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Adjuvant effect of lipopolysaccharides from *Salmonella typhimurium*

II. Induction of hapten-specific helper function for anti-DNP antibody formation

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Abstract Immunization with azobenzenearsonate-N-acetyl-L-tyrosine (ABA-Tyr)* and lipopolysaccharide from *S. typhimurium* (LPS) of guinea pigs induced ABA-specific effector functions such as delayed hypersensitivity and helper activity. As these two functions correlated each other in this particular experimental condition, it was suggested that cells responsible for each function had similar receptors for ABA-groups.

ABA-carrier proteins and ABA-Tyr were compared for their abilities to induce hapten-specific effector functions. ABA-carrier proteins induced carrier-dependent delayed hypersensitivity but not hapten-specific, nevertheless they induced moderate hapten-specific helper activity for anti-DNP antibody response.

Immunogenicity of azobenzenearsonate-N-acetyl-L-tyrosine (ABA-Tyr) in guinea pigs is dependent on the additional administration of adjuvant substances such as whole tubercle bacilli, cell walls from bacteria, LPS and peptidoglycans[1~4]. However, direct association of these adjuvant materials with ABA-Tyr is not required since LPS in FIA injected 5 days after the injection of ABA-Tyr in FIA successfully sensitized guinea pigs[4]. ABA-Tyr is a low molecular weight antigen which induces delayed hypersensitivity but does not induce appreciable amount of antibody against ABA-groups in the guinea pig. Recently, Alkan et al[5,6] and Hanna et al[7] reported that ABA-specific helper activity for antibody formation was inducible by the injection of ABA-Tyr in FCA. The helper function for the antibody formation and the delayed hypersensitivity have been regarded as different effector functions of T lymphocytes but these two function are evidently governed by genes located in the same region[8]. We compared ABA-specific helper activity and ABA-specific delayed hepersensitivity in guinea pigs immunized with various doses of LPS and ABA-Tyr in FIA. The results indicated that the two functions correlated. In contrast, when ABA-proteins were used as immunogens hapten-carrier-specific delayed hypersensitivity was induced, nevertheless, hapten-specific helper activity was demonstrable.

MATERIALS AND METHODS

Animals. Animals used in the present study was Hartley guinea pigs of both sexes weighing 300 to 500 g obtained from two breeders near Tokyo. In each experiment either male or female animals from one breeder were used.

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* Abbreviations used in this paper: ABA-Tyr; azobenzenearsonate-N-acetyl-L-tyrosine, LPS-Difco; lipopolysaccharide from *Salmonella typhimurium* (Difco), LPS-R 595; lipopolysaccharide from *Salmonella minnesota Re 595*, T-; thymus derived, FCA; Freund's complete adjuvant, FIA; Freund's incomplete adjuvant, GPA; guinea pig serum albumin, OVA; ovalbumin, BSA; bovine serum albumin, HGG; human IgG, DNP; dinitropehnyl.

Antigens. ABA-Tyr was prepared as described previously[4]. Guinea pig albumin (GPA) was prepared from guinea pig serum by precipitation with trichloroacetic acid and extraction with 80% ethanol as described by Schwert[9]. Resulted crude GPA was further purified by chromatography on a phosphocellulose column eluting with a 0.02 M sodium acetate buffer, pH 5.5. The final product gave a single precipitin arc on immunoelectrophoresis against a rabbit anti-guinea pig serum. Human IgG (HGG) was prepared by ammonium sulfate precipitation followed by DEAE-cellulose chromatography[10]. ABA_{17}-GPA and ABA_8-HGG were prepared by the method of Tabachink and Sobotka[11]. DNP_5-OVA, DNP_4-ABA_{30}-GPA and DNP_6-ABA_8-HGG were prepared by dinitrophenylation of OVA, ABA_{30}-GPA and ABA_8-HGG, respectively. Moles of ABA-groups attached to a mole of proteins were calculated based on the formula described by Tabachnik and Sobotka[11] and expressed as the sum of ABA-Tyr and ABA-histidine residues. Other antigens used were described previously[4].

Immunization protocol. To estimate the delayed hypersensitivity and the helper activity in the same individual animal the protocol of Katz and Benacerraf[12] was used throughout with slight modifications. In a typical experiment guinea pigs received 3 mg DNP-OVA in a saline solution intraperitoneally and on the day 7 they received ABA-derivatives in FIA with or without adjuvant materials in their hind foot pads. On the day 21 animals were bled by cardiac puncture and skin tested with 10 μg of ABA-BSA. On the next day, after reading delayed skin reaction, a total of 1 mg of DNP-ABA-GPA was distributed into skin and peritoneal cavity. On the day 29 animals were bled. The ABA-specific helper activity for anti-DNP antibody response was estimated by comparing anti-DNP titers of sera bled on day 29 to those on day 21.

Determination of anti-DNP antibody titer. Rabbit anti-SRBC antiserum was produced in 5 rabbits by immunization with sheep red bood cells[13]. IgG fraction of the antiserum was purified and digested with papain[14]. Resulting Fab fragment was dinitrophenylated by the method of Eisen[14]. SRBC which were attached with DNP-Fab was used as the antigen for hemagglutination test. Titration of antibody was carried out in microtiter plates (Cooke Co.).

RESULTS

Table 1 shows the effect of various adjuvant in priming ABA-specific helper activity on anti-DNP antibody response. LPS in FIA and BCG cell walls in an oil-in-water suspension, both in the absence of ABA-Tyr, did not induce ABA-specific helper activity nor ABA-specific delayed

Table 1. ABA-specific helper activity and ABA-specific delayed hypersensitivity in guinea pigs immunized with ABA-Tyr and various adjuvants

	No of animal	ABA-priming	Immediate React		Delayed React.		Anti-DNP titer ($2^n \times 10$)		
			ABA-GPA	ABA-BSA	ABA-GPA	ABA-BSA	Day 21	Day 29	Difference
Group 1	3	LPS in FIA	NT[1]	—	NT	—	2.0±0.6	2.0±0	0
Group 2	4	LPS, ABA-Tyr in FIA	NT	—	NT	13.1	2.3±0.6	5.5±0.3	3.3±0.5
Group 3	3	BCG Cw, o/w	NT	—	NT	—	3.0±0.6	2.3±0.3	-0.7±0.3
Group 4	4	BCG Cw, o/w ABA-Tyr in FIA	NT	—	NT	11.3	2.3±0.3	4.8±0.5	2.5±0.5
Group 5	4	LPS, o/w ABA-Tyr in FIA	NT	—	NT	—	1.5±0.6	2.0±0.6	0.5

1) NT: Not tested.

hypersensitivity. However, if ABA-Tyr and LPS were inoculated in the same FIA emulsion, or ABA-Tyr in FIA and BCG cell walls in an oil-in-water emulsion were administered, guinea pigs developed the helper function, thus mounted anti-DNP antibody titer on secondary challenge with DNP-ABA-GPA (groups 2 and 4). Oil-associated LPS did not work well (group 5). The latter finding suggests that either LPS did not associate with oil or oil-associated LPS does not exert its adjuvanticity. In other experiments fifty μg of dextran sulfate and 50 μl of PWM were tested for the adjuvant activity in combination with ABA-Tyr but they did not enhance anti-DNP titer. These results clearly indicate that generation of ABA-specific helper activity was accompanied by the manifestation of delayed hypersensitivity against ABA-groups.

Table 2 compares the immunogenicity of ABA-proteins to ABA-Tyr. ABA-Tyr did not induce immediate hypersensitivity against ABA-BSA or ABA-GPA, thus the delayed hypersensitivity was regarded as hapten-specific (group 1). ABA-GPA, as immunogen, induced hapten-specific immediate hypersensitivity 14 days after the immunization irrespective of the use of LPS (groups 3 and 4). The hapten specificity of the immediate reaction was deduced from the similar intensities of

Table 2. ABA-specific helper activity and ABA-specific delayed hypersensitivity in guinea pigs immunized with ABA-derivatives in the presence or absence of LPS

	No of animal	ABA-priming	Immediate React.		Delayed React.		Anti-DNP titer ($2^n \times 10$)[2]		
			ABA-GPA	ABA-BSA	ABA-GPA	ABA-BSA	Day 21	Day 29	Difference
Group 1	4	ABA-Tyr, LPS in FIA	—	—	12.9±0.8	12.6±0.7	1.3±0.3	5.0±0.8	3.8±0.6
Group 2	3	ABA-Tyr in FIA	—	—	—	—	1.7±0.9	2.7±0.3	1.0±0.6
Group 3	4	ABA-GPA, LPS in FIA	15.1±1.4	13.3±1.2	17.8±3.2	?[1]	1.5±0.3	6.5±0.6	5.0±0.7
Group 4	4	ABA-GPA in FIA	14.3±0.7	13.8±0.6	?	?	1.8±0.3	4.0±0.4	2.3±0.6
Group 5	4	ABA-BSA, LPS in FIA	17.1±1.5	15.9±1.5		20.6±1.5	2.3±0.3	4.3±0.6	2.0±0.4
Group 6	4	ABA-BSA in FIA	16.3±0.6	18.3±1.1	?	?	1.5±0.3	3.0±0	1.5±0.2

1) Delayed hypersensitivity could not be estimated because edematous reaction existed at 24 hr. However, neither erythema nor induration was detected, so that we took these reactions as negative.
2) Challenged with ABA-DNP-GPA.

Table 3. ABA-specific helper activity and ABA-specific delayed hypersensitivity in guinea gips immunized with LPS and various ABA-derivatives

	No of animal	ABA-priming	Immediate React.		Delayed React.		Anti-DNP titer ($2^n \times 10$)[2]		
			ABA-GPA	ABA-BSA	ABA-GPA	ABA-BSA	Day 21	Day 29	Difference
Group 1	4	LPS, ABA-Tyr in FIA	—	—	14.3±0.8	13.1±0.3	3.0±0.4	9.3±0.5	6.3±0.6
Group 2	4	LPS, ABA-GPA in FIA	18.3±1.2	17.8±0.3	21.6±0.9	?[1]	2.3±0.9	5.3±0.6	3.0±1.4
Group 3	3	LPS, ABA-BSA in FIA	18.7±0.2	17.8±0.4	?[1]	20.2±2.5	2.7±0.9	4.3±0.7	1.7±1.2

1) Delayed hypersensitivity could not be estimated because edematous reaction existed at 24 hr. However, neither erythema nor induration was detected, so that we took these reactions as negative.
2) Challenged with ABA-DNP-HGG

erythemas against ABA-BSA and ABA-GPA. However, delayed hypersensitivity in these groups of animals were clearly dependent on the inoculation of LPS in the immunogen and the observed reaction was not hapten-specific but hapten-carrier-specific. ABA-GPA plus LPS induced a marked helper activity but ABA-GPA without LPS did very weak helper activity (compare groups 3 and 4). On the other hand, although ABA-BSA plus LPS was an effective immunogen for the induction of hapten-specific immediate hypersensitivity and hapten-carrier-specific delayed hypersensitivity, the generation of hapten-specific delayed hypersensitivity and the generation of hapten-sprcific helper function were rather weak.

Table 4 A. ABA-specific delayed hypersensitivity in guinea pigs immunized with various doses of ABA-Tyr and LPS in FIA

Dose of ABA-Tyr (μg)	Dose of LPS (μg)			
	1000	100	10	1
1000	NT	8.0	8.3	4.5
100	7.5	11.6	11.0	5.5
10	5.3	12.7	10.0	NT

NT: Not tested.

Table 4 B. ABA-specific helper activtiy for anti-DNP antibody response in guinea pigs immunized with various doses of ABA-Tyr and LPS in FIA

Doses of ABA-Tyr (μg)	Dose of LPS (μg)			
	1000	100	10	1
1000	NT	1.0	0	−2.0
100	1.5	2.0	1.75	0
10	0	2.0	−0.33	NT

NT: Not tested.

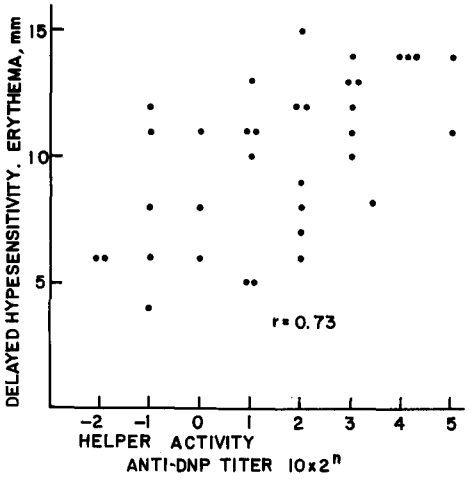

Fig. 1. Correlation of ABA-specific delayed hypersensitivity and ABA-specific helper activity in individual guinea pigs which were immunized with ABA-Tyr and LPS in incomplete Freund's adjuvant.

A question may be raised that the observed helper activity in animals which were immunized with ABA-GPA might not be hapten-specific but hapten-carrier-specific because the immunogen, ABA-GPA, possessed additional antigenic determinants which were generated by the coupling of ABA-groups. To comfirm the hapten specificity of the helper activity in these groups of animals ABA-DNP-HGG was used as a challenging antigen (Table 3). Helper activity was the highest when animals were primed with ABA-Tyr and LPS in FIA, however, ABA-GPA worked more efficiently than ABA-BSA in the induction of ABA-specific helper activity.

Next, dose dependencies of LPS and ABA-Tyr on the generation of ABA-specific helper activity and ABA-specific delayed hypersensitivity were compared. Logarithmically increased doses of LPS from 1 to 1000 μg and ABA-Tyr from 10 to 1000 μg were inoculated in FIA and administered in two hind footpads. Each group consisted of 4 animal s at the onset of the experiment but as the dose of LPS increased animals died of the toxicity of LPS so that the data presented are the average of more than two individuals in each group. In Table 4 ABA-specific delayed hypersensitivity is shown. As can be seen in the table, 10 or 100 μg of both LPS and ABA-Tyr was efficient for the induction of the delayed hypersensitivity, and a dose higher than this range was rather inactive. Similar dose dependencies for both ABA-Tyr and LPS were observed in the generation of ABA-specific helper function (Table 4 B).

Figure 1 summarizes the correlation between delayed hypersensitivity against ABA-BSA and helper activity for anti-DNP antibody response in individual guinea pigs which were immunized with ABA-Tyr and LPS inoculated in FIA 2 weeks before. The correlation efficient r was calculated as 0.73 showing that ABA-specific delayed hypersensitivity and ABA-specific helper activity correlated with a confidence limit of more than 99%.

DISCUSSION

The results presented here demonstrate that hapten-specific delayed hypersensitivity and hapten-specific helper function correlated when guinea pigs were immunized ABA-Tyr and LPS inoculated in the same FIA emulsion. These results would suggest that effector cells responsible for these two functions belong to the same subpopulation of lymphocytes, or possibly, although they belong to different subpopulations, the affinity of the effector cells would be the same.

Contrasting results were obtained when guinea pigs were immunized with ABA-proteins. Generation of hapten-specific helper function was not LPS dependent, although LPS potentiated the activity. The observed delayed hypersensitivity was not hapten-specific but hapten-carrier-specific, and the delayed hypersensitivity was LPS dependent when tested 2 weeks after the immunization.

The technique for the detection of delayed hypersensitivity in the present study may not be adequate because hapten-specific immediate hypersensitivity obscured the delayed skin reaction. In this regard, the estimation of helper function was clearly unequvocal for the detection of hapten specificity. When challenged with ABA-DNP-GPA, ABA-GPA generated the maximum helper function, whereas, when challenged with ABA-DNP-HGG, ABA-Tyr was more effective than ABA-GPA in the generation of the helper function (compare Tables 2 and 3). These results would suggest that ABA-GPA generated not only ABA-specific helper cells but also ABA-GPA specific helper cells.

These results could be related to the heterogeneity of antigenic determinants. ABA-Tyr is an univalent antigen which is capable of triggering lymphocytes rather uniformly in the presence of LPS or FCA. On the other hand, ABA-proteins may contain heterogeneous antigenic determinats and the lymphocyte receptor is clealy heterogeneous, then, immune responses to the latter antigens may be modified by various factors such as antibody[18] and suppressor cells[2].

Finally, it should be stressed that LPS potentiates T cell functions in the guinea pigs.

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