Biological Study of a Nontoxic, Water-Soluble Immunoadjuvant from Mycobacterial Cell Walls

(glycopeptide/agglutinins/precipitins/nonspecific immunity)

L. CHEDID*, M. PARANT*, F. PARANT*, R. H. GUSTAFSON†, AND F. M. BERGER†

* Institut Pasteur and Centre National de la Recherche Scientifique, Paris, France; and

† Wallace Laboratories, Cranbury, New Jersey 08512

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ABSTRACT Whole mycobacterial cells, which are used in Freund's complete adjuvant, besides inducing hypersensitivity to tuberculin, also can elicit hyperreactivity to endotoxins, lymphoid hyperplasia, and allergic polyarthritis in rats. The data reported here demonstrate that a potent water-soluble adjuvant obtained from mycobacterial cell walls is also effective in increasing the immune response to viruses, and that it is free of the toxic effects observed with whole mycobacterial cells.

Adam, Ciorbaru, Petit, and Lederer (1) have shown that a water-soluble fraction extracted from Mycobacteria is capable of increasing the amount of precipitating antibodies and of inducing hypersensitivity to ovalbumin in guinea pigs. The purpose of the present paper is to show that this water-soluble adjuvant (WSA) is also effective in increasing the immune response to viruses and does not exhibit the tuberculin sensitizing and other toxic properties that ensue upon immunization with whole bacterial cells.

All experiments reported in this paper were performed with preparations obtained at Orsay by Adam *et al.* (1). Purified cell walls of *Mycobacterium smegmatis*, *M. kansasii*, or *M. bovis* BCG strain (bacille Calmette-Guérin) were treated with lysozyme. Crude WSA (extracted from either of the three strains) was added to Freund's incomplete adjuvant (FIA) and was injected with ovalbumin; this treatment increased the titers of circulating precipitins and agglutinins within 3 weeks after administration to guinea pigs (Table 1). Additional passive hemagglutination experiments made with ovalbumin-coated sheep erythrocytes (2) confirmed (1) that WSA from *M. smegmatis* had a stronger adjuvant action than similar amounts of whole bacteria, cell wall, or wax D. Cord factor and Mycoside C were inactive in this respect.

In the following experiments, only material extracted from *M. smegmatis* was used. The purified fraction will be referred to as WSA, whereas the preparation that has not been filtered on Sephadex gel will be called crude WSA. All dosages are dry weights.

Increased antibody response to viruses

Influenza Virus. 10-gram Swiss-Webster random-bred mice were injected subcutaneously with PR-8 influenza virus inactivated by ultraviolet light in water, FIA, or FIA containing various concentrations of WSA. Groups of five mice were bled from the retro-orbital sinus at weekly intervals,

starting on day 14 after injection. Hemagglutination-inhibition titrations were done on the pooled sera (2), and the geometric mean of four titrations was calculated. As is evident in Table 2, WSA stimulated antibody response at doses of 50 and 5 µg per mouse. At 42 days, there was a 12-fold increase in antibody titers in the group that had received 50 µg.

Columbia SK Virus. Vaccine was prepared by exposing Columbia SK virus to ultraviolet irradiation. This treatment reduced the infectivity from 8.32 to 1.85 log plaque-forming units (PFU)/ml in L-cells. The vaccine was injected subcutaneously into 10-gram mice in water, FIA, or FIA with WSA. Mice were bled at 14 days and serum samples were tested for virus neutralization activity in L-cells. The titer is expressed as the reciprocal of the serum dilution that reduced the virus titer by 0.5 log units. WSA in FIA strongly stimulated the antibody response at 14 days (Table 3).

It is of particular interest that WSA can increase the immune response, not only to soluble antigens but also to viruses. Although live or killed Mycobacteria are also effective in this regard, they elicit many noxious responses that limit their use in therapeutics. Among these are tuberculin sensitivity, hyperreactivity to endotoxins (3, 4), lymphoid hyperplasia, and autoimmune responses such as adjuvant polyarthritis (5, 6). It was, therefore, important to establish whether WSA produced all or some of these untoward reactions.

Absence of induction of hypersensitivity to tuberculin

Guinea pigs were sensitized by subcutaneous injections of FIA containing 0.2 or 2 mg of phenol-killed whole cells of

TABLE 1. Humoral-antibody response to ovalbumin

	Quantitative precipita- tion*	Passive hemagglu- tination†
Freund's complete adjuvant	2350	4320
FIA (controls)	<200	830
+ crude WSA (<i>M. smeg-</i> <i>matis</i>)	2510	5600
+ crude WSA (BCG)	3940	5600
+ crude WSA (<i>M. kan-</i> <i>sasii</i>)	3950	4640

Dose of WSA was 200 µg/guinea pig.

* µg of antigen-antibody complex per ml of serum.

† Reciprocal titer of serum.

Abbreviations: WSA, water-soluble adjuvant (fraction); FIA, Freund's incomplete adjuvant.

TABLE 2. Humoral-antibody response to influenza virus (Strain PR-8)

	WSA μg/mouse	Mean hemagglutination-inhibition titer on day:				
		14	21	28	35	42
Water + virus	0	4	8	13	16	16
FIA + virus	0	11	19	32	45	64
+ virus	50	32	64	215	305	722
+ virus	5	11	32	90	108	152
+ virus	0.5	4	8	22	45	108

either *M. bovis* (BCG), *M. kansasii*, *M. smegmatis*, or WSA extracted from *M. smegmatis*.

All animals were skin-tested 21 days later by intradermal injection of 100 IU or 300 IU of old tuberculin. Glycerol was injected as a control.

Responses were uniformly positive with whole cells, even at 0.2-mg dosage, and very-strong positive reactions, with necrosis, occurred at 2-mg doses. On the contrary, no inflammation was detectable with 0.2 mg of purified WSA, which was the only dose tested, and only a slight response was noted in some animals that received 2 mg of crude WSA. We emphasize that 0.2 mg of this purified material is extracted from about 10 mg of phenol-killed whole cells.

Absence of spleen and liver hypertrophy

It is well established that intravenous administration of killed Mycobacteria to mice produces a pronounced hepatosplenomegaly that reaches a peak around day 14. In the following experiments, mice received either, 300 μg of WSA or of phenol-killed cells (either BCG or *M. smegmatis*). BCG cells were suspended in saline only, whereas *M. smegmatis* or WSA were suspended in saline or in 10% Bayol F. All materials were injected intravenously in 0.2 ml, and the animals (eight per group) were killed 14 days later.

As can be seen in Table 4, BCG produced greater hepatosplenomegaly than *M. smegmatis* in saline, but *smegmatis* in mineral oil markedly increased the size of the liver and spleen. In this experiment, an increase of the weight of the lungs was also observed (225 mg ± 31 against 160 mg ± 23.6 for the controls) when *M. smegmatis* was administered with Bayol F. Cord Factor suspended in paraffin oil is also known to produce lung granuloma (7). In contrast, injection

TABLE 3. Humoral-antibody response to Columbia SK virus

	Serum neutralizing titer at day 14
Virus in water	<2
Virus in FIA	37
Virus in FIA + WSA	181

Dose of WSA was 100 μg/mouse.

of WSA suspended in saline, or even in Bayol F, did not cause an increase in the weight or size of the liver, spleen, or lungs.

Absence of induction of hyperreactivity to endotoxin

It has been assumed that hyperreactivity to endotoxin is related to granuloma formation observed in the spleen and in various other organs (8). Also, if BCG is administered intraperitoneally instead of intravenously, neither organ enlargement nor hyperreactivity to endotoxin can be observed with dosages that are active by the intravenous route (9). Thus, it was predicted that a correlation exists between splenomegaly and susceptibility to endotoxins in BCG-treated mice.

In view of the preceding results, resistance to endotoxin was measured in mice treated in the same manner as in the previous experiment. Two additional groups received delipidated BCG cell walls suspended in saline or in Bayol F. Table 4 represents the cumulative results of several experiments. Altogether, 744 mice, distributed in groups of eight each, were challenged 14 days after treatment with at least four of the following doses of endotoxin: 0.15, 0.5, 1.5, 5, 15, and 50 μg. The median lethal dose (LD₅₀) was calculated according to the method of Reed and Muench (10).

It is clear in Table 4 that: (a) Administration of saline-suspended *M. smegmatis* cells (the strain from which WSA was prepared), sensitized much less than administration of BCG. However, mice became highly susceptible to endotoxin if they were first treated with *M. smegmatis* suspended in Bayol F. Thus, *M. smegmatis* increased the organ weight and also sensitized to endotoxin when it was suspended in Bayol F. It can also be noted that the delipidated preparations of BCG that did not sensitize to endotoxin when injected in saline could render mice hyperreactive when suspended in Bayol F. (b) Crude or purified WSA did not sensitize to endotoxin, even when suspended in Bayol F.

TABLE 4. Effect of Mycobacterial preparations on hepatosplenomegaly and on hyperreactivity to endotoxin

	Saline			Bayol F		
	Weight (mg) of		Endotoxin LD ₅₀ (μg)	Weight (mg) of		Endotoxin LD ₅₀ (μg)
	Liver	Spleen		Liver	Spleen	
Controls	1073 ± 186	123 ± 39	>50	1029 ± 159	118 ± 18	>50
BCG	1767 ± 269	388 ± 107	1.3	—	—	—
Delipidated BCG	—	—	>50	—	—	<1.5
<i>M. smegmatis</i>	1215 ± 282	194 ± 64	37.9	1761 ± 382	321 ± 89	1.1
Crude WSA	—	—	>50	—	—	>50
WSA	1065 ± 117	138 ± 36	—	1128 ± 238	157 ± 52	>50

The mice used were (CBA T6/T6 x AKR) F₁ hybrids weighing 20 g. The endotoxin used was extracted by the phenol-water procedure from *Salmonella enteritidis*, Danysz strain.

TABLE 5. *Experimental polyarthritis*

	Hind paws		Increase of body weight (%)	Albumin to globulin ratio
	Volume (ml)	Increase (%)		
Saline (controls)	4.85 ± 0.064	—	100	2.18 ± 0.16
FIA + Mycobacteria (5 mg/ml)	8.31 ± 0.471	100	42	0.70 ± 0.09
FIA + Mycobacteria (2 mg/ml)	7.34 ± 0.315	72	71	0.68 ± 0.09
Saline + WSA (2 mg/ml)	4.71 ± 0.094	0	105	1.97 ± 0.16
FIA + WSA (2 mg/ml)	5.50 ± 0.136	19	104	1.73 ± 0.07

Rats used were males of the Wistar strain weighing 150 g.

Experimental polyarthritis in the rat

A mixture of mineral oil and either killed Mycobacteria or a wax-D fraction has been used to produce polyarthritis in rats (5, 6). This reaction has been considered as a manifestation of an autoimmune disease. The arthritic changes are quite noticeable after 7 days, and are accompanied by an altered albumin to globulin ratio in the blood and by a lack of weight gain.

In the following experiment, groups of 10 rats were injected in their footpads with 0.1 ml of FIA containing either 2 or 5 mg/ml of *M. tuberculosis* H 37 Rv or 2 mg/ml of WSA. Two additional groups of controls received either saline alone or saline containing 2 mg/ml of WSA. All animals were killed 17 days later.

As can be seen in Table 5, FIA administered with Mycobacteria produced a considerable increase of the volume of paws, a lack of gain in body weight, and a reversal of the albumin to globulin ratio. No such changes were observed when WSA was administered in saline. Administered with FIA, WSA also did not meaningfully affect the albumin to globulin ratio, or the body weight, and only moderately increased the paw volume. This last effect was due to the presence of FIA, as shown by the following experiment.

Groups of 10 rats were treated with 0.1 ml of FIA, to which was added either 5 mg/ml of BCG or 5 or 0.5 mg/ml of WSA. Two groups of controls receive either saline or FIA alone. The animals were killed 14 days later and the severity of arthritis was assessed by the increase in weight of the paws. It can be seen in Table 6 that even the high dose of 5 mg/ml of WSA did not significantly affect the body weight and produced only a slight arthritis, which was equal to that observed with FIA alone. On the other hand, the weight of the limb that had been injected with Mycobacteria and FIA was increased, and the gain in body weight was sharply reduced.

DISCUSSION

Certain organisms, such as *Corynebacterium parvum* and various Mycobacteria, stimulate both specific and nonspecific immunity to infectious agents and tumors (11-14) and increase the effect of water-in-oil adjuvants such as FIA (15). Furthermore, treatment with Freund's complete adjuvant reduces the incidence of oncogenic adenovirus (16). Clinically, however, the use of such adjuvants considerably restricted, because they all may induce hyperreactivity to endotoxins, lymphoid hyperplasia, or autoimmune diseases such as polyarthritis (in rats). Mycobacteria, moreover, induce hypersensitivity to tuberculo-protein.

The experiments described in this paper confirm that the water-soluble extract of mycobacterial cell walls obtained by Adam *et al.* (1) is a potent immunological adjuvant for ovalbumin. In addition, our data demonstrate that this adjuvant activity also occurs with viral antigens and that these effects can be observed in the absence of toxic reactions, such as the increased susceptibility to endotoxin. Hyperreactivity to endotoxin in BCG-treated mice is not related to delayed or immediate hypersensitivity (17), but is probably induced by cord factor (3), which is also responsible for granuloma formation (7) and hypertrophy of various organs. Moreover, WSA, even added to FIA, does not induce tuberculin hypersensitivity nor polyarthritis. Hypersensitivity is due to the presence of tuberculin in whole mycobacterial cells and polyarthritis, in our opinion, may be due to the presence of cytotoxic components capable of liberating denatured host antigens *in situ*. All these undesirable mycobacterial factors are removed during isolation and purification of WSA. Furthermore, the biological properties of this preparation are not due to endotoxin contamination, since it is devoid of pyrogenicity in rabbits and lacks toxicity in adrenalectomized mice (unpublished data).

TABLE 6. *Experimental polyarthritis*

	Hind paws		Injected paw		Increase of body weight (%)
	Weight (g)	Increase (%)	Weight (g)	Increase (%)	
Saline (controls)	3.47 ± 0.12	—	1.74 ± 0.07	—	100
FIA (controls)	4.04 ± 0.24	28	2.31 ± 0.14	25	84
FIA + Mycobacteria (5 mg/ml)	5.62 ± 0.15	100	4.05 ± 0.14	100	32
FIA + WSA (5 mg/ml)	4.10 ± 0.17	31	2.34 ± 0.12	26	89
FIA + WSA (0.5 mg/ml)	4.02 ± 0.24	27	2.31 ± 0.13	25	95

Rats used were as in Table 5.

In conclusion, WSA is a potent immunological adjuvant that is nontoxic, nonpyrogenic, does not sensitize to tuberculin, and does not produce allergic arthritis in rats. It is thus free of the untoward effects observed with whole mycobacterial cells. In the experiments reported here, this preparation was active only in the presence of paraffin oil. The use of mineral oil parenterally is a limitation in clinical application, although paraffin oil with mycobacterial adjuvants has recently been recommended for certain vaccines (18). Experiments in progress indicate that WSA in saline potentiates the immune response to certain particulate antigens.

1. Adam, A., Ciorbaru, R., Petit, J. F. & Lederer, E. (1972) *Proc. Nat. Acad. Sci. USA* 69, 851-854.
2. Hirst, G. K. (1941) *Science* 94, 22-26.
3. Suter, E., Ullman, G. E. & Hoffman, R. G. (1958) *Proc. Soc. Exp. Biol. Med.* 99, 167-169.
4. Halpern, B. N., Biozzi, G., Howard, J. G., Stiffel, C. & Mouton, D. (1958) *C. R. Soc. Biol.* 152, 899-905.
5. Pearson, C. M. & Wood, F. D. (1959) *Arthr. Rheum.* 2, 440-453.
6. Waksman, B. H., Pearson, C. M. & Sharp, J. T. (1960) *J. Immunol.* 85, 403-417.
7. Bekierkunst, A., Levij, I. S., Yarkoni, E., Vilkas, E., Adam, A. & Lederer, E. (1969) *J. Bacteriol.* 100, 95-102.
8. Howard, J. G. (1969) in *Structure et Effets Biologiques de Produits Bactériens Provenant de Bacilles Gram-Négatifs*, ed. Chedid, L. (CNRS, Paris), pp. 331-340.
9. Chedid, L., Rousselot, C. & Parant, F. (1971) in *The Reticuloendothelial System and Immune Phenomena*, ed. DiLuzio, N. R. (Plenum Press), pp. 173-182.
10. Reed, L. J. & Muench, H. A. (1938) *Amer. J. Hyg.* 27, 493-497.
11. Stiffel, C., Mouton, D. & Biozzi, G. (1971) *Ann. Inst. Pasteur Paris* 120, 412-427.
12. Zbar, B., Bernstein, I., Tanaka, T. & Rapp, H. J. (1970) *Science* 170, 1217-1218.
13. Zbar, B. & Tanaka, T. (1971) *Science* 172, 271-273.
14. Mathé, G. (1971) *Advan. Cancer Res.* 14, 1-36.
15. Neveu, T., Branellec, A. & Biozzi, G. (1964) *Ann. Inst. Pasteur Paris* 106, 771-777.
16. Berman, L. D., Allison, A. C. & Pereira, H. G. (1967) *Int. J. Cancer* 2, 539-543.
17. Chedid, L. & Parant, M. (1971) in "Microbial Toxins," *Bacterial Endotoxins*, eds. Kadis, S., Weinbaum, G. & Ajl, S. J. (Academic Press), Vol. 5, pp. 415-459.
18. Davenport, F. M. (1968) *Ann. Allergy* 26, 288-292.