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Migration Inhibitory Factor and Interferon in the Circulation of Mice with Delayed Hypersensitivity

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When mice infected with *Mycobacterium tuberculosis* strain BCG were inoculated intravenously with old tuberculin (OT) or living BCG cells, both migration inhibitory factor (MIF) and interferon appeared in the circulation within a few hours. In such animals, which showed delayed hypersensitivity by footpad tests, as little as 1.5 mg of OT or as few as 1.7×10^6 bacteria per mouse were capable of eliciting circulating MIF and interferon. Uninfected animals inoculated with large doses of OT or living BCG cells did not produce MIF or interferon. When nonspecific stimuli such as bacterial lipopolysaccharide (LPS; from *Salmonella typhimurium* strain LT-2), heat-killed *Brucella abortus*, Newcastle disease virus (NDV), and polyinosinic acid:polycytidilic acid (poly I:C) were inoculated intravenously into BCG-infected mice, MIF was produced in the circulation of animals challenged with LPS or *Brucella* but not in those challenged with NDV or poly I:C, although all the stimuli were capable of eliciting an interferon response. The interferon elicited in BCG-infected mice by specific antigen differed in at least one important property from the viral inhibitor produced by the nonspecific stimuli. The interferon which appeared after injection of OT or living BCG cells was destroyed by treatment at *p*H 2 for 24 hr at 4C, whereas the interferons produced after injection of the nonspecific stimuli were stable under the same conditions. The MIF activity in plasma from sensitized mice inoculated with specific antigen was also destroyed by treatment at *p*H 2. When mouse plasma containing both MIF and interferon activity was filtered through Sephadex G-100, both mediators were excluded in the same peak fractions. Sensitization of mice with complete Freund adjuvant instead of infection with BCG cells produces a different pattern of response. Although hypersensitive to specific antigen by footpad swelling tests, mice sensitized with complete Freund adjuvant failed to produce MIF or interferon when they were inoculated intravenously with OT or living BCG cells.

Soluble substances with such diverse activities as migration inhibition, aggregation, chemotaxis, or viral inhibition are released when lymphocytes from animals with delayed hypersensitivity are exposed to specific antigen in vitro (3, 4, 16; S. B. Salvin et al., Fed. Proc. **28**:629, 1969). In addition, nonspecific induction of migration inhibitory factor (MIF), skin reactive factor, lymphotoxin, and chemotactic factor have been described after the stimulation of guinea pig lymphocytes with the mitogen, concanavalin A (9).

Since the discovery of interferon (6), its production has been shown not to be a response of host cells solely to infection by viruses. Interferon may be produced by animals or cell cultures exposed to a wide variety of nonviral stimuli, including substances of microbial origin (22), phytohemagglutinin (19), and synthetic polymers (7,8). In addition, immune mechanisms may be associated with interferon production. For example, on exposure to Chikungunya virus, peritoneal leukocytes from mice previously immunized with this virus produced 2 to 10 times more interferon than leukocytes from nonimmune animals (4). Additional evidence for the role of cellular immunity in interferon production was provided when lymphocytes from tuberculin-sensitive humans were shown to produce interferon after challenge with purified protein derivative (PPD) (5). Also, intra-

venous injection of PPD into mice infected with *Mycobacterium tuberculosis* strain BCG produced large amounts of circulating interferon; PPD did not elicit an interferon response in uninfected mice (13).

These findings reinforce the likelihood that a close association may exist between delayed hypersensitivity and resistance of animals to virus infection. It is also likely that, in animals stimulated under appropriate conditions, all or some of the soluble mediators which are released from lymphocytes are present in the blood or body fluids.

In this paper, experiments are reported which show that, under conditions of delayed hypersensitivity, two of these soluble mediators, MIF and interferon, are present in the circulation of experimental animals inoculated with specific antigen.

MATERIALS AND METHODS

Animals. Female Swiss Webster mice weighing 25 to 30 g were obtained from Taconic Farms, Germantown, N.Y.

Infection with M. tuberculosis BCG. Bacteria were grown in a modified Dubos-Middlebrook medium for 10 to 12 days, concentrated by centrifugation, and suspended in saline to the desired concentration by the use of a Klett colorimeter. Mice were inoculated in the tail vein with 0.1 ml of a bacterial suspension containing 5×10^6 to 10^7 viable cells. At various time intervals after infection, mice were inoculated with either old tuberculin (OT; Jensen-Salsbury Laboratories, Kansas City, Mo.) or lipopolysaccharide (LPS) from *Salmonella typhimurium* strain LT-2, and extracted and purified by the method of Westphal and Jann (18). Bleedings were carried out by cardiac puncture with heparinized syringes, and plasma from 8 to 10 mice was pooled for each determination of biological activity. The results obtained in MIF and interferon assays described below were similar when serum or plasma was used.

Footpad tests. Footpad tests to determine delayed hypersensitivity were done as follows. Mice were inoculated in one hind footpad with 0.05 ml of saline containing 30 μg of PPD (Parke-Davis, Detroit, Mich.) and in the other hind footpad with saline alone. At least 14 mice were used for each determination. The thickness of the footpads was measured at 24 hr with a calipers-micrometer (Schnelltäster, Systems-Kröplin, Schlüchtern, Hessen, West Germany). The average thickness of the footpads inoculated with PPD was compared to the average thickness of footpads inoculated with saline; an increase of greater than 10% in thickness was considered an indication of the presence of delayed hypersensitivity.

Migration inhibition of PE cells. Five to ten days before cell harvest, each donor mouse was inoculated intraperitoneally with 3 ml of light petrolatum. The peritoneal cavity was washed out with phosphate-buffered saline or Hanks solution, and the peritoneal exudate (PE) cells were washed three times by centrifugation in Hanks solution for 10 min at 1,000 rev/min. The cells then were concentrated in a 12-ml conical centrifuge tube at 1,200 rev/min for 10 min and were drawn up into a Pasteur pipette, and a tiny drop was planted on the surface of an agar substrate. This substrate consisted of medium 199, 0.5% Ionagar, penicillin (100 units/ml), and streptomycin (100 μg/ml); 20% by volume of the mouse plasma sample to be assayed was added to the liquid substrate at 45 C, and 3-ml volumes were dispensed into 30-ml Falcon plastic tissue culture flasks (11). The flasks were incubated at 37 C for 48 hr, at which time the radius of migration was measured with the aid of an ocular micrometer using low-power magnification. The distance determined was from the edge of the implantation site to the point of maximal cell movement away from the site. In addition, the cells were examined by high-power phase microscopy to study morphological changes. The migration of PE cells from uninfected donor mice was the same in the presence or absence of antigen. The percentage of migration inhibition was expressed as follows: percent migration inhibition = 100 − (migration distance of PE cells with experimental plasma/migration distance of PE cells with control plasma). The percent migration inhibition was based on a mean of data from at least four replicate determinations with each sample. An average inhibition of 20% or more was considered evidence for the presence of MIF.

Interferon assays. Interferon assays were done by the plaque-reduction method using a continuous line of mouse L cells (clone 929) and vesicular stomatitis virus (VSV) as the challenge virus (24). Cultures of L cells in 60-mm petri dishes were exposed for 20 hr at 37 C to 3 ml of twofold serial dilutions of plasma in growth medium (Eagle minimal essential medium plus 4% calf serum) and then were challenged with 40 to 60 plaque-forming units of VSV. Three dishes were used for each dilution. The titers of viral inhibitor were determined by plotting on probit paper the percent inhibition against the different dilutions and were expressed as the reciprocal of the dilution of the sample which reduced the plaque count to 50% of the control plaque count. A standard reference interferon sample was included in each assay; this standard varied within a twofold range.

RESULTS

Delayed hypersensitivity in mice infected with M. tuberculosis BCG. Control and BCG-infected mice were tested for footpad reactions to PPD at different times after infection. The footpads of uninfected control mice had the same thickness whether they had been inoculated with saline (0.05 ml) or PPD (30 μg in 0.05 ml of saline). However, in BCG-infected mice, the footpads inoculated with PPD showed a significant thickening. One week after infection with BCG cells, PPD

produced a thickening of 11% compared to the saline-injected footpads; at 2 weeks after infection a 30% difference existed. The average increase in footpad thickness at 3, 4, and 5 weeks was 30, 25, and 24%, respectively. These data show that delayed hypersensitivity, as indicated by footpad swelling in response to specific antigen, was present at least from 1 to 5 weeks after infection of mice with BCG cells.

Mice infected with BCG cells also had an enhanced responsiveness to the lethal effects of LPS. When LPS was injected intravenously into uninfected mice, the mean lethal dose (LD_{50}) was 270 μg. In infected mice, in contrast, the LD_{50} at 1, 2, 3 and 4 weeks postinfection was <10 μg, 6 μg, 2 μg, and 39 μg, respectively. This enhanced toxicity of LPS is another indication of the hyperresponsiveness of mice infected with BCG cells (14, 24).

Production of MIF and interferon in mice infected with M. tuberculosis BCG. At different intervals after infection with BCG cells, groups of infected or control mice were inoculated intravenously with 50 mg of OT. Blood samples were obtained by cardiac puncture at 4 hr, the time of maximal interferon production by this stimulus (13). The pooled plasmas were tested for interferon and for MIF activity by the techniques which have been described above. The data in Table 1 show that intravenous injection of OT failed to elicit an interferon response in control mice, whereas high titers of interferon (5,000 units) were produced in animals challenged 2 and 3 weeks after infection with BCG cells. The observation that maximal production of interferon occurred 2 to 3 weeks after infection coincides with earlier data (13). By 4 weeks after infection, the interferon response elicited in infected animals by the injection of OT was significantly lower (1,300 units), and at later times the interferon response was markedly reduced or absent altogether.

Table 1 also shows that in BCG-infected mice inoculated intravenously with OT, the MIF activity in the plasma paralleled the interferon titers. One week after BCG infection, OT failed to produce MIF or interferon; at 2 and 3 weeks after infection, maximal MIF activity was elicited in plasma. The MIF activity declined only slightly by week 6, was absent in plasmas of animals given OT 15 weeks after infection, and rose temporarily to significant levels at 19 weeks, when a small increase in interferon activity also was seen.

TABLE 1. *Influence of time after infection on interferon and macrophage migration inhibitory activity of plasmas from BCG-infected and control mice inoculated intravenously with 50 mg of old tuberculin*

Weeks after infection with BCG cells	Activity of plasma		
	Interferon (units/3 ml)		Migration inhibition[a] (%)
	Control mice	BCG-infected mice	
1	<32	<128	14
2	<32	5,000	62
3	<32	5,000	60
4	<32	1,300	38
5	<32	280	44
6	<32	Not done	28
11	<32	120	16
15	<32	<32	0
19	<32	128	52
23	<32	<32	30

[a] BCG-infected mice. See Materials and Methods for details.

Dose response data: MIF and interferon production in BCG-infected mice inoculated with different amounts of OT or living BCG cells. Eighteen days after mice were infected with BCG cells, groups of infected and control animals were inoculated intravenously with different doses of OT or living BCG cells. Pooled plasma samples obtained 4 hr later were tested for MIF and interferon activity. The plasmas of uninfected control mice inoculated with OT or living BCG cells did not exhibit any MIF or interferon activity. However, infected animals given different doses of OT showed a variation in MIF and interferon production (Table 2). The smallest dose of OT used (1.5 mg/mouse) still induced MIF and interferon activity in plasma. When living BCG cells were the stimulus, the intravenous injection of as few as 1.7×10^6 bacteria induced a rise in interferon titer and in MIF activity in the plasma.

In addition to confirming the striking correlation between production of interferon and production of MIF by specific antigen in mice with delayed hypersensitivity, the data demonstrate the minimal effective doses of OT and living BCG cells required to obtain a response. The positive responses evoked only in infected animals by as few as 1.7×10^6 living organisms tend to eliminate the possibility that nonspecific factors are involved in these phenomena.

Comparison of MIF and interferon production by specific and nonspecific stimuli in BCG-infected mice. The data which have

TABLE 2. *Production of MIF and interferon in the plasmas of mice infected with BCG and challenged 18 days later with varying doses of old tuberculin (OT) or living BCG cells*

Stimulus	Dose[a] per mouse	Activity of plasma tested for:	
		Interferon[b] (units)	Migration inhibition (%)
OT	50 mg	7,000	79
	25 mg	6,400	72
	12.5 mg	2,200	61
	6 mg	2,500	64
	3 mg	350	49
	1.5 mg	350	47
BCG (viable bacteria)	1.7×10^7	1,200	39
	1.7×10^6	50	25
	1.7×10^5	<32	0
	1.7×10^4	<32	0
Saline		<32	0

[a] Intravenous injection.

[b] Plasma from uninfected mice challenged with OT or living BCG cells had interferon titers of <32.

been presented show a correlation of MIF and interferon production by specific antigen in BCG-infected mice. Experiments were conducted to determine whether both MIF and interferon activity are produced in the plasma of BCG-infected mice after inoculation with nonspecific materials which are known to be potent stimuli of interferon production in animals.

In the case of LPS, the increased lethality produced by this material in BCG-infected mice (14, 25) is accompanied by an increased capacity of small doses of LPS to stimulate interferon production (25). At different intervals after infection with BCG cells, groups of infected or control mice were inoculated intravenously with 1 μg of LPS. Blood samples were obtained at 2 hr, the time of maximal interferon production by this stimulus, and tested for MIF and interferon activity. An enhancement of interferon titers in BCG-infected mice occurred from 1 to 5 weeks after infection (Table 3). Thereafter, the interferon titers in BCG-infected and control mice were essentially the same. In the case of MIF, a striking correlation existed between the appearance of MIF and the presence of enhanced interferon titers in BCG-infected mice; both MIF and enhanced interferon production occurred only during the first 5 weeks after infection. Despite the fact that interferon activity was present in control mice given LPS, at no time was MIF activity detected; migration zones in agar were not different from those seen with plasmas from uninoculated control animals.

Additional nonspecific materials known to produce interferon in mice were tested for their ability to stimulate the production of MIF in BCG-infected animals. Two weeks after infection with BCG, infected and control mice were inoculated with OT, LPS, heat-killed (85 C for 60 min) *Brucella abortus* (strain 456), Newcastle disease virus (NDV, Herts strain), or a synthetic double-stranded polyribonucleotide (polyinosinic acid:polycytidilic acid [poly I:C]). The methods used to prepare these materials for inoculation have been presented in detail elsewhere (23–25). Groups of 10 mice were bled at the time of maximal interferon production by each stimulus (Table 4).

In agreement with the data previously presented in Tables 1 and 3, OT and LPS evoked both MIF and interferon in the plasmas of BCG-infected mice; in the case of LPS, interferon production in uninfected mice was not accompanied by the appearance of MIF. Plasmas from BCG-infected mice challenged with heat-killed *B. abortus* showed MIF activity but did not show any enhanced interferon activity. When NDV or poly I:C was used as a stimulus, the plasmas of both BCG-infected and uninfected control mice showed very high titers of interferon; however, these stimuli failed to elicit any MIF activity in the plasma.

The production of MIF in BCG-infected

TABLE 3. *Influence of time after infection on interferon production and macrophage-migration inhibitory activity of plasmas from BCG-infected and control mice inoculated intravenously with 1 μg of lipopolysaccharide*

Weeks after infection with BCG cells	Activity of plasma		
	Interferon titer (units/3 ml)		Migration inhibition[a] (%)
	Control mice	BCG-infected mice	
1	100	250	25
2	500	1,200	14
3	600	1,000	37
4	450	1,000	26
5	650	1,800	24
6	Not done	Not done	0
11	500	560	0
15	680	320	0
19	1,000	600	0
23	600	600	0

[a] BCG-infected mice.

TABLE 4. *Interferon production and macrophage migration inhibitory activity in the plasmas of BCG-infected and control mice inoculated intravenously with different stimuli*[a]

Stimulus	Dose[b]	Time of bleeding[c] (hr)	Activity of plasma tested for:			
			Interferon (units)		Migration inhibition[d] (%)	
			Control mice	BCG-infected mice	Control mice	BCG-infected mice
Saline	0.1 ml	2	<64	<64	0	0
Old tuberculin	50 mg	4	<64	7,000	0	64
LPS	1 μg	2	350	2,300	0	41
B. abortus (heat-killed)	2×10^9	5	4,000	2,800	0	29
Newcastle disease virus	2×10^8 PFU[e]	8	33,000	17,000	0	0
Poly I:C	50 μg	3	7,400	18,000	0	0

[a] Migration zones in the presence of plasma from control and BCG-infected mice without stimuli were similar.

[b] Intravenous injection.

[c] Time of peak titer of circulating interferon.

[d] Migration inhibition is based on migration of cells in the presence of plasma from BCG-infected mice or plasma from infected mice not given any of the above-listed stimuli.

[e] PFU, plaque-forming units.

mice challenged with heat-killed *B. abortus* is of significance in the light of the finding that this organism contains two separate stimuli of interferon, one of them being *Brucella* LPS (21). The MIF response to *Brucella* may be due to the LPS component of the bacterial cell wall (J. S. Youngner and G. Keleti, *unpublished data*). The data in Tables 3 and 4 illustrate that MIF activity accompanies the production of interferon only in BCG-infected mice and that the only stimuli capable of eliciting this double response are specific antigens (OT or living BCG cells), purified bacterial LPS, or intact bacteria (*B. abortus*) which contain LPS.

A comparison was made of several characteristics of the interferon which appeared in the plasmas of BCG-infected mice inoculated with the specific and nonspecific stimuli listed in Table 4. The viral inhibitors produced in response to the various stimuli had the following common characteristics: (i) they were species specific, i.e., they did not inhibit plaque formation by VSV in primary chicken embryo cell cultures; (ii) they were inactivated by incubation at 37 C for 3 hr with 50 μg of crystalline trypsin per ml; (iii) they required host (L cell) ribonucleic acid (RNA) synthesis for production of resistance to VSV, i.e., incubation of L cells with the different inhibitors in the presence of 2 μg of actinomycin D per ml blocked the development of resistance to subsequent challenge with VSV, an RNA virus which can replicate in the presence of the antibiotic; (iv) they did not neutralize directly the infectivity of VSV; and (v) they were not dialyzable. These properties are consistent with the characteristics required to classify a virus inhibitor as interferon (15).

One important characteristic differentiated the interferon produced in response to OT or living BCG cells from the viral inhibitors produced by the nonspecific stimuli LPS, killed *B. abortus* organisms, Newcastle disease virus, and poly I:C (Tables 2 and 4): when the different inhibitors were diluted 1:32 in saline and dialyzed against glycine-hydrochloride buffer, *p*H 2, for 24 hr at 4 C, the activity of the interferon produced in response to OT was completely destroyed, whereas the interferons elicited by the nonspecific stimuli were not affected by the acid treatment. After this observation, several additional plasma pools of interferons produced in BCG-infected mice inoculated with the different stimuli were tested for their stability at *p*H 2. In every case, only the inhibitor present in the plasmas of mice given OT or living BCG cells was destroyed by exposure to buffer at *p*H 2.

The stability of MIF at *p*H 2 was also determined. Plasma samples diluted 1:16 with saline were dialyzed against buffer at *p*H 7 or *p*H 2 at 4 C for 24 hr. Both samples were then dialyzed against buffer at *p*H 7 and assayed for MIF. The plasma sample dialyzed against *p*H 7 buffer produced migration inhibition at a

dilution of 1:64. In contrast, the plasma sample dialyzed against *p*H 2 buffer did not show any residual MIF. Thus, the MIF in mouse plasma is nondialyzable and unstable at *p*H 2.

Fractionation of mouse plasma containing MIF and interferon activity. A plasma pool containing both MIF and interferon activity was prepared in the following manner. Mice, infected 14 days previously with BCG, were inoculated intravenously with 50 mg of OT and bled 4 hr later. The plasma pool, when tested by the usual procedures, had an interferon titer of 2,000 units and significant MIF activity (49% inhibition). An attempt was made to separate the two activities by means of filtration through Sephadex G-100.

A column of Sephadex G-100 (91 by 2.5 cm) was prepared with a buffer of 0.01 M sodium phosphate, 0.15 M sodium chloride, *p*H 7.4, with a flow rate of 14 ml/hr. A sample of the mouse plasma pool (2 ml) was carefully added to the column. After the void volume had passed through the column, consecutive samples in tubes containing 100 drops each were collected, and absorbancy at 280 nm of the contents of each tube was determined. On the basis of absorbancy, the samples were pooled in seven groups (as indicated in Fig. 1), lyophilized, and stored at 4 C. Prior to assay for MIF and interferon activity, the lyophilized samples were reconstituted in 3 ml of deionized water, centrifuged at 3,000 rev/min for 30 min to remove insoluble materials, and then dialyzed against 0.15 M sodium chloride at 4 C.

Maximal MIF (51% inhibition) and interferon activity (900 units) were present in fraction pool IV. When compared to the exclusion volumes of proteins of known molecular weight passed through the same column, materials in this fraction had a molecular weight of 45,000 to 80,000. Lower levels of MIF and interferon activity were noted in fraction pool II (24% inhibition and 192 units) and fraction pool III (16% inhibition and 192 units). Biological activity in the other fractions was absent or not significant. Fractionation of similar mouse plasma pools by filtration through Sephadex G-100 was carried out on two other occasions with similar results. When a plasma pool from uninfected mice inoculated with 50 mg of OT was filtered through Sephadex G-100, fraction pools did not exhibit MIF or interferon activity although an apparently identical absorbancy pattern at 280 nm was obtained.

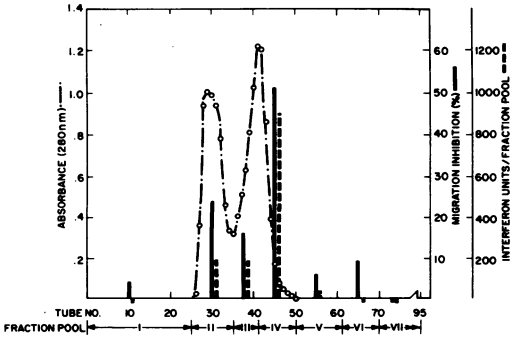

FIG. 1. *Fractionation by filtration through Sephadex G-100 of MIF and interferon activity in a plasma pool from BCG-infected mice inoculated intravenously with OT.*

Delayed hypersensitivity induced with complete Freund adjuvant: effect on MIF and interferon production. Mice were inoculated both in the front footpads and subcutaneously in the nape with 2 mg of killed, acetone-dried *M. tuberculosis* (Jamaica strain) in Freund adjuvant (0.25 or 0.5 ml). The injection of 30 μg of PPD into the hind footpads 14 to 49 days after sensitization produced a 24 to 37% increase in thickness, compared to the opposite hind footpad tested with 0.05 ml of saline.

When OT was used as the stimulus, neither interferon nor MIF activity was detected in plasma from mice sensitized with complete Freund adjuvant or in plasma from unsensitized animals. In the case of LPS, the interferon titers measured in the plasmas of sensitized and control mice were not significantly different; MIF was not measurable in any of the plasmas tested. These results are in marked contrast to the results with plasmas from mice sensitized by infection with living BCG cells (Tables 2–3). Animals infected with BCG not only showed delayed hypersensitivity by footpad tests, but after intravenous inoculation with OT or LPS also produced both MIF and enhanced amounts of interferon in the plasma.

DISCUSSION

The injection of specific antigen into BCG-infected mice with delayed hypersensitivity results in the release of MIF into the circulation. Until recently (20), MIF was produced only in vitro in cultures of lymphocytes from sensitized donors belonging to one of several species, e.g., human, guinea pig, or mouse.

Some important questions are raised by the finding that MIF, as well as interferon, is re-

leased into the circulation of BCG-infected mice after intravenous injection of OT or living BCG cells. Firstly, do these biological activities reside in the same or different molecules? Although the data presented do not answer this question completely, they show that the properties of the two mediators are very similar. The maximal production of the two mediators occurred 2 to 3 weeks after infection of mice with BCG; maximal induction of the two mediators occurred 3 hr after an intravenous injection of 50 mg of OT (S. B. Salvin and J. S. Youngner, *unpublished data*). Filtration of mouse plasma containing both MIF and interferon through columns of Sephadex G-100 showed that both activities were in the same exclusion volumes, which corresponded to a molecular weight of 45,000 to 80,000. Preliminary data have been obtained which indicate that MIF and interferon activity cannot be separated by passage of mouse plasma containing both mediators through columns of diethylaminoethyl-cellulose (W. H. Lederer, *unpublished results*), i.e., the two mediators cannot be distinguished solely on the basis of their charge.

In addition, the MIF and interferon activities of mouse plasma are unstable at *p*H 2. This observation is of special significance in the case of interferon. In contrast to the interferon produced by inoculation of specific antigen into mice with delayed hypersensitivity, the interferon activity in plasma from BCG-infected mice inoculated with LPS, virus (NDV), heat-killed *B. abortus*, or poly I:C was stable at *p*H 2. Thus, the interferon produced by inoculation of specific antigen into mice with delayed hypersensitivity is different in an important physicochemical property from the viral inhibitors produced in hypersensitive mice in response to nonspecific stimuli. Stinebring and Absher (13) described several properties of the interferon produced by PPD in BCG-infected mice but did not include stability of the inhibitor at *p*H 2. The stability at *p*H 2 of the interferon produced by PPD in macrophage-lymphocyte cell cultures from human donors with delayed hypersensitivity has been tested (2). This interferon was unstable at *p*H 2 for 18 hr at 4 C, lending additional support to the finding that in animals with delayed hypersensitivity the interferons produced by specific antigens are different from the interferons produced in response to nonspecific stimuli.

A second question raised by the data presented in this paper concerns the relationship of delayed hypersensitivity, as indicated by skin-test reactivity, to the production of MIF and interferon in the circulation of animals given specific antigen. When the reactions of mice inoculated with complete Freund adjuvant were compared to the reactions of mice infected with living BCG cells, different patterns of reactivity were observed. Although both groups of animals developed delayed hypersensitivity, as indicated by footpad reactions to PPD, only the animals infected with BCG produced MIF and interferon when OT was inoculated intravenously. A qualitative difference may therefore exist in the types of delayed hypersensitivity induced by complete Freund adjuvant or by infection with BCG. The nature of the cellular responses elicited by these stimuli may be so different that only in the case of infection with BCG does an MIF and interferon response follow the intravenous inoculation of specific antigen. On the other hand, the possibility cannot be eliminated that the differences seen are quantitative, i.e., a difference exists only in the number, not the type, of the specific cells which are found in animals with delayed hypersensitivity induced by complete Freund adjuvant or living BCG. In this case, the MIF and interferon responses after injection of OT into animals sensitized with complete Freund adjuvant may be too low to be measured by the assay methods employed. The precise significance of skin reactivity, in this case footpad swelling, as an index of delayed hypersensitivity, cellular resistance, or MIF production may thus be open to question.

If the production of soluble mediators and their release into the circulation under the conditions of the foregoing experiments are associated with enhanced resistance, then the mere presence of a skin reaction to specific antigen is not in itself indicative of this enhanced resistance. Typically, vaccination with living BCG cells results in the development of both delayed (tuberculin) hypersensitivity and enhanced cellular resistance to infection with virulent tubercle bacilli (1). Nevertheless, the exact relationship between the two is highly controversial (10).

LPS has been shown to elicit MIF and enhanced amounts of interferon in the circulation of BCG-infected mice. The mechanism of production of these mediators is not known, although delayed hypersensitivity may be involved. Hyperreactivity of BCG-infected mice to the toxic effects of LPS may result from the development of delayed hypersensitivity to BCG antigens immunologically related to

the LPS of gram-negative bacteria (12, 17).

Additional questions are raised by the experiments reported in this paper. (i) Are the MIF and interferon produced in the circulation of hypersensitive mice identical to or different from the mediators produced in vitro when cultures of sensitized lymphocytes are exposed to specific antigen? (ii) Are MIF and interferon produced in the circulation of hypersensitive animals of species other than the mouse, e.g., guinea pig or man? (iii) What role, if any, do MIF and interferon play in delayed hypersensitivity and in resistance to infection by agents such as bacteria, fungi, and viruses, or in rejection of allogeneic or tumor grafts? With the procedures described for producing MIF and interferon directly in the circulation of BCG-infected animals by antigenic stimulation, experiments are being carried out to answer some of these questions.

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