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Tuberculin Shock in Mice: Relationship Between Systemic Reactions to Tuberculin and Endotoxin and Lack of Correlation Between Tuberculin Shock and Cellular Immunity to Tuberculin (38024)

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Generalized tuberculin reactions, first described some 80 yr ago (1) have been reported to occur in laboratory animals (2–8) as well as in man (9–11). The controversy as to the immunological nature of tuberculin shock and the paucity of information about its pathophysiological mechanisms prompted us to attempt a more precise definition of the conditions under which systemic sensitivity to tuberculin occurs in mice. In an earlier study (12), the symptomatology and associated cardiovascular parameters during the systemic tuberculin reaction were compared with those of anaphylactic shock, anaphylaxis-like reactions following histamine-serotonin injection, and endotoxin shock in normal or BCG-pretreated mice. In contrast to anaphylactic shock and endotoxin shock in normal mice, the systemic tuberculin reaction and endotoxin shock in mice pretreated with BCG were characterized by a delayed onset, a general convulsive condition leading to death within a few minutes, and normal values of blood pressure and blood volume. This finding suggested that nonimmunological mechanisms might be involved in the pathogenesis of tuberculin shock. Further evidence in support of this hypothesis is provided by the results of the present study, in which the conditions necessary for induction of systemic tuberculin reactivity and of cellular immunity to purified protein derivative (PPD), such as delayed skin reactions, lymphocyte transformation and the inhibition of leukocyte migration, were systematically compared.

Material and Methods. The mice and the mycobacterial strain used in these experiments were the same as those described in a previous communication (12).

Induction and elicitation of systemic reactivity to tuberculin. Mice were inoculated either once or twice with washed, living *Mycobacterium bovis* of the BCG strain, suspended in 0.5 ml saline containing 10% tween-albumin medium. A standard procedure consisted in a single ip injection of 0.1 mg BCG followed by 0.3 mg BCG given iv 14 days later (1 mg wet weight contained $0.96–1.20 \times 10^8$ viable units). For s.c. injections, the bacterial suspension was incorporated into equal amounts of adjuvant (0.85 part Marcol 52 and 0.15 part Arlacel C), and 0.1 ml of the emulsion was distributed among the left hind and the two front foot pads (FP). Systemic tuberculin reactions were elicited by iv injection of 2 mg purified protein derivative (PPD, State Serum Institute, Copenhagen) dissolved in 0.5 ml phosphate-buffered saline (0.14 *M* NaCl, 0.01 *M* $NaH_2PO_4 \cdot H_2O$ adjusted to pH 7.2 with 10 *M* NaOH) (PBS). This amount of PPD had no effect when injected into normal mice. All the animals challenged with PPD were observed for several hours and mortality was recorded 24 hr after challenge. As described previously (12), PPD shock can be distinguished from anaphylaxis by its symptoma-

tology and the time of occurrence. PPD, as well as bacterial endotoxin were always injected between 9 and 11 a.m.

Systemic reactivity to endotoxin. Lipopolysaccharide from *Escherichia coli* 0111: B4 (Difco) dissolved in PBS was administered iv. Twenty-four hr thereafter mortality was recorded. Median lethal doses were estimated from semi-log plots of doses against mortality. One to 3 experiments were carried out and 10–15 mice were used per dose. Similarly, the lethal effects of endotoxin and PPD were quantitated in actinomycin D-treated mice (13), as well as in embryonated eggs. Except for minor modifications, the technique described by Finkelstein (14) was used. Test inocula were made into an allantoic vein of chick embryos after 10–11 days of incubation. Mortality was determined by candling 1–3 days after inoculation. Finally, the pyrogenicity of endotoxin or PPD was evaluated by standard pharmaceutical procedures using acclimatized rabbits. Body temperature was measured every 30 min for 6 hr by means of rectally inserted thermocouples connected to a multi-channel direct readout telethermometer (Honeywell). The average body temperature of 3–5 rabbits per group was plotted against time. The area beneath the fever curve was taken as the fever index (15), a numerical expression of both the height and duration of fever. Median effective doses of endotoxin or PPD were estimated from semi-log plots of doses and the percentage of the maximum fever index obtained with an optimal dose of endotoxin.

Delayed skin reactivity to tuberculin. PPD (0.03 mg in 0.05 ml PBS) was injected into the right hind foot pad. The width and thickness of each foot was measured with microcalipers (Schnelltaster Kröplin) prior to challenge and 4, 24, and 48 hr after the injection. The results are indicated in terms of the change in cross-sectional area, which was assumed to be the product of width times thickness (8). Statistical evaluation was performed by Student's *t*-test.

In vitro stimulation of lymphocytes by PPD. Cell suspensions from pooled mesenterial, axillary and inguinal lymph nodes were prepared and cultured as described elsewhere (16). To triplicate cultures containing 10^6 cells (1 ml) 5 μl of PPD (1, 10 or 100 μg) was added at the beginning of the 72 hr culture period. DNA synthesis was estimated by measuring ^{3}H-thymidine incorporation (17). The results are expressed as stimulation ratios (^{3}H-thymidine incorporation of PPD-stimulated cultures/incorporation of nonstimulated control cultures). The culture system was regularly checked for cell viability and responsiveness to phytohemagglutinin (10 μg, Difco) and concanavalin A (4 μg, Calbiochem).

Inhibition of the migration of peritoneal exudate cells by PPD. Peritoneal cells from mice which had been injected ip three days before with 2 ml 10% sterile gelatin were collected in ice-cold Hanks' balanced salt solution (BSS) containing 0.5 units of heparin per ml. The cells were washed twice with BSS (250 × *g*, 10 min) and resuspended in Eagle's minimum essential medium supplemented by 1% L-glutamin, penicillin-streptomycin (50 U/ml and 50 μg, respectively) and 15 per cent fetal bovine serum (Rehatuin, Reheis Chemical Company, Chicago). Migration inhibition was assessed using the capillary tube technique of Bloom and Bennet (18). Per cent inhibition was calculated as follows:

$$\frac{\text{average migration area without antigen} - \text{average migration area with antigen}}{\text{average migration area without antigen}} \times 100$$

inhibition greater than 25% was considered to be significant.

Results. 1. *Conditions necessary to induce systemic reactivity to tuberculin.* As is shown in Fig. 1 and seen in other experiments not reported here, a single iv injection of live BCG only induced a lethal tuberculin shock in a small, varying percentage of the mice challenged. Doses of less than 1 mg were always without effect. Likewise, single ip injections of 0.1–3 mg BCG did not induce systemic reactivity to PPD, whereas 30 mg BCG proved sufficient to provoke fatal PPD shock in ap-

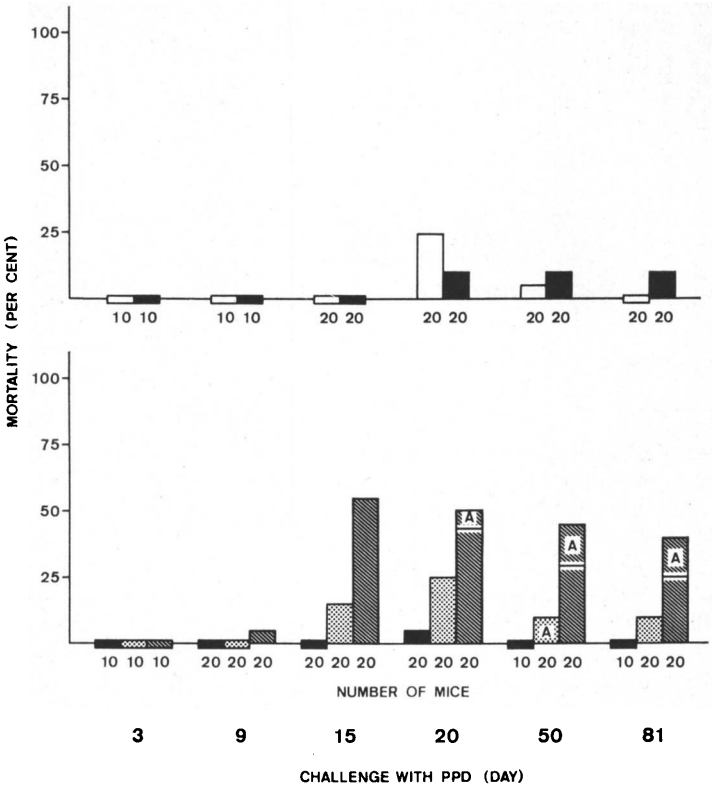

FIG. 1. Systemic tuberculin reactions at different times after a single iv injection (top) or a single ip injection (bottom) of living mycobacteria: mg BCG injected on Day 0: 1, 3, 10, 30. Challenge with 2 mg PPD (iv) on the day indicated. The proportion of anaphylactic deaths are denoted by A.

proximately half the animals challenged on day 15. Later in the course of infection, systemic reactivity to tuberculin decreased. Some of the mice revealed fatal anaphylaxis, which was shown to be due to antibodies to BSA (unpublished).

Subcutaneous injection of 0.1 and 1 mg BCG in Freund's adjuvant did not induce systemic reactivity to tuberculin (Fig. 2). However, in a large percentage of the mice anaphylactic shock occurred in response to PPD after inoculation with 10 or 50 mg of BCG in adjuvant. In contrast, two injections of BCG (0.1 mg ip on day 0 and 0.3 mg or 1 mg iv 2–20 days later) induced systemic reactivity to PPD in a great number of animals. As can be seen from Fig. 3, the maximum incidence of PPD shock occurred when the injections of mycobacteria were spaced 2–3 weeks apart. In an experiment identical to the one outlined in Fig. 3, except that the first injection of BCG was given iv and the second ip, similar results were obtained. Systemic reactivity to tuberculin induced by two injections of BCG lasted for a limited period of time. Thus, approximately 40 days after the second injection of mycobacteria delayed PPD shock was only elicited in a comparatively small number of animals.

In 18 experiments carried out within several months, more than 600 mice were injected ip with 0.1 mg BCG and iv with 0.3 mg BCG 14 days later. Under these conditions the average incidence of lethal tuberculin shock in mice challenged with PPD 8–9 days after the second BCG treatment (Day 22–23) was 83 ± 16%. On the other hand, only 1–5% of the animals died when challenged 4 weeks after the second BCG injection (Day 42–45). Results of experiments using different batches

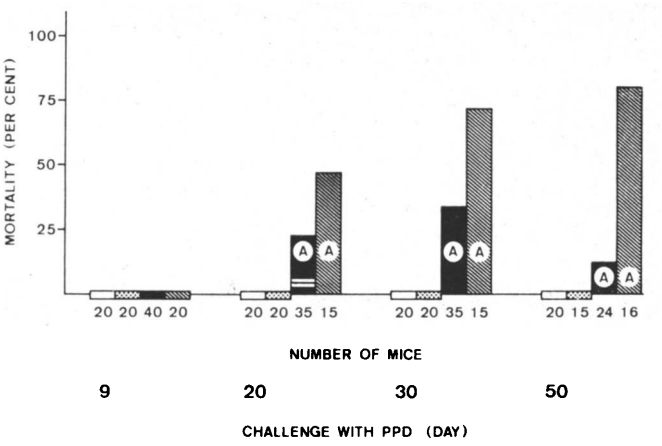

FIG. 2. Systemic tuberculin reactions at different time after a single s.c. injection of living mycobacteria in Freund's adjuvant; mg BCG injected on Day 0: ▭ 0, 1, ⁝⁝⁝ 1, ▬ 10, ▨ 50. Challenge with 2 mg PPD (iv) on the day indicated. Anaphylactic deaths are denoted by A.

of PPD did not reveal any significant differences. Possible trace-contamination with reagents used in the extraction procedure is unlikely to be responsible for these results, since we never observed untoward side effects of large doses of PPD administered to normal control mice.

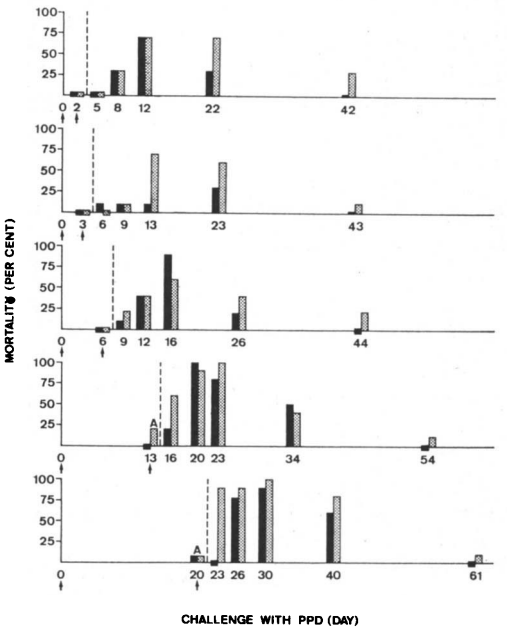

FIG. 3. Systemic tuberculin reactions at different times after two variously spaced injections (↑) of living mycobacteria: mg BCG injected ip on Day 0: 0.1 mg BCG injected iv on days 2, 3, 6, 13 or 20: ▬ 0.3, ⁝⁝⁝ 1 anaphylactic death occurring at the second injection of BCG are denoted by A. Challenge with 2 mg PPD (iv) on the day indicated. 15–20 mice per group.

2. *Sensitivity to bacterial endotoxin after pretreatment with BCG.* One injection of BCG in Freund's adjuvant or a single ip administration of BCG did not significantly alter sensitivity to endotoxin, whereas two injections of BCG at an interval of 14 days greatly augmented endotoxin sensitivity (Fig. 4). Within a few days of the second injection of mycobacteria, the LD_{50} values were reduced by a factor greater than 10^3. This situation lasted for approximately one week, and sensitivity steadily then decreased to reach near-normal levels by Day 42. At the height of endotoxin sensitivity, the endotoxin shock also changed in quality and became indistinguishable from PPD shock by the criteria described earlier (12).

3. *Demonstration of endotoxin or endotoxin-like activity in PPD.* As is shown in Table I, a bacterial lipopolysaccharide and PPD were quantitatively evaluated in four different test systems. In BCG-infected mice, actinomycin D-pretreated mice and chick embryos, the LD_{50} of PPD differed from that of endotoxin by a factor ranging from 1.6×10^3 to 10^4, whereas in the rabbit pyrogenicity test there was a 1.5×10^5 fold difference between the ED_{50} values. These findings show that PPD contains contaminants exhibiting endotoxin activity.

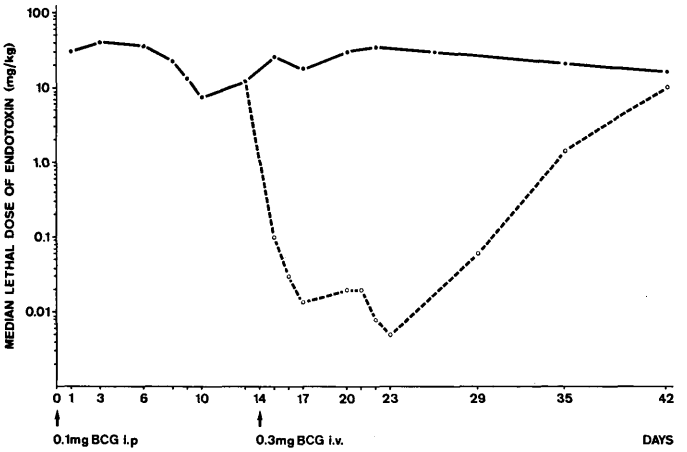

FIG. 4. Susceptibility to endotoxin of BCG-infected mice: Single injection of 0.1 mg BCG (ip) on day 0: ●—●, two injections of BCG, 0.1 mg (ip) on Day 0 and 0.3 mg (iv) on Day 14: ○- - - -○.

4. *Delayed skin reactions to PPD after various pretreatments with BCG.* Groups of mice pretreated with BCG were skin-tested with PPD at different intervals after the injection of mycobacteria. The animals were challenged only once and then excluded from the experiment. The skin reactions observed 24 hr after PPD were always greater than those seen after 48 hr, whereas reactivity after 4 hr was similar to that seen in noninfected control animals. As is indicated in Fig. 5C, the greatest swellings of the foot-pads were observed 3 weeks after the injection of 1 mg BCG in Freund's adjuvant, a pretreatment which was shown to be ineffective in inducing systemic reactivity to PPD (Fig. 2). In contrast delayed skin reactions were clearly less marked in the groups treated with either a single ip dose of BCG (Fig. 5A) or by the standard procedure adopted to induce systemic PPD reactivity (Fig. 5B).

5. *In vitro PPD-stimulation of lymphocytes from BCG-pretreated mice.* As is shown in Fig. 6, PPD-induced ^{3}H-thymidine incorporation of lymphocytes was not significantly increased in the groups pretreated either once or twice with mycobacteria without adjuvant (Figs. 6A and 6B, respectively). There was no difference from the group which had not been infected with BCG (Fig. 6D). On the other

TABLE I. Systemic Effect of Endotoxin and of PPD.

Test systems		LD_{50} or ED_{50} (mg/kg)		
		LPS 0111:B4 (a)	PPD (b)	b/a
Lethality:	normal mice	40		
Lethality:	BCG-infected mice[a]	0.005[b]	8[b]	1.6×10^3
Lethality:	Actinomycin D-treated mice[c]	0.002	20	10^4
	Chick embryo	0.0001[d]	0.4[d]	4×10^3
Pyrogenicity:	rabbits	0.0001	15	1.5×10^5

[a] Two injections of BCG: .1 mg (ip) on Day 0, 0.3 mg (iv) on Day 14.

[b] Determined on Day 23.

[c] Cosmegen (Merck Sharp & Dohme) 0.5 mg/kg (iv, simultaneously injected with endotoxin or PPD).

[d] mg.

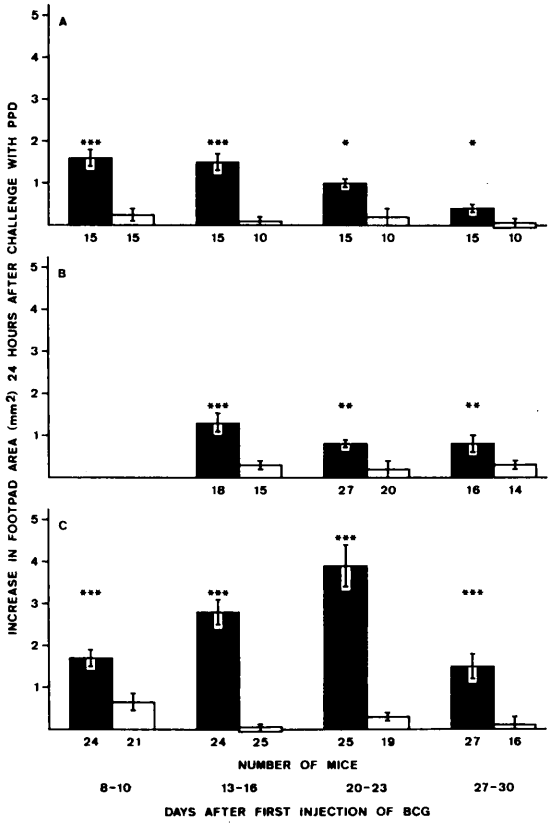

FIG. 5. Delayed (24 hr) skin reactions to PPD in BCG-infected mice A: single injection of 0.1 mg BCG (ip) on day 0 B: two injections of BCG, 0.1 mg (ip) on day 0 and 0.3 mg (iv) on day 14. C: single s.c. injection of 1 mg BCG in Freund's adjuvant on day 0. Vertical bars denote standard errors of the mean and stars represent the level of statistically significant differences ($P < 95\%$:*, 99%:**, 99.9%:***) between experimental groups ■ and controls □.

hand, stimulation ratios greater than 10 were obtained with cells from mice which had been injected with 1 mg BCG in Freund's adjuvant three weeks before (Fig. 6C). Later in the course of infection, however, the stimulation ratios decreased to control levels. No difference was evident between the three doses of PPD tested.

6. *PPD-induced inhibition of the migration of peritoneal exudate cells from BCG-pretreated mice.* As is shown in Table II, the migration of peritoneal cells from mice pretreated with one single ip injection of BCG one to two weeks prior to the experiment was only marginally inhibited by PPD. In the group given the standard pretreatment to induce PPD shock no significant inhibition of migration could be observed at all. However, migration was greatly suppressed with cells from mice which had been pretreated with 1 mg BCG in Freund's adjuvant. Nevertheless, it should be noted that pretreatment of mice with BCG appeared to increase migration of peritoneal cells in the absence of added antigen. (Mean area of migration in untreated mice 4.8 ± 0.33 in a total of 50 experiments.)

Discussion. Tuberculin shock in mice has been observed by several workers (3, 5, 6, 8, 12) but only few attempts have been made to study systematically the conditions necessary for its induction. The virulence, quantity and route of injection of myco-

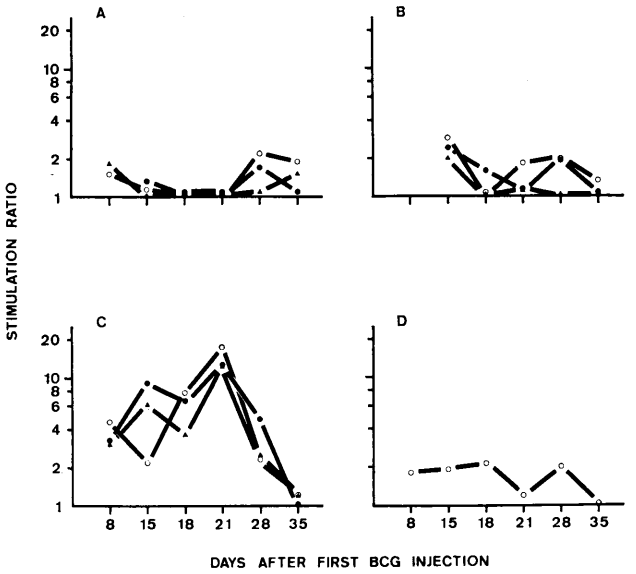

FIG. 6. *In vitro* stimulation by PPD of lymphocytes from BCG-infected mice. A–C: same as in Fig. 5. D: not infected with BCG. Stimulation with PPD (μg): ▲—▲ 1, ●—● 10, ○—○ 100.

bacteria were found to be important parameters. Single injections of small amounts of virulent bacteria (3, 5, 6) proved to be sufficient, a single ip administration of rather large amounts of BCG (3, 5, 8) gave irregular results, whereas treatment with killed mycobacteria was either ineffective (6, 8, Dietrich unpublished) or inferior to live bacteria (3). In the present study, single iv doses of approximately 10^8 to 3×10^8 infectious units of BCG were effective in only a few mice. Ten times as many mycobacteria given ip sensitized only approximately half of the animals, whereas s.c. injections of BCG incorporated into Freund's adjuvant were ineffective. Pretreatment with 10^9 mycobacteria in adjuvant induced anaphylactic reactivity rather than PPD shock. On the other hand, two injections of living mycobacteria prepared the majority of mice for lethal PPD shock.

Systemic sensitivity to tuberculin has a finite duration. In our model it reached maximum levels about 10 days after the second injection of bacteria and vanished almost completely 40 days thereafter. The duration of systemic PPD sensitivity parallels the period of greatly increased susceptibility to bacterial lipopolysaccharide. During this time the endotoxin shock becomes not only quantitatively and qualitatively different from systemic endotoxin effects in normal mice, but also indistinguishable from tuberculin shock (12). On the other hand, single pretreatments with 0.1 to 1 mg of BCG (ip, iv or adjuvant), which failed to induce systemic tuberculin reactivity, were also ineffective with respect to endotoxin sensitization. Such mice, however, showed delayed cutaneous PPD sensitivity and, in the case of adjuvant-pretreated animals, other manifestations of cell-mediated immunity to tuberculin or to its constituents. In contrast, cells from mice prepared for systemic PPD reactivity by two injections of BCG could not be stimulated to more than a marginal extent and showed no inhibited migration. These findings clearly reveal a lack of correlation between the conditions necessary to induce systemic reactivity to PPD and cell-mediated immunity to PPD. This conclusion and the fact that systemic PPD reactivity parallels the increased susceptibility to bacterial lipopolysaccharide suggest that PPD shock is probably a nonimmunological phenomenon.

TABLE II. PPD-Induced Migration Inhibition of Peritoneal Exudate Cells from BCG-Pretreated Mice.

Injection of BCG	PPD (μg/ml) in incubation medium	Area of migration[a] Days after first injection of BCG				Migration inhibition (%) days after first injection of BCG			
		7–8	14–16	21–23	27–27	7–8	14–16	21–23	27–28
0.1 mg ip	none	4.2 ± 0.57 (10)	7.2 ± 0.52 (12)	4.6 ± 0.21 (12)	4.6 ± 0.25 (10)				
	10	2.6 ± 0.32 (12)	5.2 ± 0.17 (12)	4.8 ± 0.26 (15)	5.0 ± 0.38 (10)	38	28	–4	–9
0.1 mg ip 0.3 mg iv	none	n.d.	7.0 ± 0.40 (21)	4.8 ± 0.20 (25)	5.2 ± 0.08 (20)				
	10	n.d.	6.6 ± 0.51 (20)	4.4 ± 0.20 (25)	5.6 ± 0.32 (25)	n.d.	9	17	–8
	50	n.d.	6.2 ± 0.45 (20)	4.2 ± 0.16 (23)	4.8 ± 0.12 (25)	n.d.	11	12	8
1 mg s.c. (in Freund's adjuvant)	none	5.6 ± 0.53 (28)	12.4 ± 0.59 (30)	8.6 ± 0.36 (25)	6.8 ± 0.29 (30)				
	10	6.0 ± 0.49 (30)	4.2 ± 0.29 (29)	5.4 ± 0.32 (25)	4.6 ± 0.37 (30)	–7	66	37	32
	30	4.8 ± 0.34 (24)	5.4 ± 0.59 (25)	4.0 ± 0.44 (24)	3.6 ± 0.22 (24)	14	57	54	48
	100	4.0 ± 0.53 (30)	4.6 ± 0.13 (25)	3.0 ± 0.33 (24)	4.4 ± 0.26 (25)	29	63	65	35

[a] On Day 14.

[b] Average in mm^2 ± SE (number of cultures).

Nevertheless, manifestations of cell-mediated immunity could have been obscured by blocking antibodies. This aspect remains to be further elucidated. On the other hand, the presence in PPD of contaminants exhibiting endotoxin activity and the greatly increased susceptibility to endotoxin known to occur in mycobacterial infections (19, 20) would explain one facet of systemic tuberculin reactivity.

Summary. Systemic reactivity to tuberculin was regularly induced after two injections of living BCG. In contrast, only a small proportion of mice was fatally shocked with PPD after a single iv or ip inoculation of mycobacteria. Single treatments with high doses of bacteria given either ip or into the foot pads together with Freund's adjuvant caused anaphylaxis rather than delayed tuberculin shock upon challenge with PPD.

With regard to the conditions of induction and the time of occurrence there was a strict correlation between systemic hypersensitivity to PPD and sensitivity to endotoxin. In the period of increased sensitivity to endotoxin, PPD shock and endotoxin shock had an identical symptomatology. On the other hand, there was a complete lack of correlation between systemic tuberculin reactivity and manifestations of cell-mediated immunity such as skin reactivity, *in vitro* transformation of lymphocytes and inhibition of the migration of peritoneal exudate cells. These facts, as well as the demonstration of a small but decisive endotoxin activity in the PPD preparations used in this study, corroborate the view that systemic tuberculin reactivity is fundamentally a nonimmunological phenomenon.

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