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## Adjuvant effect of lipopolysaccharides from *Salmonella typhimurium*

I. Delayed hypersensitivity to  
azobenzene-*o*-arsenate-N-acetyl-L-tyrosine  
in guinea pigs

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**Abstract** Azobenzene-*o*-arsenate-N-acetyl-L-tyrosine (ABA-Tyr\*) and lipopolysaccharides from *S. typhimurium* (LPS) inoculated in Freund-type water-in-oil emulsions successfully sensitized guinea pigs to ABA-groups. Such guinea pigs showed exclusively delayed hypersensitivity against ABA-BSA. The sensitization was dependent on the physical forms of the LPS administered, since LPS inoculated in FIA exerted the adjuvant activity but LPS in saline was not effective. However, the direct association of the ABA-Tyr and LPS in the same water-in-oil emulsion was not required. In addition, LPS administered 5 days after ABA-Tyr injection exerted an adjuvant action in the same experimental system. From these results it was suggested that ABA-Tyr lacked any intrinsic adjuvant activity and LPS provided a signal (s) for the differentiation and proliferation of ABA-specific lymphocytes.

Azobenzene-*o*-arsenate-N-acetyl-L-tyrosine (ABA-Tyr) is a low molecular weight chemical which sensitizes guinea pigs if injected with FCA (1). ABA-Tyr emulsified with FIA does not sensitize guinea pigs but leads the animal to tolerant to subsequent immunization with ABA-Tyr in FCA (2). ABA-Tyr has been regarded as a T-lymphocyte directed antigen since it induced little (3) or no (1, 4) antibody but induced hapten-specific delayed hypersensitivity as well as hapten-specific helper activity (4, 5). Therefore, ABA-Tyr, as an antigen, may have an advantage for the analysis of T-lymphocyte functions in guinea pigs.

The study presented here was initially undertaken in order to find substances which could replace for tubercle bacilli in FCA. Among various T and B lymphocyte mitogens tested LPS was the most effective adjuvant for this purpose. LPS and ABA-Tyr emulsified in the same FIA induced strong ABA-specific delayed hypersensitivity, however, direct association of the two was not required. Similarly, separate administrations of ABA-Tyr in FIA and BCG cell walls in an oil-in-water emulsion, if they were injected in such a way to drain into the same regional lymph nodes, rendered the animal delayed hypersensitive to ABA-BSA.

### MATERIALS AND METHODS

ABA-Tyr was prepared according to the method of Tabachnicki and Sobotka (7). Six hundred and fifty mg of arsanilic acid was dissolved in 1 N HCl to which 206 mg of NaNO<sub>2</sub> dissolved in

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\* Abbreviations used in this paper: ABA-Tyr; azobenzene-*o*-arsenate-N-acetyl-L-tyrosine, FCA; Freund's complete adjuvant, FIA; Freund's incomplete adjuvant, T-; thymus derived, LPS; lipopolysaccharide from *Salmonella typhimurium*, BSA; bovine serum albumin, PPD; purified protein derivatives of tuberculin, PHA; phytohemagglutinin, PWM; pokeweed mitogen, Con A; concanavalin A.

6 ml de-ionized water was added over 10 min in an ice bath. Potassium bromide was included to prevent self-coupling of the diazonium reagent. After stirring the mixture for an additional 1 hr in the same ice bath resulting diazonium reagent was diluted to 100 ml with ice cold water. An aliquot of the above diazonium reagent (25 ml) was added slowly to a N-acetyl-L-tyrosine solution in which 350 mg were dissolved in 10 ml 0.01 M borate buffer. During the addition of the diazotized arsanilic acid the pH of the mixture was maintained at 9.5 by 1 N NaOH. Then the mixture was brought to a cold room and continued stirring for 4 h in the dark. Resulted deep red solution was acidified by 1 N HCl to precipitate the azo-compounds. The precipitates formed was collected by centrifugation and then dissolved again with 1% NH<sub>4</sub>OH. The acid precipitation of azo-compounds was repeated twice and finally dissolved in 1% NH<sub>4</sub>OH. The partially purified material was chromatographed in a Sephadex G-15 column (2.5 × 95 cm) according to the method of Alkan et al (4). Fractions which had the ratio of optical densities at 490 and 550 nm being 1.5 were collected and rechromatographed in the same column.

ABA-BSA was generously supplied by Dr. R. Sakai of this institute. PPD was obtained from National Institute of Health, Tokyo.

FIA consisted of 8.5 ml Drakeol (Pennsylvania Biological Lab.) and 1.5 ml of Arlacel A (Atras Chemical Ind.) to which an equal volume of an antigen solution was added and emulsified. For the preparation of FCA a suspension of heat killed *Mycobacterium tuberculosis* H<sub>37</sub>RV 1 mg/ml, was added. The final content of mycobacteria in 0.1 ml FCA emulsion was 25 µg.

BCG cell walls were obtained through the courtesy of Dr. E. Ribi, Rocky Mountain Laboratory, NIH. An oil-in-water adjuvant of BCG cell walls was prepared by the method of Zbar et al (6). The final content of BCG cell walls was 1 mg/0.4 ml.

Other adjuvant substances used in this report were obtained from various sources; LPS (*S. typhimurium*) and PHA were purchased from Difco, PWM from GIBCO and Con A was kindly supplied by Dr. Y. Mizuno of this institute.

Hartley guinea pigs weighing 300 to 500 g were immunized in their legs subcutaneously with total 0.2 ml of FIA or FCA emulsions. When oil-in-water adjuvant of BCG cell walls was used 0.4 ml of the suspension was distributed in two legs subcutaneously.

Skin tests were carried out 2 weeks after the immunization. Fifty or 10 µg of ABA-BSA and 10 or 20 µg of PPD both in 0.1 ml saline were used as test antigens. Reactions were read at 3 and 24 hr of the injection. Delayed skin reactions at 24 hr which consisted of erythema of more than 10 mm in diameter accompanied by an apparent induration was taken as positive.

## RESULTS

As ABA-Tyr inoculated in FCA is known as a potent immunogen for ABA-specific delayed hypersensitivity in guinea pigs the initial experiment was undertaken to confirm the adjuvanticity of FCA and BCG cell walls. Table 1 summarizes results of several experiments. ABA-Tyr in FIA repeatedly failed to sensitize guinea pigs but the same antigen in FCA sensitized against ABA-groups. Replacement of tubercle bacilli for BCG cell walls did not change the adjuvant activity of the emulsion. An immunogen in which BCG cell walls were associated with oil droplets and suspended in 0.2% tween 80 solution and ABA-Tyr was dissolved in the water phase was found to be inactive. The same result was obtained when ABA-Tyr and BCG cell walls were associated with oil droplets and then suspended in 0.2% tween 80, probably because ABA-Tyr dissociated from oil into water phase during the preparation of the suspension due to strong negative charge of ABA-Tyr. By contrast, when ABA-Tyr in FIA and BCG cell walls in an oil-in-water emulsion

were injected separately in two hind legs, 5 out of 8 animals responded against ABA-BSA. These results would indicate that, for the sensitization of guinea pigs with ABA-Tyr, (a) ABA-Tyr should be given in insoluble forms and (b) direct association of ABA-Tyr and mycobacterial preparation is not required provided that the both antigen and adjuvant were given in such a way to drain in the same regional lymph nodes. Con A (50 to 500  $\mu\text{g}$ ) and PWM (50  $\mu\ell$ ) did not show adjuvant activity (Table 2) and PHA showed weak adjuvanticity only when 1 mg was used (Table 3).

LPS inoculated in FIA was a good adjuvant for ABA-Tyr (Table 4). Five hundred  $\mu\text{g}$  of LPS and 100  $\mu\text{g}$  of ABA-Tyr in FIA sensitized all 6 guinea pigs tested. A lower dose of LPS was less effective. Administration of LPS alone did not sensitize guinea pigs against ABA-groups as well as PPD, showing that the adjuvant action of LPS used in this experiment was not due to the contamination of mycobacterial components.

**Table 1.** Delayed hypersensitivity in guinea pigs sensitized with 100  $\mu\text{g}$  of ABA-Tyr and mycobacteria<sup>1)</sup>

Preparation of Immunogen <sup>2)</sup>	Number of Animal	Number of Positive Reaction <sup>3)</sup>		
		ABA-Tyr	PPD	Saline
ABA-Tyr in FIA	25	0		NT <sup>4)</sup>
ABA-Tyr in FCA (H 37R V)	2	2(18.0)		NT
ABA-Tyr and BCG-CW in FIA	4	4(12.1 $\pm$ 0.4)	4(13.3 $\pm$ 0.8)	NT
ABA-Tyr in water (oil-in-water) BCG-CW in oil	7	0	7(16.4 $\pm$ 1.0)	0
ABA-Tyr in oil (oil-in-water) BCG-CW in oil	4	0	4(15.4 $\pm$ 0.8)	0
ABA-Tyr in FIA BCG-CW in oil-in-water	8	5(10.6 $\pm$ 1.3)	8(15.3 $\pm$ 0.9)	0

- 1) Guinea pigs were immunized with immunogens in their two hind feet two weeks before the skin test. Fifty  $\mu\text{g}$  of ABA-BSA and 20  $\mu\text{g}$  of PPD, each of 0.1 ml in saline, were injected intradermally in the back. No immediate reaction at 3 hr was observed.
- 2) Doses of immunogens; 0.2 ml Freund-type emulsion was divided into two parts and distributed in hind feet and/or 0.4 ml of oil-in-water suspension was divided into two parts and distributed into hind feet.
- 3) In parenthesis are given average diameter (mm) of erythema and standard error of the average at 24 hr in all animals of the group tested.
- 4) NT; not tested.

**Table 2.** Delayed hypersensitivity in guinea pigs sensitized with 100  $\mu\text{g}$  of ABA-Tyr and mitogens<sup>1)</sup>

Immunogen <sup>2)</sup>	Number of Animal	Number of Positive Reaction <sup>3)</sup>		
		ABA-BSA	PPD	Saline
Con A 500 $\mu\text{g}$ + ABA-Tyr	3	0	0	0
Con A 50 $\mu\text{g}$ + ABA-Tyr	3	0	0	0
PWM 50 $\mu\ell$ + ABA-Tyr	3	0	0	NT <sup>4)</sup>

- 1) Guinea pigs were immunized with immunogens in their hind feet two weeks before skin test. Fifty  $\mu\text{g}$  of ABA-BSA and 20  $\mu\text{g}$  of PPD, each in 0.1 ml saline, were injected intradermally in the back. No immediate skin reaction was observed at 3 hr.
- 2) Two tenth ml of Freund-type emulsion was distributed in two hind feet.
- 3) Read at 24 hr.
- 4) NT; not tested.

As the results presented in Table 4 established the adjuvant activity of LPS and the results presented in Table 1 suggested that the direct association of ABA-Tyr with adjuvant materials was not important, LPS emulsified in FIA was injected on different days from ABA-Tyr injection. On day 0, 100  $\mu\text{g}$  of ABA-Tyr in FIA was distributed in two hind foot pads. On days -5, -1, 0, 1 and 5, 200  $\mu\text{g}$  of LPS in FIA was injected in the same site as ABA-Tyr injection. Two weeks after the injection of ABA-Tyr skin test was carried out. As presented in Figure 1 all 5 guinea pigs which received ABA-Tyr and LPS on the same day showed the strong delayed hypersensitivity against ABA-BSA. Injection of LPS prior to ABA-Tyr was rather inactive but if

**Table 3.** Delayed hypersensitivity in guinea pigs sensitized with ABA-Tyr and PHA emulsified in FIA<sup>1)</sup>

PHA <sup>2)</sup> ( $\mu\text{g}$ )	ABA-Tyr <sup>2)</sup> ( $\mu\text{g}$ )	Number of Animal	Number of Positive Reaction <sup>3)</sup>		
			ABA-BSA	PPD	Saline
100	10	3 <sup>3)</sup>	0	0	0
100	100	6	0	0	0
1000	10	3	0	0	0
1000	100	6	4 (12.2 $\pm$ 2.5)	0	0
3000	100	3	0 <sup>6)</sup>	0	0
1000	0	3	0	0	0
1000	100	3 <sup>4)</sup>	2 ( 9.9 $\pm$ 1.0)	NT <sup>7)</sup>	NT
0	100	3 <sup>4)</sup>	0 <sup>6)</sup>	NT	NT

- 1) Guinea pigs were immunized with immunogens in their two hind feet. Fifty  $\mu\text{g}$  of ABA-BSA and 20  $\mu\text{g}$  of PPD, each in 0.1 ml, were injected intradermally in their back. No immediate reaction was observed at 3 hr.
- 2) Two tenth ml of PHA and ABA-Tyr emulsified in FIA were distributed in two legs.
- 3) Skin test was carried out 2 weeks after immunization.
- 4) Skin test was carried out 7 weeks after immunization.
- 5) In parenthesis average diameter (mm) of erythema and standard error of the average are given in all animals tested at 24 hr.
- 6) One out of 3 guinea pigs responded with positive induration but erythema was less than 10 mm.
- 7) NT; not tested.

**Table 4.** Delayed hypersensitivity in guinea pigs sensitized with ABA-Tyr and LPS emulsified in FIA<sup>1)</sup>

LPS <sup>2)</sup> ( $\mu\text{g}$ )	ABA-Tyr <sup>2)</sup> ( $\mu\text{g}$ )	Number of Animal	Number of Positive Reaction <sup>3)</sup>		
			ABA-BSA	PPD	Saline
500	100	6	6 (12.2 $\pm$ 0.8)	0	0
50	100	3	2 ( 9.0 $\pm$ 1.7)	0	0
500	0	3	0	0	0

- 1) Guinea pigs were immunized with immunogens in their two hind feet two weeks before. Fifty  $\mu\text{g}$  of ABA-BSA and 20  $\mu\text{g}$  of PPD, each in 0.1 ml saline, were injected intradermally in the back. No immediate skin reaction was observed at 3 hr.
- 2) Two tenth ml of LPS and 0.2 ml of ABA-Tyr in FIA emulsions were distributed in two feet.
- 3) In parenthesis are given average diameter (mm) of erythema and standard error of the average in all guinea pigs tested at 24 hr.

LPS was injected later than ABA-Tyr almost 80% of the animals was rendered delayed-hypersensitive. The results of the next experiment revealed that if injection of LPS was delayed up to 2 weeks after the administration of ABA-Tyr, LPS did not show its adjuvant activity (Figure 2). Combining these results one could explain in the following way; for triggering ABA-specific lym-

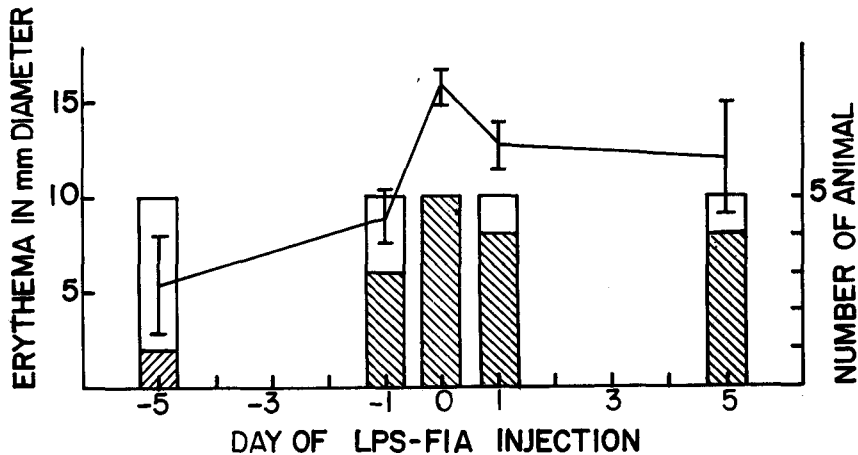


Fig. 1. ABA-specific delayed hypersensitivity in guinea pigs who received LPS in FIA and ABA-Tyr in FIA on different days.

Guinea pigs received 100  $\mu$ g of ABA-Tyr on day 0. Each group received 200  $\mu$ g of LPS in FIA on -5, -1, 0, 1 and 5 days of ABA-Tyr injection. Skin test with 50  $\mu$ g ABA-BSA in 0.1 ml was carried out on the day as indicated in the figure. Hatched columns represent fraction of animals showing positive reactions. A solid line represents mean diameters of erythema in all the animals tested and vertical bars standard errors of the mean.

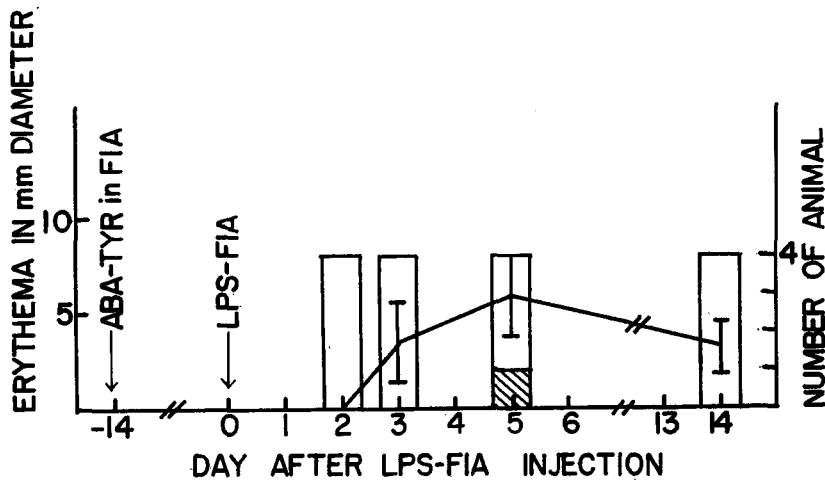


Fig. 2. ABA-specific delayed hypersensitivity in guinea pigs which received LPS in FIA two weeks after ABA-Tyr in FIA.

Guinea pigs received ABA-Tyr in FIA and two weeks later were injected 100  $\mu$ g of LPS in FIA in their feet pads. Skin tests with 10  $\mu$ g ABA-BSA was carried out 2, 3, 5 and 14 days after LPS injection. Hatched columns represent number of animals with positive skin reaction and open columns number of negatively reacted animals tested. A solid line represents mean diameters of erythema and vertical bar standard errors of the mean.

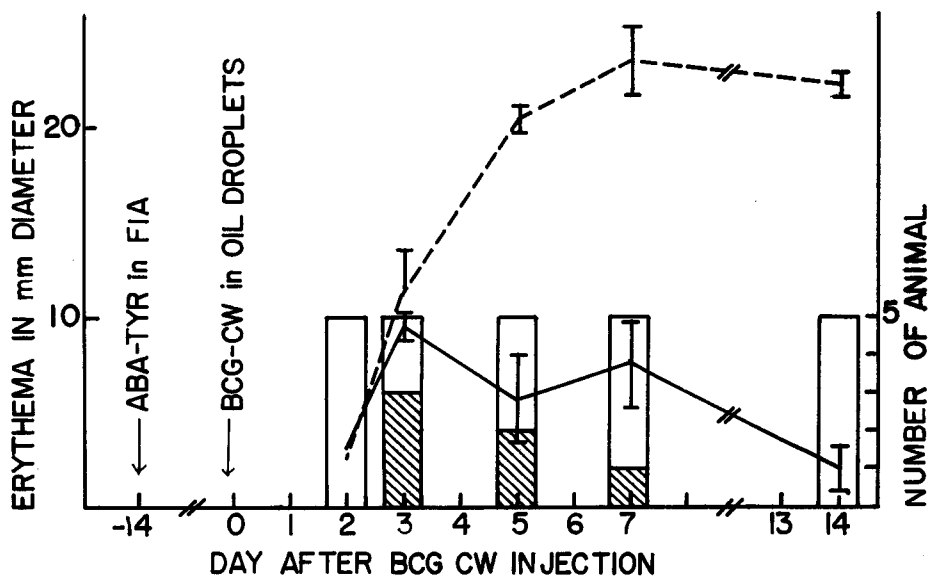


Fig. 3. Delayed hypersensitivity against ABA-BSA and PPD in guinea pigs which received BCG cells walls two weeks after ABA-Tyr in FIA.

Guinea pigs received BCG cell walls in 0.4 ml oil-in-water suspension two weeks after the injection of ABA-Tyr in FIA. Hatched columns represent the number of animals with positive delayed reaction against ABA-BSA and open columns the number of negatively reacted animals tested. A solid line represents mean diameter of erythema of ABA-specific delayed reaction and an interrupted line represents mean diameter of tuberculin reaction. Vertical bars represent standard errors of the mean.

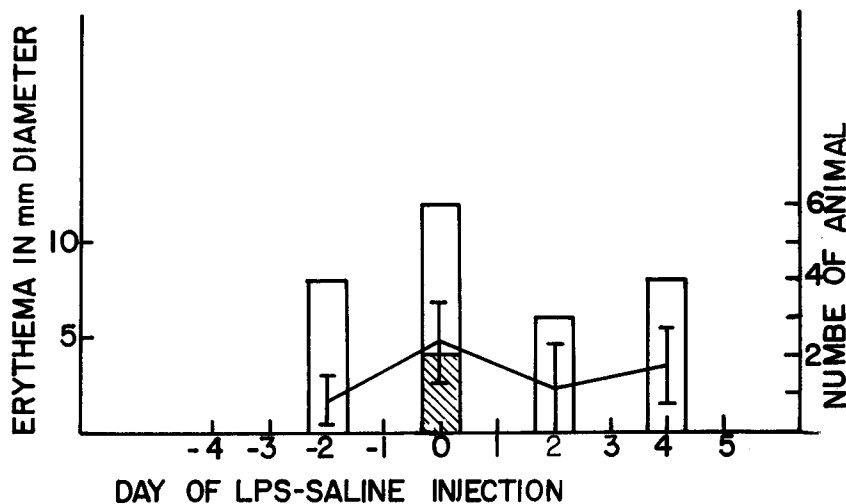


Fig. 4. ABA-specific delayed hypersensitivity in guinea pigs who received LPS in saline and ABA-Tyr in FIA on separate days.

Animals received 100  $\mu$ g of ABA-Tyr in FIA on day 0. Each group of animals received 100  $\mu$ g of LPS in saline suspension on -2, 0, 2 and 4 day of ABA-Tyr injection. Skin test was carried out with 10  $\mu$ g of ABA-BSA on day 14 of ABA-Tyr injection. Open columns represent number of animals tested and hatched columns number of animals with positive reaction. A solid line represents mean diameters of erythema and vertical bar standard errors of the mean.

phocytes ABA-Tyr and LPS interact with the cells independently. It is possible that ABA-Tyr interacted with the lymphocytes first and then LPS interacted.

It is known that ABA-Tyr inoculated in FIA without any adjuvant substances leads guinea pigs unresponsive to further sensitization with ABA-Tyr in FCA (2). The results presented in Figure 2 may be explained by a possible function of suppressor cells. Injection of ABA-Tyr in FIA lead guinea pigs tolerant to ABA-groups. In these animals suppressor cells were generated but some lymphocytes were on the way of stimulation, the latter received the antigen signal but not signals from LPS. Two weeks later, when LPS was injected, the partially stimulated lymphocytes differentiated to effector cells for delayed hypersensitivity. However, in the mean time, suppressor cells expanded and the animal became insensitive to ABA-BSA.

Almost identical result was obtained when 1 mg of BCG cell walls was injected 2 weeks after the ABA-Tyr injection (Figure 3). Delayed hypersensitivity against PPD reached the maximum on the day 7 of BCG injection and not changed thereafter. However, delayed hypersensitivity against ABA-BSA reached the peak on the 3rd day of BCG injection and then disappeared quite rapidly.

To test whether LPS exert its adjuvanticity in an aqueous form or not an additional experiment was undertaken. On day 0, 100  $\mu$ g of ABA-Tyr in FIA was injected in two hind footpads and on days -2, 0, 2 and 4, 100  $\mu$ g of LPS in saline suspension was injected in the same sites. Two weeks after the ABA-Tyr injection skin test with ABA-BSA was carried out. One third of the animal which received ABA-Tyr and LPS on the same day showed weak positive reactions and the rest of the animal in the same group and all animals in other groups were negative (Figure 4).

## DISCUSSION

The results of the present investigation demonstrate that LPS is an excellent adjuvant for ABA-Tyr sensitization in guinea pigs. Other mitogens did not exert themselves as adjuvant or, if they did, their activity was rather weak. Adjuvant activity of LPS was comparable to tubercle bacilli and the range of the effective doses was wide (8). The association of ABA-Tyr with adjuvant substances was not essential for the sensitization since separate administrations of ABA-Tyr and LPS or BCG cell walls were effective in sensitizing guinea pigs with ABA-Tyr. Furthermore, 4 out of 5 guinea pigs which received LPS inoculated in FIA 5 days after the ABA-Tyr exhibited delayed reaction against ABA-BSA. However, when LPS in FIA was injected 2 weeks after the ABA-Tyr injection, guinea pigs were not sensitized with ABA-Tyr, or if they were sensitized, the hypersensitivity was transient. Similar but a little more exaggerated result was obtained when BCG cell walls in an oil-in-water suspension were injected 2 weeks after ABA-Tyr in FIA. Three out of 5 animals which were tested on the day 3 of BCG injection responded to ABA-BSA but no animal in another group which was tested on the day 14 of BCG injection responded.

Mechanisms of adjuvant action of LPS has remained open. LPS is a well known B lymphocyte mitogen (9-11) but increasing body of evidences suggested that LPS acted as adjuvant by potentiating T lymphocyte functions both in vitro and in vivo (2, 12-15). The results of the present investigation are consistent with the latter view. However, physical form of LPS administered was found to be important for the adjuvant activity of LPS. Thus LPS emulsified in FIA was a strong adjuvant but LPS suspended in saline was not a strong adjuvant. LPS precipitated with alum was also a good adjuvant for ABA-Tyr (M. K., unpublished). Histological examination of regional lymph nodes revealed that LPS in saline suspension induced massive germinal centers in the cortex but the lymph nodes of LPS-FIA injection were characterized by wide-spread granulomatous changes

accompanied by nuclear debris of polymorphonuclear leukocytes and appearance of mature epithelioid cells.<sup>D</sup>

There may be raised a question, then, if LPS used in the present study contained two factors, one was mitogen for B lymphocyte and the other adjuvant for T lymphocytes, or not. Lipid A of LPS is the candidate for B lymphocyte mitogen (15) and peptidoglycans from cell walls of bacteria are the adjuvant-active moiety (16, 17). Recently, we observed that LPS from *S. minnesota R 595* and lipid A portion of LPS from *S. typhimurium* had adjuvant activity in the same experimental system as the present paper.<sup>D</sup> Thus, adjuvant activity for T lymphocyte functions is ascribed to the property of lipid A of LPS.

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