rI-rC) was injected into BCG-sensitized mice after the challenge with BCG-CW, no significant suppression of IFN production by these type I IFN-α/β inducers was observed (Fig. 2B).

Characterization of IFN induced by BCG-CW in BCG-sensitized mice during the hyporeactive state. All IFN induced by BCG-CW in BCG-sensitized mice that were previously injected with PBS completely lost antiviral activity by dialysis against pH 2.0 buffer, and the IFN titers were not affected by treatment with anti-mouse IFN-α/β serum (Table 1). However, we noticed that IFN-α/β determined on the basis of both pH 2.0 stability and antigenicity was significantly produced by the second challenge with BCG-CW on day 2 after the challenge with BCG-CW. However, on days 4 or 7, IFN induced by the second challenge with BCG-CW was IFN-γ. We repeated this experiment several times. In rare cases, we could detect very low antiviral activity of about 30 IU/ml by the second challenge with BCG-CW on day 1 after the challenge with BCG-CW in BCG-sensitized mice. Such preparations contained IFN-α/β. We also investigated the time course of IFN-α/β production after the second challenge with BCG-CW, using mice that had received the first challenge with BCG-CW 2 days earlier. IFN-α/β production peaked 3 or 4 h after administration of BCG-CW, as did IFN-γ production by the same antigen (data not shown).

To confirm these results, we repeated the experiments to check the acid stability and antigenicity of the IFN specimens obtained. Antiviral activities of all samples detected in sera from BCG-sensitized mice challenged twice with BCG-CW at 2-day intervals remained after pH 2.0 treatment (31 to 55%) and were largely neutralized by anti-mouse IFN-α/β serum (83 to 87%) (Table 2). In control studies, all IFN preparations produced by mice that had been sensitized with BCG and had received one challenge with BCG-CW had completely lost antiviral activity after the acid treatment, but were not affected by the treatment with anti-mouse IFN-α/β serum.

DISCUSSION

IFN induced by specific antigen in BCG-sensitized mice completely loses its antiviral activity after treatment at pH 2.0 and is not neutralized by anti-mouse IFN-α/β serum. This is a typical IFN-γ-producing system in vivo. In our study, however, IFN-α/β defined by virtue of its acid stability and antigenicity could be induced by BCG-CW in BCG-sensitized mice that had been previously challenged with the same antigen. Production of IFN-α/β was recognized at the early stage after the first challenge with the specific antigen. Sensitization with BCG is necessary to induce IFN-α/β as well as IFN-γ (data not shown). In most sera obtained from mice challenged with BCG-CW on day 2 after the first challenge with the same antigen, the amount of IFN-α/β identified by acid stability was less than that defined by anti-mouse IFN-α/β serum. We conclude that some of the IFN-α/β in the sera as well as IFN-γ could be inactivated by interacting with some components in the sera during dialysis. Actually, NDV-

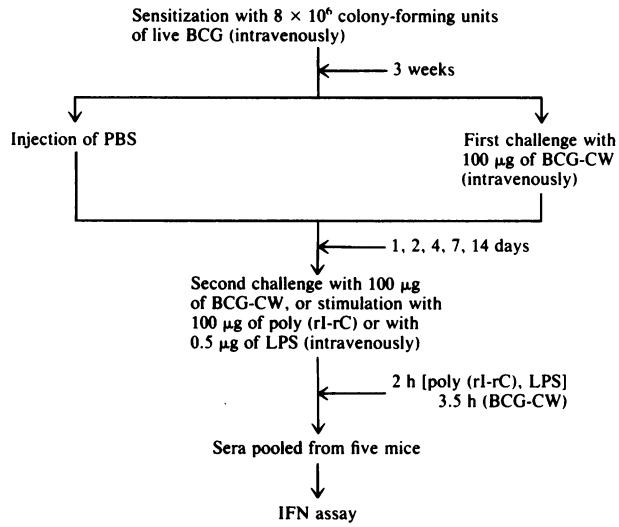

FIG. 1. Experimental schedule.

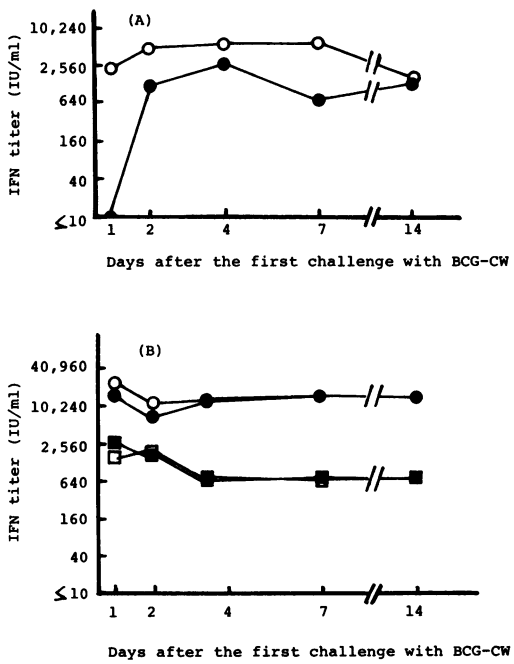

FIG. 2. IFN responses of BCG-sensitized mice by BCG-CW or different IFN-α/β inducers on each day after the challenge with BCG-CW. Three weeks after inoculation of BCG live cells, the sensitized mice were challenged with 100 μg of BCG-CW (challenged group) or PBS (control group). (A) IFN titers of pooled sera from control (○) or challenged (●) group rechallenged with BCG-CW. (B) IFN titers of pooled sera from control (○) or challenged (●) group injected with poly(rI-rC) or from control group (□) or challenged group (■) injected with LPS.

induced mouse serum IFN-α/β was partially inactivated by pH 2.0 treatment, although all activity was recovered after such treatment in NDV-induced L-cell IFN (Table 2). We confirmed these results for human IFN-α in sera from patients with certain diseases (Nakane and Minagawa, unpublished data).

It may be considered that IFN-α/β production by BCG-CW in BCG-sensitized mice correlates with the restoration of the hyporeactive state. Induction of IFN by BCG-CW was completely abolished in BCG-sensitized mice on day 1 after the first challenge with the specific antigen. Although restoration of IFN production was apparently observed on day 2 after the first challenge, most of the IFN induced by BCG-CW was type I IFN-α/β. On day 4 after the first challenge, substantial IFN-γ production was restored. These results suggest that there are two stages of IFN production induced by the second challenge with BCG-CW. IFN-α/β could be produced to compensate for the lack of IFN-γ production in the early stage. In the late stage, restoration of IFN-γ production may follow the diminished production of IFN-α/β.

Sonnenfeld and co-workers (7) suggested that IFN-γ in BCG-sensitized mice may be produced by T cells, especially suppressor or cytotoxic (Ly-2,3$^+$) T lymphocytes in cooperation with B lymphocytes and macrophages. Youngner and Salvin (13) reported that hyporeactivity for the specific antigen, but not for a nonspecific agent (bacterial LPS), developed in BCG-sensitized mice challenged with old tuberculin. We confirmed their results of IFN responses by LPS after challenge of BCG-sensitized mice with BCG-CW. Similarly, poly(rI-rC) could induce the same levels of IFN in these mice as well as in

TABLE 1. Characterization of IFN in sera from BCG-sensitized mice challenged with BCG-CW on each day after stimulation with the same antigen

First challenge with:	Days after first challenge	IFN titer (IU/ml)		
		Induced by second challenge with BCG-CW	After pH 2.0 treatment[a]	After neutralization with anti-IFN-α/β[b]
PBS	1	1,360	<10 (<1)[c]	1,320 (97)[d]
BCG-CW	1	<10	ND[e]	ND
PBS	2	4,800	<10 (<1)	4,720 (98)
BCG-CW	2	300	50 (17)	60 (20)
PBS	4	4,800	<10 (<1)	4,600 (96)
BCG-CW	4	610	<10 (<2)	580 (95)
PBS	7	4,000	<10 (<1)	3,900 (98)
BCG-CW	7	140	<10 (<1)	140 (100)
NDV-induced mouse L-cell IFN		2,000	1,960 (98)	90 (4)

[a] Samples were dialyzed against 0.2 M KCl-HCl buffer (pH 2.0) at 4°C for 48 h, followed by dialysis against minimal essential medium at 4°C for 24 h.

[b] Anti-IFN-α/β serum was obtained from sheep-immunized NDV-induced mouse L-cell IFN. The neutralization test was carried out as described in the text.

[c] Numbers in parentheses are percentage of residual IFN activity after pH 2.0 treatment.

[d] Numbers in parentheses are percentage of residual IFN activity after treatment with anti-IFN-α/β.

[e] ND, Not done.

the control group. It was reported that B lymphocytes or macrophages produced IFN by poly(rI-rC) (4) or LPS (3). Therefore, although T lymphocytes seem to become unresponsive to the homologous antigen, it may be assumed that at least non-T lymphocytes and macrophages continue to share sufficient ability to induce IFN even after BCG-CW challenge in BCG-sensitized mice. Based on these findings, a possible explanation for IFN-α/β production in BCG-sensitized mice may be proposed. In the normal state of BCG-sensitized mice, only IFN-γ is induced by the specific antigen because T lymphocytes are fully capable of producing IFN-γ. Soon after the challenge with the specific antigen, however, decreased response of T lymphocytes may render non-T lymphocytes or macrophages (or both) able to produce IFN-α/β by the response to the specific antigen. More study is necessary to explain the phenomenon reported here.

Our studies demonstrated that IFN-γ was induced by *L. monocytogenes* in human peripheral blood mononuclear leukocyte cultures (6) or mouse spleen cell cultures (Nakane and Minagawa, manuscript in preparation). In human peripheral blood mononuclear leukocyte cultures, whole cells or E rosette-forming T cells

TABLE 2. Characterization of IFN in sera from BCG-sensitized mice challenged with BCG-CW on day 2 after stimulation with the same antigen

Expt no.	First challenge with:	IFN titer (IU/ml)		
		Induced by second challenge with BCG-CW	After pH 2.0 treatment[a]	After neutralization with anti-IFN-α/β[b]
1	PBS	1,600	<10 (<1)[c]	1,520 (95)[d]
	BCG-CW	60	30 (50)	7 (12)
2	PBS	2,400	<10 (<1)	2,380 (99)
	BCG-CW	240	105 (44)	40 (17)
3	PBS	1,100	<10 (<1)	1,020 (91)
	BCG-CW	420	130 (31)	70 (17)
4	PBS	1,840	<10 (<1)	1,780 (97)
	BCG-CW	190	105 (55)	30 (16)
NDV-induced L-cell IFN		2,000	1,960 (98)	90 (4)
NDV-induced mouse serum IFN[e]		2,000	1,350 (68)	80 (4)

[a–d] See Table 1.

[e] Mice were injected with 7×10^6 PFU of NDV, and the sera were collected 6 h later.

cooperating with macrophages can produce IFN-γ by *Listeria*. However, IFN-α production arises by *Listeria* in non-T, non-B lymphocyte cultures when T lymphocytes or macrophages are removed. In mouse spleen cell cultures, IFN-γ can be produced by cooperation between T lymphocytes and macrophages by *Listeria*. On the other hand, IFN-α/β production arises by *Listeria* in macrophage-depleted cultures. These facts may extend a hypothesis that there is an alternative or compensatory producing mechanism between IFN-α/β and IFN-γ in vivo as well as in vitro in response to some bacterial antigens.

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