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Characterization of Two Different Antibody Specificities Recognizing Distinct Antigenic Determinants in Free Lipid A of *Escherichia coli*

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Antisera were raised in rabbits with acid-treated Re mutant bacteria from *Salmonella minnesota* and *Escherichia coli* and tested in a passive hemolysis assay with di- and monophosphorylated free lipid A of *E. coli* (LipA-Ac and LipA-HCl, respectively) coated onto sheep erythrocytes. Depending on the acid used to prepare the immunogen (acetic versus hydrochloric acid), different antibody specificities were obtained. Antiserum prepared against HCl-treated bacteria was found to react with both antigens to the same extent (i) in the passive hemolysis test, (ii) in the passive hemolysis inhibition test, and (iii) in absorption experiments, suggesting that antibodies in this antiserum recognize an antigenic determinant equally present in LipA-Ac and LipA-HCl. Antiserum raised against acetic acid-treated bacteria reacted with the homologous antigen (LipA-Ac) in the passive hemolysis and passive hemolysis inhibition test as well as in absorption experiments. However, the antiserum failed to react with the heterologous antigen (LipA-HCl) in the hemolysis test and during absorption, whereas in inhibition studies interaction of this antiserum with both antigens was observed. The inhibiting capacity of LipA-Ac was lower compared with that of LipA-HCl, indicating that the antigenic determinant of LipA-Ac is partly expressed by LipA-HCl in solution, but not when fixed on the surface of sheep erythrocytes. The role of glycosidically linked phosphate in lipid A is discussed with respect to antigenicity.

Lipopolysaccharides (LPS) are integral constituents of the cell wall of gram-negative bacteria. Because of their numerous biological effects in higher organisms such as fever, induction of the Shwartzman phenomenon, and hemodynamic changes leading to shock and death, LPS are also termed endotoxins (18, 20, 25, 31). LPS are amphipathic, highly aggregated molecules consisting of a heteropolysaccharide (O-specific chain and the core oligosaccharide) covalently linked to a lipid portion (lipid A), the latter being responsible for most of the endotoxic activities of LPS (20, 32).

The deleterious effects of endotoxins during gram-negative infections prompted investigations on the antigenicity of lipid A to induce antibodies against the toxic principle that could confer protection against endotoxicity of LPS. Since all lipid A preparations derived from various gram-negative bacteria are all made up by the same architectural principle [a diphosphorylated D-glucosamine disaccharide with ester- and amide-linked (*R*)-3-hydroxy fatty acids (32)], it was expected that such antibodies would protect against a broad spectrum of gram-negative bacteria.

In the early seventies, Galanos et al. discovered the antigenicity of lipid A (11). The main antigenic and immunogenic properties of lipid A were characterized by this group. They found that the lipid A antigen is present in a cryptic form in LPS and that removal of the polysaccharide moiety is a prerequisite for the exposure of the lipid A antigenic determinant. The same held true for the immunogenicity of lipid A, which was best effected by treating rough mutant bacteria with dilute acid, leading to the exposure of immunogenic lipid A on the bacterial cell surface.

In the same report (11), the authors showed that all lipid A preparations with the same architectural principle mentioned above exhibited serological cross-reactivity and, moreover, that all lipid A antibodies seemed to recognize the same antigenic determinant which was later shown by several groups to require the presence of a glucosamine disaccharide with at least one amide-linked 3-hydroxy fatty acid (6, 7, 12, 14, 21).

The possible biological relevance of antibodies to lipid A was studied by many groups with respect to protection against gram-negative infection, endotoxicity of LPS, or both. Lipid A antibodies were thus shown to opsonize *Escherichia coli* bacteria for subsequent intraperitoneal phagocytosis in mice (11) and to protect mice against the experimental infection with *Salmonella typhimurium* (6). In rabbits, lipid A antibodies were found under certain experimental conditions to suppress the pyrogenic activity of LPS and lipid A and to inhibit the local Shwartzman reaction (30). In addition, antibodies to lipid A were protective against the abortive effects of endotoxin (33). However, there also exist reports showing no protective effect of these antibodies (3, 24, 26).

Although lipid A antibodies have been studied for more than a decade, the field is still relatively new compared with that of other extensively investigated regions of LPS, e.g., the O-specific chain and the core oligosaccharide (19). In the present paper, we will extend the knowledge on the antigenicity and immunogenicity of lipid A by describing two different antibody specificities which recognize distinct antigenic determinants in the lipid A molecule.

MATERIALS AND METHODS

Bacterial LPS and lipid A. LPS was obtained from an *E. coli* Re mutant (strain F515) by the phenol-chloroform-petroleum ether method (10), purified, and converted to the uniform triethylammonium salt as reported earlier (9). Free lipid A was obtained after hydrolysis (100°C for 1 h) of LPS in 0.1 M acetate buffer (pH 4.4), and monophosphorylated lipid A was prepared by hydrolysis in 0.1 M HCl at 100°C for

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TABLE 1. Hemolytic antibody titers in rabbits after immunization with acid-treated Re mutant bacteria of *E. coli* and *S. minnesota*

Animal no.	Immunizing bacterial strain	Acid treatment[a]	Hemolytic antibody titer against SRBC coated with:[b]	
			E. coli LipA-Ac	*E.coli* LipA-HCl
1	*S. minnesota*	HAc	2,048	<4
2	*S. minnesota*	HAc	1,024	<4
3	*S. minnesota*	HAc	512	<4
4	*S. minnesota*	HAc	512	<4
5	*E. coli*	HAc	256	<4
6	*E. coli*	HAc	1,024	<4
7	*E. coli*	HCl	512	512
8	*E. coli*	HCl	256	128
9	*S. minnesota*	HCl	256	128
10	*S. minnesota*	HCl	1,024	512

[a] Bacteria were treated with either 1% acetic acid (HAc) or 0.1 M HCl at 100°C for 1 h.

[b] SRBC (200 μl of packed cells) were sensitized with 80 μg of the respective antigen.

1 h. Both preparations were solubilized by the addition of triethylamine; they will be referred to as LipA-Ac and LipA-HCl, respectively.

Anti-lipid A antisera. Polyclonal antibodies against lipid A were raised in rabbits (New Zealand White) by immunization with Re mutant bacteria of *E. coli* (strain F515) or *Salmonella minnesota* (strain R595). Bacteria were treated with 1% acetic acid or 0.1 M HCl (100°C for 1 h), washed with and suspended in distilled water, and lyophilized. For immunization, acid-treated bacteria were suspended in distilled water at a concentration of 1 mg/ml; the schedule was as previously described (11). Antisera were stored at −20°C after absorption with sheep erythrocytes (SRBC) and labeled anti-LipA-Ac and anti-LipA-HCl, respectively.

Passive hemolysis and passive hemolysis inhibition tests. Antibodies and antigens were titrated by the passive hemolysis and passive hemolysis inhibition test, respectively, in microtiter plates as previously described (11). Quantitative inhibition studies were performed in the following way. Serial dilutions of antigen (100 μl) were incubated with appropriately diluted antiserum (100 μl) in plastic tubes in a water bath (37°C for 30 min). Antigen-coated SRBC (1 ml) and guinea pig complement (25 μl) were added, and after mixing, incubation was carried out for 1 h at 37°C. After cooling in an ice bath, the tubes were centrifuged, and the supernatants were read in a spectrophotometer at 546 nm against an appropriate blank. The test erythrocytes were adjusted to give an absorption value of A_{546} = 1.25 after complete lysis. The antiserum was diluted to give 80% of lysis (A_{546} = 1.0) without the addition of antigen. This value was set at 100%, and inhibition is expressed as a percentage of this value.

Absorption experiments. Antiserum (500 μl; 1:2 dilution) was incubated (37°C for 30 min) with glutardialdehyde-fixed SRBC (25 μl) coated with the respective antigen. After centrifugation, the supernatant was tested by the passive hemolysis test.

Hydrolysis kinetics. Samples of LipA-Ac (500 μg) were hydrolyzed (100°C) in 0.1 M HCl (100 μl) for various lengths of time. The precipitated lipid A was removed by centrifugation, washed twice with distilled water, resolved in water (500 μl) by the addition of triethylamine, and tested by the passive hemolysis inhibition test. The supernatant was used to determine the amount of inorganic phosphate.

Chemical analysis. LipA-Ac and LipA-HCl were analyzed for phosphate (17), D-glucosamine (36), 2-keto-3-deoxyoctonic acid (2), and fatty acids (37). In the case of LipA-HCl, D-glucosamine was also determined after reduction with sodium borohydride ($NaBH_4$) at 56°C overnight (13).

RESULTS

Chemical analysis. Chemical analysis performed on LipA-Ac and LipA-HCl from *E. coli* Re mutant LPS revealed the presence of D-glucosamine, phosphate, and fatty acids in a molar ratio of 2:2:5.4 and 2:1:5.2, respectively. The determination of D-glucosamine, after reduction with $NaBH_4$ at 56°C, indicated that no D-glucosamine could be reduced in LipA-Ac, whereas 50% of the total D-glucosamine was reduced in LipA-HCl. An estimation of 2-keto-3-deoxyoctonic acid gave values of less than 10 nmol of LipA-Ac or LipA-HCl per mg.

Hemolytic activity of rabbit antisera prepared by immunization with acid-treated bacteria. Anti-lipid A antisera were prepared in rabbits by immunization with Re mutant bacteria of *E. coli* and *S. minnesota* which had been treated with acetic or hydrochloric acid. The hemolytic titers of these antisera were tested by the passive hemolysis test with LipA-Ac and LipA-HCl, respectively, as a sensitizing antigen for SRBC. The results are shown in Table 1.

Antisera raised against acetic acid-treated bacteria reacted exclusively with the homologous antigen (LipA-Ac) and not with the heterologous antigen (LipA-HCl). The results were similar with both immunizing bacterial strains, the hemolytic titers ranging from 256 to 2,048. In contrast, antisera elicited with HCl-treated bacteria reacted with both antigens to the same extent with titers of 128 to 1,024, and again comparable titers were obtained with both bacterial strains.

Hemolytic activity of anti-LipA-Ac and anti-LipA-HCl with SRBC coated with various amounts of antigen. To see how the different hemolytic activities of these antisera were depending on the amount of antigen used for the sensitization of SRBC, both types of antisera (anti-LipA-Ac and anti-LipA-HCl) were titrated against SRBC which had been coated with various amounts of antigen.

All anti-LipA-Ac and anti-LipA-HCl antisera behaved in a

TABLE 2. Hemolytic antibody titers of two differently prepared anti-lipid A antisera (anti-LipA-Ac and anti-LipA-HCl) obtained with SRBC coated with various amounts of LipA-Ac or LipA-HCl from *E. coli*

Sensitizing antigen	Amt of antigen (μg)/200 μl of SRBC	Hemolytic antibody titer obtained with:	
		anti-LipA-Ac[a]	anti-LipA-HCl[a]
E. coli LipA-Ac	4	<4	<4
	8	128	256
	40	2,048	1,024
	80	2,048	1,024
	200	2,048	1,024
	400	2,048	1,024
E. coli LipA-HCl	4	<4	<4
	8	<4	64
	40	<4	256
	80	<4	512
	200	128	512
	400	256	1,024

[a] Anti-LipA-Ac and anti-LipA-HCl are antisera from animals no. 1 and 10, respectively, in Table 1.

similar way; thus, the results (Table 2) are representative for the other sera not shown. Anti-LipA-Ac (animal no. 1 in Table 1) gave maximal titers (2,048) with LipA-Ac-coated SRBC with a sensitizing dose of 40 μg of antigen per 0.2 ml of SRBC or higher. When the same antiserum was reacted with LipA-HCl-coated SRBC, no hemolytic activity was observed up to 80 μg/0.2 ml of SRBC. With 200 and 400 μg, very low titers of 128 and 256, respectively, were obtained. Amounts higher than 400 μg could not be tested, since nonspecific lysis occurred.

The anti-LipA-HCl antiserum (animal no. 10 in Table 1) gave the same titer of 1,024 with LipA-Ac-coated SRBC at a concentration of 40 to 400 μg of antigen per 0.2 ml of SRBC. This antiserum reacted with LipA-HCl-coated SRBC already at 8 μg/0.2 ml with a titer of 64, which increased to 256 at 40 μg/0.2 ml and to 512 with 80 and 200 μg/0.2 ml. Again, with more than 400 μg of antigen, nonspecific lysis took place. Thus, higher amounts were not tested. For further experiments, 80 μg/0.2 ml of SRBC was used as the sensitizing dose.

Absorption experiments. The hemolytic activity of anti-LipA-Ac and anti-LipA-HCl antiserum (animals no. 1 and 10 respectively, in Table 1) was tested before and after absorption with the homologous and the heterologous antigen coated onto glutardialdehyde-fixed SRBC. The absorbed antisera were tested with LipA-Ac- and LipA-HCl-coated SRBC, and the titers were compared to those of the nonabsorbed sera. The results are shown in Table 3.

The hemolytic activity of anti-LipA-Ac antiserum against LipA-Ac-coated SRBC (2,048) could effectively be absorbed with the homologous antigen (LipA-Ac) but not with the heterologous preparation (LipA-HCl). Since this antiserum did not react with LipA-HCl-coated SRBC at the concentration used for sensitization (80 μg of antigen per 0.2 ml of SRBC), it was tested also with SRBC which had been coated with higher amounts of antigen (200 μg/0.2 ml), resulting in a hemolytic titer of 128 (see Table 2). This hemolytic activity could be absorbed with both the homologous and heterologous antigens (data given in parentheses in Table 3). The activity of anti-LipA-HCl antiserum against both antigens could be completely abolished by absorption with both the homologous and heterologous antigens.

TABLE 3. Hemolytic activities of anti-LipA-Ac and anti-LipA-HCl antisera after absorption with LipA-Ac and LipA-HCl

Antiserum[a]	Hemolytic titer against SRBC coated with:[b]	
	LipA-Ac	LipA-HCl
Anti-LipA-Ac		
Before absorption	2,048	<4 (128)[c]
Absorbed with LipA-Ac[d]	<4	ND[e] (<4)
Absorbed with LipA-HCl	1,024	ND (<4)
Anti-LipA-HCl		
Before absorption	1,024	1,024
Absorbed with LipA-Ac	<4	<4
Absorbed with LipA-HCl	<4	<4

[a] Anti-LipA-Ac and anti-LipA-HCl were from animals no. 1 and 10, respectively, in Table 1.

[b] SRBC were coated with 80 μg of antigen per 0.2 ml of packed cells.

[c] Titers obtained when SRBC were coated with 200 μg of antigen per 0.2 ml of packed cells.

[d] Absorption was carried out with glutardialdehyde-fixed SRBC coated with the respective antigen (80 μg per 0.2 ml) of packed cells. Antiserum (500 μl; 1:2 dilution) was incubated at 37°C for 30 min with antigen-coated SRBC (25 μl).

[e] ND, Not determined.

TABLE 4. Passive hemolysis inhibition tests of LipA-Ac and LipA-HCl of *E. coli* in the hemolytic antigen-antibody system of LipA-Ac–anti-LipA-Ac and LipA-HCl–anti-LipA-HCl

Inhibitor	Inhibition value (ng) obtained in the hemolytic system of:[a]	
	LipA-Ac–anti-LipA-Ac	LipA-HCl–anti-LipA-HCl
LipA-Ac	8	8
LipA-HCl	63	8

[a] Three hemolytic units of antibody were used.

Inhibition experiments. The different antigenic properties of LipA-Ac and LipA-HCl and the different antibody specificities were investigated by the passive hemolysis inhibition test. Two different antigen-antibody test systems were employed, LipA-Ac–anti-LipA-Ac and LipA-HCl–anti-LipA-HCl. The results of the microtiter assay are shown in Table 4; the hemolytic system of LipA-HCl–anti-LipA-HCl is inhibited to the same extent (8 ng) by both antigens, LipA-Ac and LipA-HCl. However, in the antigen-antibody system of LipA-Ac–anti-LipA-Ac, different results were obtained. In this system, the homologous antigen (LipA-Ac) caused 50% inhibition of lysis with 8 ng, and the heterologous antigen (LipA-HCl) yielded the same inhibition with 63 ng. Thus, the homologous test system is inhibited more effectively by a factor of 8.

Since, from a chemical point of view, the difference between these two antigen preparations is restricted to the presence of the glycosidically linked phosphate group, kinetic studies were performed under conditions which are known to release this phosphate group. LipA-Ac was subjected to hydrolysis (0.1 M HCl at 100°C) for various lengths of time. Inorganic phosphate was determined in the supernatant, and the sedimented precipitate was used as an

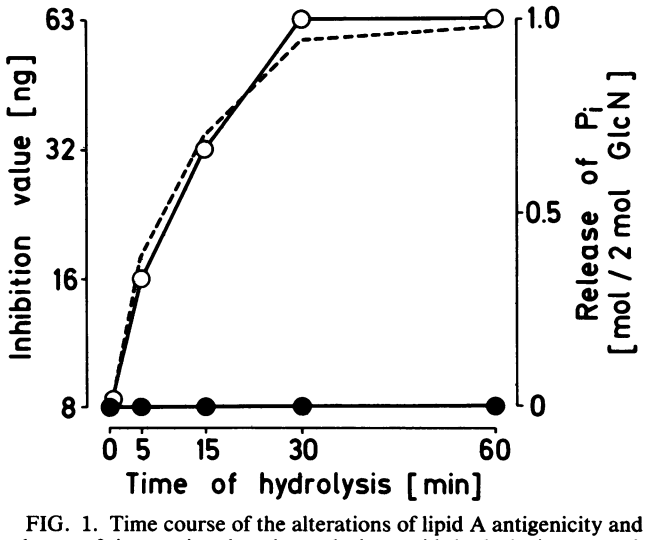

FIG. 1. Time course of the alterations of lipid A antigenicity and release of inorganic phosphate during acid hydrolysis. *E. coli* LipA-Ac was hydrolyzed in 0.1 M HCl at 100°C for the times indicated on the abscissa. The resulting precipitate was assayed by the passive hemolysis inhibition test with the antigen-antibody systems of LipA-Ac–anti-LipA-Ac (○) and LipA-HCl–anti-LipA-HCl (●). Inorganic phosphate (P_i) was determined in the supernatant; the result is expressed as moles of P_i per 2 mol of D-glucosamine (GlcN) (dashed line).

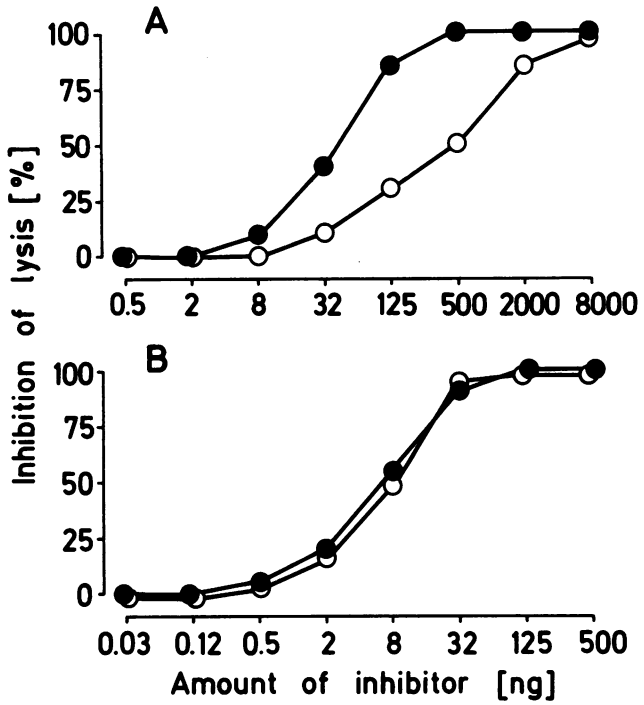

FIG. 2. Quantitative inhibition assay obtained with LipA-Ac (●) and LipA-HCl (○) of *E. coli* in the hemolytic antigen-antibody systems of LipA-Ac–anti-LipA-Ac (A) and LipA-HCl–anti-LipA-HCl (B). The passive hemolysis test was used with an amount of antibody causing 80% of lysis (A_{546} = 1.0). The inhibition of various amounts of antigen (indicated on the abscissa) is expressed as a percentage of this value.

inhibitor for the two antigen-antibody systems. The results are illustrated in Fig. 1. The inhibition values obtained with the hydrolyzed LipA-Ac samples for the hemolytic system of LipA-Ac–anti-LipA-Ac changed considerably during hydrolysis. The starting material exhibited an inhibition value of 8 ng, which increased to 16 ng after 5 min and to 32 ng after 15 min of hydrolysis. A plateau value of 63 ng was reached after 30 min of hydrolysis, which did not change thereafter. When the hemolytic system of LipA-HCl–anti-LipA-HCl was used, the inhibiting capacity of the antigen was not altered by hydrolysis, as indicated by a constant inhibition value of 8 ng over the observed period of time.

The determination of inorganic phosphate (dashed line in Fig. 1) indicated that 1 mol of phosphate per 2 mol of D-glucosamine was released within the first 30 min, and 50% of the total D-glucosamine could be reduced with $NaBH_4$ at that time. Thus, the changes observed in the serological assay are paralleled by the release of glycosidically linked phosphate. Since in the passive hemolysis test, as carried out in microtiter plates, an excess of antibody is used (usually 2 to 3 hemolytic units), further characterization of the two different lipid A antigens was performed by the quantitative passive hemolysis inhibition test, with a limited amount of antibody causing 80% inhibition of lysis of the test erythrocytes without the addition of inhibitor. The inhibition curves obtained with LipA-Ac and LipA-HCl in both hemolytic test systems (LipA-Ac–anti-LipA-Ac and LipA-HCl–anti-LipA-HCl) are shown in Fig. 2A and B, respectively.

In the hemolytic system of LipA-HCl–anti-LipA-HCl (Fig. 2B), a similar inhibition curve was obtained for LipA-Ac and LipA-HCl, whereas in the LipA-Ac–anti-LipA-Ac system a significant difference was observed (Fig. 2A). Whereas LipA-Ac exhibited 50% inhibition with ca. 40 ng, the same amount of LipA-HCl caused only 10% inhibition of lysis, and 50% inhibition was achieved with 500 ng. The shape of the inhibition curve obtained with LipA-HCl was more flattened than that with LipA-Ac.

DISCUSSION

For more than a decade, it has been known that lipid A, the endotoxic principle of bacterial LPS, represents an antigen which is shared by many gram-negative bacteria (11). Until now, all lipid A preparations seemed to exhibit the same antigenic determinant, the minimal structural requirements of which have been postulated as a β1.6-linked D-glucosamine disaccharide with one amide-linked, 3-hydroxy long chain fatty acid (6, 7, 12, 14, 21). In addition, it was proposed that all antisera against lipid A had the same antibody specificity obviously recognizing the aforementioned antigenic determinant (6, 7, 11).

However, we have observed during our studies on the antigenicity of lipid A that the mode of hydrolysis (acetic or hydrochloric acid) used to yield free lipid A from LPS can alter its antigenicity (unpublished observations). Since it is well known that dilute mineral acid cleaves the glycosidically linked phosphate group of lipid A (13), we hypothesized that the observed differences could be related to the presence or absence of this very phosphate group. The results of the present paper support this hypothesis.

In the first step of our study, we immunized rabbits with rough mutant bacteria (Re mutants of *S. minnesota* and *E. coli*) which had been treated with either acetic or hydrochloric acid, expecting the exposure of immunogenic di- and monophosphorylated lipid A, respectively. According to this expectation, we prepared di- and monophosphorylated lipid A (LipA-Ac and LipA-HCl, respectively) as antigens to sensitize SRBC which were used as test erythrocytes in a passive hemolysis assay.

It was found that all antisera raised against acetic acid-treated bacteria (anti-LipA-Ac) reacted exclusively with the homologous antigen (LipA-Ac-coated SRBC) but not with the heterologous preparation (LipA-HCl-coated SRBC), whereas all antisera elicited with HCl-treated bacteria (anti-LipA-HCl) reacted with both antigens (Table 1).

From these results, we made the preliminary conclusions that (i) acetic acid-treated bacteria induce an antibody specificity being directed against a determinant of diphosphorylated lipid A (LipA-Ac) which is not present in monophosphorylated lipid A (LipA-HCl) and (ii) HCl-treated bacteria elicit antibodies which recognize a determinant present in both antigen preparations. This working hypothesis was in accordance with results of absorption experiments, whereby it was demonstrated that the hemolytic activity of anti-LipA-HCl antiserum against both LipA-Ac- and LipA-HCl-coated SRBC could be absorbed completely with LipA-Ac or LipA-HCl. On the other hand, anti-LipA-Ac antiserum could only be absorbed with the homologous antigen (LipA-Ac), and absorption with LipA-HCl had no effect on the hemolytic activity of this antiserum. In the hemolysis test, the latter also reacted with LipA-HCl-coated SRBC when these were sensitized with a higher amount of antigen than usually used (200 instead of 80 μg); however, the titer of 128 was very low compared with that of 2,048 obtained with the homologous antigen. This marginal hemolytic activity did not seem to be due to a cross-reaction of the same antibody specificity but to a second antibody which had a similar specificity as the anti-LipA-HCl antiserum; like the latter, it

could be absorbed with LipA-Ac and LipA-HCl. It is stressed that hemolytic activity with such a low titer did not affect the results of the following inhibition experiments for two reasons. First, in the inhibition tests, SRBC were coated with a low dose of antigen (80 μg of LipA-HCl), with which no hemolytic activity of anti-LipA-Ac was observed; second, the low hemolytic activity of 128 is diluted out at the antiserum dilution used (1:400) to give 3 hemolytic units with LipA-Ac-coated SRBC.

Next, we performed inhibition studies with two different hemolytic antigen-antibody systems consisting of LipA-Ac–anti-LipA-Ac and LipA-HCl–anti-LipA-HCl. The latter was expected to be specifically inhibited by the antigenic determinant present in both lipid A antigens. Actually, LipA-Ac and LipA-HCl were equally active inhibitors, yielding the same inhibition value of 8 ng. This result underlines that one antigenic determinant is expressed by mono- and diphosphorylated lipid A.

When LipA-HCl was tested in both hemolytic systems, an inhibition value of 8 ng was obtained in the homologous test system; however, the heterologous antiserum was also inhibited, although to a lower degree (63 ng). This was unexpected, according to results of the hemolysis test and the absorption experiments (cf. Tables 1 and 3) which indicated that anti-LipA-Ac interacts with an antigenic determinant present exclusively in diphosphorylated LipA-Ac.

Since in the passive hemolysis inhibition test, when carried out in microtiter plates, an excess of antibody is used (usually 2 to 3 hemolytic units), the possibility arises that in complex systems in which more than one antigen-antibody reaction takes place, one antibody is inhibited and the second still causes complete hemolysis. Therefore, we tested the two lipid A antigens also in a quantitative assay in which limiting amounts of antibody (causing 80% of hemolysis) were used. The results were similar to those obtained by the semi-quantitative microtiter assay showing the specific inhibition of LipA-Ac–anti-LipA-Ac by both antigens. In addition, another important observation could be made in this assay. The inhibition curves obtained with LipA-Ac and LipA-HCl in the hemolytic system of LipA-Ac–anti-LipA-Ac did not only differ quantitatively but also qualitatively (Fig. 2A). LipA-HCl as an inhibitor gave a more flattened sigmoid inhibition curve than did LipA-Ac, suggesting a lower affinity of LipA-HCl compared with LipA-Ac for the anti-LipA-Ac antiserum. That this difference was not due to different physicochemical properties of the two antigens (e.g., solubility, aggregation, or conformational changes) was seen in the antigen-antibody system of LipA-HCl–anti-LipA-HCl in which superimposed inhibition curves were obtained (see Fig. 2B). Therefore, the anti-LipA-Ac antiserum seems to recognize an antigenic determinant in LipA-Ac which is also partly expressed by LipA-HCl in solution (inhibition test) but not in SRBC-bound antigen (hemolysis and absorption experiments).

It should be kept in mind that the interaction of antibody with antigen fixed onto the surface of an SRBC (as used in the hemolysis test and for absorption) can be completely different from that with the same antigen in solution, as in the case of the inhibition test. In the fixed antigen, parts of the antigenic determinant could be sterically hindered and thus would not be optimally accessible for the interaction with antibody.

In this context, we refer to the results of Banerji and Alving (1), who demonstrated the role of ester-linked fatty acids for the expression of lipid A antigenicity in a liposome model (15, 16) and who stressed the possible importance of the geometric placement of lipid A for exhibiting antigenic and immunogenic properties. A different geometric placement of lipid A in the bacterial outer membrane as used for immunization, in the membrane of an SRBC as used in this study, on the plastic surface of a microtiter plate in an enzyme-linked immunosorbent assay (5, 22, 23), on the membrane of liposomes (34), or on a nitrocellulose sheet in immunoblotting experiments may lead to the exposure of different antigenic determinants. This would also explain how some authors were able to show the interaction of lipid A antibodies with LPS by the enzyme-linked immunosorbent assay and the immunoblotting technique (4, 26, 27) which we and others never observed by means of the passive hemolysis test.

Despite this, the experiments show that LipA-Ac and LipA-HCl exhibit at least two—perhaps overlapping—antigenic determinants, depending on the presence of glycosidically linked phosphate. To sustain the hypothesis on the role of this phosphate group, kinetic studies were performed under conditions which are known to split off this very phosphate group from LipA-Ac (13). The release of inorganic phosphate was determined, and in parallel, the resulting (partially dephosphorylated) lipid A was subjected to inhibition tests with the above hemolytic antigen-antibody systems. It came out that the inhibition value in the LipA-HCl–anti-LipA-HCl system was not altered by hydrolysis, whereas in the hemolytic system of LipA-Ac–anti-LipA-Ac, the inhibition value increased from 8 to 63 ng within the first 30 min of hydrolysis. This increase was paralleled by the release of 1 mol of inorganic phosphate (calculated on 2 mol of D-glucosamine). Differential determination of D-glucosamine and D-glucosaminitol after reduction of LipA-HCl (after 60 min of hydrolysis) confirmed that the glycosidically linked phosphate group had been removed during hydrolysis.

At present, it cannot be decided how this phosphate group is involved in the antigenic specificity of LipA-Ac and whether phosphate as such is important or could be replaced by another glycosidically linked substituent. The physicochemical properties are changed by the presence or absence of this phosphate group, leading perhaps to a completely different attachment of lipid A to the cell surface of SRBC.

We have noticed that the presence of the glycosidically linked phosphate group in lipid A and synthetic lipid A analogs has also been shown to alter their endotoxic activities (8, 28, 29). However, it is not yet clear whether these different biological activities are due to a change in solubility or to the alteration of a hypothetical toxophore group as previously suggested (28).

Much more experience will be needed to understand the various mechanisms involved in the interaction of antibodies (free or B cell-bound immunoglobulin) with different epitopes of the lipid A molecule. Further insight into these mechanisms is expected from studies on synthetic *E. coli* lipid A and part structures thereof (35), from alternate techniques to quantify antigen-antibody reactions of the lipid A system (such as the enzyme-linked immunosorbent assay technique [5, 23] and the liposome model [1, 15, 16]), and from the production of monoclonal antibodies against defined epitopes of lipid A.

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