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## Delayed Hypersensitivity Induced in Guinea Pigs with Tuberculoprotein from *M. bovis* (BCG)\* (33813)

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No reports have appeared in the literature showing tuberculoproteins to be capable of inducing delayed hypersensitivity in animals. Gell and Benacerraf (1) state that attempts to produce delayed tuberculin sensitivity with tuberculoproteins alone have consistently failed. Other recent reviews dealing with delayed hypersensitivity (2, 3) make no reference to the ability of tuberculoproteins to act in this manner. Raffel (4) demonstrated that tuberculoprotein alone was incapable of provoking delayed hypersensitivity. Boyden (5) studied the effect of unheated tuberculin upon guinea pigs and concluded that when this material was injected subcutaneously in either saline or oil, only immediate sensitivity was induced. The guinea pigs were sensitized with 0.1 ml of tuberculin and tested 42 days later by intradermal injection of 20  $\mu$ g of heated or unheated tuberculoprotein.

Previously, we have shown that protoplasts of acid-fast bacilli were able to elicit delayed reactions in sensitized guinea pigs and rabbits (6, 7) although they failed to induce delayed hypersensitivity in such animals (8). The two present experiments demonstrate that a fraction derived from *M. bovis* (BCG) protoplasm and labeled "C protein" is capable of inducing a state of delayed hypersensitivity in guinea pigs when it is incorporated in incomplete Freund's ad-

juvant and injected subcutaneously into the foot pads.

*Materials and Methods. Animals.* Female Hartley strain guinea pigs weighing about 300 g were used. These were obtained from a supplier in Hamilton, Montana.

*Antigens.* Old tuberculin (OT) was generously supplied by Parke Davis and Company, Detroit, Michigan. Protoplasm was obtained from viable BCG bacilli by methods previously described (7). The fraction, "C protein," was prepared from this protoplasm by the following method. This procedure is a modification of the technique described by Seibert and Affronti (9). All manipulations were carried out at 4° and all materials and equipment were cooled to this temperature prior to their use.

Whole BCG protoplasm in phosphate buffered saline (PBS), pH 7.2, was adjusted to pH 4.5 with 1 *M* acetic acid. After 5 min of stirring, the solution was centrifuged at 12,100g for 10 min and the resulting brownish translucent precipitate was dissolved in PBS. The pH was adjusted to 7.6 with 1 *N* NaOH and ethanol was rapidly added to a final concentration of 40%. After 10 min of stirring, the suspension was centrifuged at 12,100g for 10 min. The clear but slightly opalescent supernatant was adjusted to pH 4.5 with 1 *M* acetic acid. After 5 min, the suspension was again centrifuged at 12,100g for 10 min and the resulting precipitate was dissolved in PBS.

*Sensitization.* Animals in Experiment 1

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were sensitized with 20  $\mu\text{g}$  of "C protein" contained in 0.2 ml of incomplete Freund's adjuvant. The emulsion was made as follows: 2 parts Arlacel A (0.5 ml) was mixed with 8 parts light mineral oil (2.0 ml) in a mortar and pestle, then 10 parts (2.5 ml) of a saline suspension of "C protein" containing 20  $\mu\text{g}/0.1$  ml was added drop by drop with constant stirring. One-tenth-ml amounts of the resulting emulsion were injected into both hind foot pads. Animals in Experiment 2 were sensitized in the same manner except that those in groups A, B, C, and D received 0.8, 4.0, 20, or 100  $\mu\text{g}$  of "C protein," respectively.

*Skin tests.* These tests were performed at selected intervals following sensitization. In Expt. 1, 4 or 6 animals were tested 5, 7, 10, and 13 days after sensitization. In Expt. 2, 3 animals from each group were tested 7, 14, 25 and 40 days after immunization. Animals were skin tested only once. The size of the reactions was measured at 4, 6, 24, and frequently 48 hr after intradermal injection of the antigen. The volumes of the lesions were calculated by the following formula suggested by Waksman *et al.* (10): Diameter  $\times$  diameter  $\times$   $\frac{1}{2}$  thickness  $\times$   $\frac{3}{4}$ . In both experiments, 20-, 4-, 0.8-, and 0.16- $\mu\text{g}$  amounts of "C protein," contained in 0.1 ml of saline and injected intradermally, were employed for skin testing. In addition, in Expt. 1, identical amounts of protoplasm as well as 0.1-ml amounts of OT diluted 1:20 and 1:100 in saline were used.

*Tests for presence of antibodies.* Three animals from each group of Expt. 2 were bled 12, 15, 19, 22, and 40 days after immunization. Passive cutaneous anaphylaxis (PCA) tests were done with serums obtained from animals in Expt. 2 twenty-two days after sensitization. Three normal guinea pigs were injected intradermally with 0.1 ml of each of 12 serums and 4 hr later, were injected intravenously with a mixture of 1.0 ml of saline containing 4 mg of protoplasm and 1.0 ml of 0.5% Evans blue. Thirty min later, the skin was examined for the presence of extravasated dye. Precipitin tests (ring tests) were also done with serum taken at selected inter-

TABLE I. Volume of Lesions 4, 6, 24, and 48 hr after Intradermal Injection of Graded Doses of C Protein and Protoplasm from BCG Bacilli and of Old Tuberculin (OT) in Guinea Pigs Sensitized with 20  $\mu\text{g}$  of C Protein in Incomplete Freund's Adjuvant and Tested 5, 7, 10, and 13 Days Later.

Skin test antigen	Amount ( $\mu\text{g}$ )	Day after sensitization tests were performed												
		5			7			10			13			
		(hr):			Volume ( $\text{mm}^3$ ) of lesion at			Volume ( $\text{mm}^3$ ) of lesion at			Volume ( $\text{mm}^3$ ) of lesion at			
C protein	20	7.9 <sup>a</sup>	21.6	75.6	50.4	16.3 <sup>a</sup>	28.6	104.1	2.4 <sup>a</sup>	17.1	100.8	77.9 <sup>b</sup>	92.4	232.7
	4	2.9	5.8	23.8	23.1	5.5	16.9	30.0	—	3.5	37.6	25.2	28.7	67.9
	0.8	0.3	0.8	3.5	6.1	1.6	1.9	5.1	—	0.4	4.7	7.8	9.8	49.0
	0.16	—	0.3	0.2	0.5	0.2	0.4	2.0	—	—	2.3	2.6	2.6	5.6
Protoplasm	20	—	—	—	—	19.2	33.1	92.8	3.9	42.5	97.8	45.7 <sup>c</sup>	44.4	18.6
	4	—	—	—	—	4.6	12.5	26.8	0.9	6.3	36.5	14.4 <sup>d</sup>	12.9	33.2
	0.8	—	—	—	—	1.7	3.6	9.4	—	1.2	5.0	—	—	—
	0.16	—	—	—	—	0.3	0.8	1.5	—	—	0.8	—	—	—

<sup>a</sup> Average of 4 animals.

<sup>b</sup> Average of 6 animals.

<sup>c</sup> OT diluted 1:20 in saline.

<sup>d</sup> OT diluted 1:100 in saline.

vals from animals in the second experiment. The antigen used for this purpose was a saline solution containing 4 mg of protoplasm/ml.

*Results. Expt. 1.* The results of Expt. 1 are shown in Table I. Delayed hypersensitivity was established within 5 days and continued throughout the period of observation. In addition, the degree of sensitivity increased with time. Not only was there an increase in the volume of the reactions elicited by intradermal injection of 20- and 4- $\mu$ g amounts of antigen, but definite delayed reactions following injection of 0.8 and 0.16  $\mu$ g of antigen appeared as time progressed. Delayed reactions obtained with protoplasm were similar to those elicited by "C protein." Reactions elicited by OT were of the delayed type.

In order to demonstrate the changes occurring in the skin reactions 4, 6, and 24 hr after injection of the antigen, the actual dimensions of four typical examples are given in Table II. The reactions presented are from Expt. 1 and were observed in the first guinea pig of each group tested after 5, 7, 10, and 13 days. As shown, both the diameter and thickness of the lesion increased progressively. At 4 hr, the lesions were pale pink and doughy in consistency but by 24 hr, they were red and firm. They were evenly distributed about the site of injection and the edges rose sharply from the surrounding skin. In some lesions, necrosis was noted at 24 hr.

*Expt. 2.* Groups of 12 guinea pigs were sensitized with 100, 20, 4, or 0.8  $\mu$ g (groups D, C, B, and A, respectively) of "C protein" contained in incomplete Freund's adjuvant. Lots of 3 animals from each group were skin tested 7, 14, 25, and 40 days after sensitiza-

tion with graded amounts of the same antigen. The results are shown in Table III. Sensitivity to "C protein" had developed by day 7 in all groups of guinea pigs. Animals in groups A and B reacted only to 20  $\mu$ g of antigen while those of groups C and D reacted to 4  $\mu$ g of antigen as well. On day 14 animals of group D reacted vigorously to 0.8  $\mu$ g and moderately to 0.16  $\mu$ g of antigen. The highest level of sensitivity was elicited on day 25. At this time, the smallest amount of antigen to which the animals of groups A and B gave a positive delayed response was 4.0  $\mu$ g, while in groups C and D it was 0.16  $\mu$ g. Reactions observed at 40 days were not as marked as those at 25 days.

No precipitins were detected at any time in the serums of guinea pigs sensitized with 4 or 0.8  $\mu$ g of antigen. Precipitins were demonstrated in the serum of  $\frac{1}{3}$  of the animals of group C tested 19 and 22 days after sensitization and in all 3 serums from group C animals tested after 40 days. The serums from all the animals in group D tested on days 15, 19, 22, and 40 contained precipitins, while negative results were obtained with serums obtained on day 12. Serums taken 22 days after sensitization from animals in each of the groups were tested for the presence of antibodies capable of producing PCA reactions in guinea pigs. Only the 3 serums from animals in group D yielded positive results.

*Discussion.* In one of the original reports (8) on the biological activity of cell wall and protoplasm fractions of acid-fast bacilli, the statement was made that intradermal injection of 1 mg of protoplasm failed to induce delayed hypersensitivity in rabbits. The present studies indicate that 0.8  $\mu$ g of "C

TABLE II. Changes in Skin Reactions after Injection of Antigen.

Day tested	Hours after injection of antigen					
	4		6		24	
	Dimensions	Volume (mm <sup>3</sup> )	Dimensions	Volume (mm <sup>3</sup> )	Dimensions	Volume (mm <sup>3</sup> )
5	7 × 6 × 0.2	3.2	10 × 9 × 0.4	13.5	14 × 16 × 0.9	75.6
7	10 × 11 × 0.2	8.3	11 × 11 × 0.3	13.6	19 × 20 × 0.9	128.3
10	8 × 11 × 0.1	3.3	9 × 11 × 0.3	11.1	16 × 16 × 1.2	115.2
13	15 × 16 × 0.7	63.0	15 × 17 × 0.8	76.5	24 × 25 × 1.9	427.5

TABLE III. Volume of Lesions 4, 6, 24, and 48 hr after Intradermal Injection of 20, 4, 0.8, and 0.16  $\mu\text{g}$  of C Protein into Guinea Pigs Sensitized by Injection into the Hind Foot Pads of 100, 20, 4, or 0.8  $\mu\text{g}$  of C Protein Contained in Incomplete Freund's Adjuvant and Tested 7, 14, 25, and 40 Days Later.

Group	Sensitizing dose ( $\mu\text{g}$ )	Skin test dose ( $\mu\text{g}$ )	Day after sensitization tests were performed															
			7				14				25				40			
			Volume (mm <sup>3</sup> ) of lesion at			Volume (mm <sup>3</sup> ) of lesion at			Volume (mm <sup>3</sup> ) of lesion at			Volume (mm <sup>3</sup> ) of lesion at						
			4	6	24	48	4	6	24	4	6	24	48	4	6	24	48	
A	0.8	20	7.3	5.4	9.4	19.8	8.2	3.3	7.5	1.1	2.1	49.3	15.5 <sup>c</sup>	8.2	4.2	32.2	17.3	
		4 <sup>a</sup>	0.8	0.2	—	—	2.3	3.2	1.7	1.1	0.2	11.2	2.3	3.5	1.9	8.0	5.3	
B	4.0	20	3.2	8.8	11.9	1.9	3.7	3.2	4.4	2.5	31.6	51.0	22.5	12.8	19.3	47.8	17.9	
		4	1.3	2.6	1.6	—	0.3	0.5	—	1.0	3.5	16.5	2.3	2.2	5.4	12.3	4.7	
		0.8 <sup>b</sup>	—	—	—	—	—	—	—	—	0.4	5.8	—	—	—	1.8	—	
C	20.0	20	13.4	12.6	45.8	11.2	12.3	21.1	48.6	143.2	117.4	575.1	319.1	47.7	81.9	256.5	135.5	
		4	2.9	6.4	19.9	2.2	2.2	4.3	21.6	57.0	78.1	141.8	45.4	20.6	35.7	126.1	54.1	
		0.8	—	1.0	3.4	0.2	0.5	1.4	5.4	25.4	19.2	84.9	30.6	2.9	17.0	38.3	16.4	
		0.16	—	—	—	—	—	—	0.7	17.9	8.4	30.6	8.6	0.9	4.4	15.9	8.2	
D	100.0	20	11.1	11.4	37.1	25.2	52.4	91.3	79.9	131.9	267.3	588.0	142.6 <sup>d</sup>	82.5	70.3	150.9	45.4	
		4	2.3	3.8	17.4	1.8	24.6	32.9	39.2	44.2	70.3	150.8	51.4	35.3	41.1	57.7	17.0	
		0.8	0.5	0.4	3.1	0.4	9.3	18.0	17.9	25.2	20.5	59.5	8.7	9.0	10.6	17.3	6.3	
		0.16	—	—	—	—	3.2	8.0	8.0	14.1	7.4	14.5	0.8	—	1.3	6.8	0.2	

<sup>a</sup> Negative results following injection of 0.8 and 0.16  $\mu\text{g}$  of antigen.

<sup>b</sup> Negative results following injection of 0.16  $\mu\text{g}$  of antigen.

<sup>c</sup> Animal 1 of this group had lesions measuring  $3 \times 4 \times 0.1$  mm at 4 hr and  $15 \times 18 \times 0.5$  mm at 24 hr.

<sup>d</sup> Animal 1 of this group had lesions measuring  $16 \times 16 \times 1.8$  mm at 4 hr and  $25 \times 27 \times 3.0$  mm at 24 hr with central necrosis.

protein" from the protoplasm of BCG bacilli is capable of producing this state in guinea pigs provided the protein is incorporated into incomplete Freund's adjuvant. Delayed reactions can be elicited by intradermal injection of the homologous antigen 5 days after the animals have been sensitized and this type of hypersensitivity persists for at least 40 days. Whole protoplasm and OT can also elicit delayed reactions. We have recently found that whole protoplasm also induces such delayed hypersensitivity when incorporated into incomplete Freund's adjuvant (unpublished results).

The degree of sensitivity detected by skin tests appears to be related to the amount of antigen employed for sensitization and to the amount used as the eliciting dose. The original experiment was done following the methods of Benacerraf and Gel (11) who sensitized guinea pigs with 20  $\mu\text{g}$  of protein antigen. The second experiment demonstrates that 0.8- to 100- $\mu\text{g}$  amounts of "C protein" may also be used for this purpose. When animals are tested 7 days after sensitization, those immunized with 0.8 or 4.0  $\mu\text{g}$  of antigen react only to 20  $\mu\text{g}$  of antigen but those immunized with larger amounts of antigen develop equally large reactions to injections of 4.0  $\mu\text{g}$  of antigen. Animals tested 40 days after sensitization show a similar difference in the degree of sensitivity. Those immunized with smaller amounts of antigen react to injections of 4.0 or 20.0  $\mu\text{g}$  of material while those immunized with 20.0 or 100.0  $\mu\text{g}$  of antigen react to 0.8 and 0.16  $\mu\text{g}$  of material as well.

None of the guinea pigs immunized with small amounts of "C protein" produced sufficient circulating antibodies to elicit precipitin reactions with protoplasm but those injected with 100  $\mu\text{g}$  of antigen developed precipitins within 15 days. PCA reactions were demonstrable after 22 days but only with serums from animals given 100  $\mu\text{g}$  of

antigen. Such results indicate that guinea pigs sensitized with 20  $\mu\text{g}$  or less of "C protein" apparently develop a pure state of delayed hypersensitivity. The early reactions elicited by large doses of antigen in animals sensitized with 200 or 100  $\mu\text{g}$  of "C protein" may be interpreted as part of an extremely intense delayed reaction. Studies in progress show that it is possible to transfer hypersensitivity to normal guinea pigs with peritoneal exudate cells from sensitized ones.

*Summary.* Delayed hypersensitivity was produced in guinea pigs with 0.8 to 100  $\mu\text{g}$  of "C protein" from protoplasm of BCG bacilli incorporated in incomplete Freund's adjuvant and injected into the foot pads of guinea pigs. Delayed reactions were elicited as early as 5 and as late as 40 days after sensitization. In highly sensitized animals, 0.16  $\mu\text{g}$  of antigen elicited definite delayed reactions.

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