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Reaginic Antibody Production to Protein Antigens of *Escherichia coli* and *Pseudomonas aeruginosa* by Mice

PAMELA JONES DANNEMAN* AND J. GABRIEL MICHAEL

Department of Microbiology, School of Medicine, University of Cincinnati, Cincinnati, Ohio 45267

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Water-soluble antigens isolated from acetone-dried, gram-negative bacteria elicited reaginic antibody formation in mice. Antibodies specific for *Escherichia coli* antigens reacted with antigens isolated from several enterobacterial species tested, but not with antigens isolated from *Pseudomonas aeruginosa*. Reaginic antibodies induced by antigens isolated from a *P. aeruginosa* strain reacted with antigens isolated from several *P. aeruginosa* serotypes as well as with a purified protein component of the envelope of *P. aeruginosa*. The anti-*Pseudomonas* reagins did not cross-react with enterobacterial antigens. Antigenicity of the bacterial extracts was destroyed by trypsin treatment and reduced by heating, which suggested that the antigens were protein in nature. Whole bacterial cells adsorbed out reaginic antibodies, indicating that the antigens are located at or near the surface of the bacteria.

Gram-negative bacteria possess numerous antigens. The most widely investigated of these antigens has been the endotoxin or lipopolysaccharide (LPS), due to its role in virulence (30), its pyrogenicity (36), its mitogenicity for lymphocytes (3), and its role in immune protection (25). Other antigens of gram-negative bacteria, such as the small-molecular-weight lipoprotein found in the outer membrane of *Escherichia coli* (6, 14), and antigens of *Neisseria* species (15, 16, 19) have been subjects of many recent investigations. The common enterobacterial antigen, originally described by Kunin et al. (24), has also been of interest lately because the functional significance of such cross-reacting antigens in protection is currently in dispute (10, 13, 20, 28, 29, 32).

We have recently reported that the LPS of gram-negative bacteria failed to induce the production of reaginic antibodies specific for LPS in mice (8), even though LPS functioned as an effective adjuvant in the production of reaginic antibodies to a protein antigen, hen egg albumin. The purpose of the work presented here was to extend the observations concerning the reaginic response of mice to gram-negative bacteria to other, non-LPS, antigens. We identified an antigen(s), apparently protein in nature, common to many gram-negative species which induced the formation of reaginic antibodies in mice. Our work was performed with antigenic preparations isolated from *E. coli* and *Pseudomonas aeruginosa*. A partial characterization of the antigens was also undertaken and is included in this report.

MATERIALS AND METHODS

Bacterial antigens. The following bacterial species were used as sources of antigens in our study. *E. coli* O127, *Klebsiella pneumoniae*, *Enterobacter aerogenes*, and *Proteus mirabilis* were obtained from our own stock cultures; *Salmonella minnesota* strains (SF1111 and SF1167) were obtained from Otto Luderitz, Max Planck Institute, Freiberg, Germany; *P. aeruginosa* PL509 (type VIII) was isolated from a urinary tract infection by Thomas Korfhagen, University of Cincinnati; and *P. aeruginosa* serotypes IV, V, VI, and VII were kindly given to us by Myron Fisher, Parke-Davis & Co., Detroit, Mich. Bacteria were cultured in Trypticase soy broth (Difco Laboratories, Detroit, Mich.) for 18 to 24 h in a shaking 37°C water bath, followed by three washings in distilled water. When whole bacteria were used for immunization, the cultures were heat-killed (70°C, 1 h). When used as a source of antigenic extract, the bacteria were dried with acetone, resuspended in distilled water to approximately a 10% suspension (vol/vol), and incubated at room temperature for 4 h, followed by overnight incubation at 4°C, as described originally by Whiteside and Baker (44). The bacteria were centrifuged (12,000 × *g*), the supernatants were decanted and saved, and the bacterial pellet was once more subjected to aqueous extraction, as above. The two supernatants were combined and lyophilized.

The antigenic extracts of *E. coli* 0127 (termed *E. coli* antigen or EcA) and of *P. aeruginosa* PL509 (*P. aeruginosa* antigen or PaA) were used to immunize mice. The remaining bacterial species served as sources of antigenic extracts to determine antigenic cross-reactivity.

Original endotoxin protein (OEP) of *P. aeruginosa* was provided by J. Y. Homma, University of Tokyo, Tokyo, Japan. The chemical and biological

properties of OEP have been described previously by Abe et al. (1) and Homma and Suzuki (17).

LPS W from *E. coli* O127:B8 was purchased from Difco Laboratories, Detroit, Mich. Pseudogen, a heptavalent LPS preparation from *P. aeruginosa* serotypes I to VII (2), was the gift of M. Fisher, Parke-Davis & Co., Detroit, Mich. *P. aeruginosa* type II (Rx-X41594) and type VII (Rx-X41599) LPS preparations (Parke-Davis & Co.) as well as rabbit antibody to LPS types I to VII (Rx-X41867, Parke-Davis & Co.) were provided by J. D. Stinnett, University of Cincinnati. Solutions of LPS in normal saline were heated for 30 min in a 100°C water bath before use.

Protein determinations of the antigenic extracts were performed according to the method of Lowry et al. (27), using bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) as standard. Endotoxin content, as measured by the *Limulus* amoebocyte lysate assay (39), was determined by George Cole, Parke-Davis & Co., Detroit, Mich.

Nonbacterial reagents. Hen egg albumin, 5× crystallized, was purchased from Miles Laboratories, Kankakee, Ill. Aluminum hydroxide gel was used as an adjuvant and was prepared according to the methods of Levine and Vaz (26).

Immunization of mice. Female, 8- to 12-week-old, CBA/J, A/J, and HRS/J (hairless) mice were obtained from Jackson Laboratory, Bar Harbor, Me. Groups of mice (five mice per group), A/J or CBA/J, were immunized intraperitoneally (i.p.) in one of the following ways designed to elicit the production of reaginic antibody (immunoglobulin E [IgE]) to EcA or PaA. (i) Heat-killed (70°C, 1 h) bacteria (*E. coli* O127 or *P. aeruginosa* PL509) were injected i.p. alone or in conjunction with 8.6 mg of $Al(OH)_3$ gel. The numbers of bacteria injected were as indicated in the Results section. (ii) Acetone-dried bacteria (100 or 125 μg [dry weight]) suspended in 0.1 M phosphate-buffered saline (PBS), pH 7.2, were combined with 8.6 mg of $Al(OH)_3$ gel and injected i.p. (iii) Lyophilized aqueous antigenic extracts (100 μg of EcA or PaA) of acetone-dried bacteria were dissolved in PBS, pH 7.2, and injected i.p. alone or in conjunction with either 10 μg of *E. coli* O127 LPS or 8.6 mg of $Al(OH)_3$ gel. (iv) Fifty micrograms of OEP was dissolved in PBS, pH 7.2, and injected alone or in combination with $Al(OH)_3$ gel. (v) *E. coli* O127 LPS (10 or 50 μg), diluted in PBS, pH 7.2, was injected i.p. (vi) *P. aeruginosa* LPS (Pseudogen), 3.3 or 10 μg, was administered i.p.

Mice were bled via the retroorbital plexus, as described previously (8), or by decapitation 9 days after primary immunization. The blood from all members of the same group was pooled, and the serum was stored at −70°C, pending reaginic antibody determinations.

PCA determination of serum reaginic antibody. Passive cutaneous anaphylaxis (PCA) tests for measuring reaginic antibodies were performed as described previously (8). Each serum dilution (0.05 ml) was injected intracutaneously (i.c.) in two to four recipient HRS/J mice. Forty-eight hours later, PCA reactions were elicited by intravenous injections of 0.5 ml of 0.25% Evans blue dye (Matheson, Coleman and Bell, Norwood, Ohio) containing the appropriate antigen (500 μg of bacterial extract, 500 μg of LPS or 50 μg of OEP) dissolved in PBS, pH 7.2. Skin reactions were observed 15 to 30 min later. It was found that heating the sera at 56°C for 4 h completely destroyed the 48-h PCA reactivity of positive sera, which is the criterion used to place these homocytotropic antibodies in the mouse IgE class (34). The serum titers represent the reciprocal of the highest dilution yielding a positive PCA reaction (i.e., 5 mm or greater diameter of blueing) in at least two recipient mice. Because samples of pooled sera were used in assessing PCA titers, the results were not amenable to statistical analysis. However, it was found that the PCA titers of several serum pools did not vary by more than one dilution (i.e., twofold) when tested repeatedly. Therefore, a two dilution (i.e., fourfold) difference in titer between two serum pools was considered to be significant.

Adsorption of reaginic antibody. Adsorption experiments, using reagin-positive antiserum to EcA or PaA [induced by injecting EcA or PaA in combination with $Al(OH)_3$ gel], were carried out as follows: a 1:5 dilution (in PBS, pH 7.2) of antiserum was mixed with the appropriate bacterial suspension or antigenic extract (as designated in the Results section) and incubated at 37°C for 2 h, followed by overnight incubation at 4°C. The antigen-antibody mixtures were centrifuged (3,600 × *g*, 4°C) for 30 min to remove particulate antigen and/or precipitated immune complexes, and the supernatants were assessed for residual reaginic antibody by the mouse PCA test.

Trypsin treatment of bacterial antigens. Trypsin (Worthington Biochemical Co., Freehold, N. J.), 100 μg, was mixed with the bacterial antigens (1 mg) in a volume of 0.5 ml in PBS, adjusted to pH 8.2 with 0.1 N NaOH. The mixture was incubated overnight (20 h) at 37°C and then tested for residual antigenic activity in the mouse PCA test by using high-titered reaginic antiserum specific for the antigen. Controls consisted of treating the antigen extracts just as above, but adding no trypsin.

Heat lability of bacterial antigens. The antigenicity of PBS solutions of EcA and PaA (1 mg/ml) before and after heating was determined in the mouse PCA test. The antigen solutions were heated in a boiling (100°C) water bath for 30 min. Heated and unheated antigenic extracts were mixed with Evans blue dye, and the mixtures were used to challenge HRS/J mice previously sensitized (i.c. injections) with dilutions of anti-EcA- or anti-PaA-positive sera.

Hemagglutination inhibition (HI) of bacterial antigens. To determine the concentration of LPS antigenic determinants present in EcA and PA, the antigens were tested for their ability to inhibit specific LPS/anti-LPS antibody reactions in vitro. Sheep erythrocytes were coated with *E. coli* O127 LPS, *P. aeruginosa* type II LPS, or *P. aeruginosa* type VII LPS, as described previously (8). The antibody used in the *E. coli* LPS test system was induced in CBA/J mice 9 days after i.p. immunization with 10 μg of *E. coli* O127 LPS. Rabbit antiserum to *P. aeruginosa* LPS (multivalent, types

I to VII) was used in both the type II and type VII LPS test systems. The antisera were mixed with the appropriate antigen (homologous or heterologous LPS, homologous or heterologous bacterial antigen) diluted to concentrations of 0.1 to 250 μg/ml. Incubation of antisera with PBS, pH 7.2, served as controls. After 30 min of incubation at 37°C, the antigen-antibody mixtures were diluted in serial twofold steps. The LPS-coated or uncoated sheep erythrocyte suspension was added, and the mixtures were incubated for 2 h at 37°C, followed by overnight incubation at 4°C. All tests were performed in duplicate. The concentrations of LPS present in EcA and PaA were determined by comparing the inhibition of hemagglutination obtained with the specific purified LPS with that obtained with EcA or PaA.

RESULTS

Immunogenicity of bacteria and bacterial antigens. The results presented in Table 1 demonstrate the ability of an aqueous antigenic extract (EcA) of acetone-dried *E. coli* O127 to elicit the production of reaginic antibodies in mice when injected along with homologous LPS, 10 μg, or $Al(OH)_3$ gel. In contrast, the LPS (10 or 50 μg) was ineffective in inducing specific reagins, confirming previously reported results (8). The EcA, when injected alone (100 μg into CBA/J mice) did not elicit anti-EcA reaginic antibodies. Heat-killed *E. coli* also failed to induce detectable anti-EcA reagins unless mixed first with $Al(OH)_3$ gel. The acetone-dried bacteria, when injected in combination with $Al(OH)_3$ gel, were effective inducers of anti-EcA reaginic antibodies (1:40 titer). Table 1 also shows that *E. coli* O127 LPS failed to elicit a PCA reaction in mice that had received i.c. injections of the EcA-positive reaginic sera. In another set of experiments (data not presented), a second strain of mice, A/J, was tested for its ability to respond to EcA and whole *E. coli*. The A/J mice produced reaginic antibodies to EcA in a pattern similar to that by CBA/J mice. Anti-EcA antibody present in all sera was heat-labile (56°C, 4 h), confirming the IgE-like nature of the homocytotropic antibodies detected by the 48-h PCA test (34).

Table 2 depicts the results obtained after immunization of CBA/J mice with *P. aeruginosa* antigens. In contrast to EcA, 100 μg of the aqueous extract (PaA) of acetone-dried *P. aeruginosa* PL509 induced reaginic antibodies, even in the absence of exogenously added adjuvant. A higher concentration of antibody was induced by PaA in the presence of $Al(OH)_3$ (1:320 titer), but mixing PaA with *E. coli* O127 LPS did not result in enhanced production of anti-PaA reagins over that induced by PaA alone. Heat-killed as well as acetone-dried *P. aeruginosa* PL509 bacteria proved to be effective in eliciting anti-PaA reaginic antibody in mice. Immunization of mice with *P. aeruginosa* LPS (Pseudogen) failed to elicit reaginic antibodies specific either for Pseudogen or PaA, and anti-PaA reagins did not react with *P. aeruginosa* LPS determinants. Anti-PaA antibody detected by the 48-h mouse PCA test was shown to be heat labile.

Protein and endotoxin contents of antigenic extracts. EcA, PaA, and OEP were tested in our laboratory for protein content, and endotoxin activity was kindly determined for us by George Cole (Parke-Davis & Co.), using the

TABLE. 1. *Immunogenicity of E. coli O127 antigenic extract (EcA) and related bacterial antigens in CBA/J mice*

Antigen used in i.p. immunization (plus adjuvant, if any)	Amt of antigen injected/mouse	Reaginic antibody titer (48-h PCA)		
		EcA challenge[a]		*E. coli* O127 LPS challenge[b]
		Unheated serum	Heated serum (4 h, 56°C)	
E. coli O127 anti-EcA, alone	100 μg	<5	<5	<5
EcA + 10 μg of *E. coli* O127 LPS	100 μg	20	<5	<5
EcA + 8.6 mg of $Al(OH)_3$ gel	100 μg	160	<5	<5
E. coli O127 whole bacteria, acetone-dried	100 μg	40	<5	<5
+ 8.6 mg of $Al(OH)_3$ gel	125 μg	40	<5	<5
E. coli O127 whole bacteria, heat-killed (70°C, 1 h), alone	5×10^8	<5	<5	NT[c]
E. coli O127 whole bacteria, heat-killed + 8.6 mg of $Al(OH)_3$ gel	5×10^8	10	<5	<5
E. coli O127 LPS, alone	10 μg	<5	<5	<5
	50 μg	<5	<5	<5

[a] *E. coli* O127 antigenic extract (500 μg) dissolved in 0.5 ml of 0.25% Evans blue dye was used in the PCA challenge.

[b] *E. coli* O127 LPS (500 μg) in 0.5 ml of 0.25% Evans blue dye was used in the PCA challenge.

[c] NT, Not tested.

TABLE 2. *Immunogenicity of P. aeruginosa PL509 antigenic extract (PaA) and related bacterial antigens in CBA/J mice*

Antigen used in i.p. immunization (plus adjuvant, if any)	Amt of antigen injected/mouse	Reaginic antibody titer		
		PaA challenge[a]		*P. aeruginosa* LPS challenge[b]
		Unheated serum	Heated serum (4 h, 56°C)	
P. aeruginosa PL509 (type VII) PaA, alone	100 μg	40	<5	<5
PaA + 10 μg of *E. coli* O127 LPS	100 μg	40	<5	<5
PaA + 8.6 mg of $Al(OH)_3$ gel	100 μg	320	<5	<5
P. aeruginosa PL509 whole cells, acetone-dried, + 8.6 mg of $Al(OH)_3$ gel	125 μg	80	<5	<5
P. aeruginosa PL509 whole cells, heat-killed (70°C, 1 h), alone	1.5×10^8	<5	<5	<5
	6.0×10^8	40	<5	<5
P. aeruginosa PL509 whole cells, heat-killed, plus 8.6 mg of $Al(OH)_3$ gel	1.5×10^8	40	<5	<5
P. aeruginosa LPS (Pseudogen), alone	3.3 μg	<5	<5	<5
	10.0 μg	<5	<5	<5
OEP alone	50 μg	<5	<5	<5
OEP + 8.6 mg of $Al(OH)_3$ gel	50 μg	160	<5	<5

[a] *P. aeruginosa* PL509 antigenic extract (500 μg) dissolved in 0.5 ml of 0.25% Evans blue dye was used in the PCA challenge.

[b] *P. aeruginosa* LPS (Pseudogen, Parke-Davis) (500 μg) in 0.5 ml of 0.25% Evans blue dye was used in the PCA challenge of recipient HRS/J mice.

Limulus amoebocyte lysate assay. The concentration of LPS in the antigens was also measured by the HI assay. The results presented in Table 3 show that EcA was 87% protein and contained 0.04 to 1.0% endotoxin (*Limulus* assay) and 2% LPS (HI test). PaA was found to contain only 71% protein but 10 to 33% endotoxin (*Limulus* test), depending on the LPS standard used, and 16% LPS (HI test). Thus, there are striking differences in the nature of the two antigenic extracts, PaA and EcA, even though identical isolation procedures were used. The OEP provided by J. Y. Homma was found to contain 76% protein, 10 to 33% endotoxin, and 7% LPS.

Cross-reacting antigens of *Enterobacteriaceae*. Results shown in Table 4 demonstrate the presence of antigens in all enterobacterial species tested (*S. minnesota* SF1111, *S. minnesota* SF1167, *E. aerogenes*, *K. pneumoniae*, and *P. mirabilis*) which cross-reacted with the antigens present in EcA. The reaginic antibody titer obtained with heterologous enterobacterial challenge was not significantly different from the titer induced with homologous (EcA) challenge. However, no cross-reactivity with PaA or OEP antigens was detected.

In further experiments PaA-induced reaginic antibody was tested for its ability to react with antigenic extracts of four *P. aeruginosa* serotypes (IV to VII), with OEP, and with extracts of enterobacterial species (Table 4). Cross-reactivity was observed with all four *P. aeruginosa* serotypes tested as well as with OEP. However, antigens isolated from the enterobacterial species tested did not cross-react with the IgE-like antibodies prepared against the PaA antigenic extract. OEP-induced reaginic antibody reacted with PaA in the PCA test, but did react upon challenge with EcA.

TABLE 3. *Protein and endotoxin concentrations found in EcA, PaA, and OEP antigen preparations*

Antigen prepn	% Protein[a]	% Endotoxin[b]	% LPS[c]
EcA	87	0.04–1.0	2
PaA	71	10–33	16
OEP	76	10–33	7

[a] Protein determined by the method of Lowry et al. (27).

[b] Endotoxin determined by G. Cole (Parke-Davis) using the *Limulus* amoebocyte lysate assay (39). Estimates of percentage of LPS varied with the LPS used as standards in the assay.

[c] LPS concentrations were measured by the ability of the antigen to inhibit in a homologous passive hemagglutination test.

Adsorption or neutralization of reaginic antibodies. To ascertain whether the antigens extracted from *E. coli* and *P. aeruginosa* were cell surface associated, we utilized a modification of the technique described by Whiteside and Baker (44). The antibody induced by EcA [in the presence of $Al(OH)_3$ gel] was tested by mouse PCA after adsorption with homologous and heterologous antigenic extracts and whole

TABLE 4. *Cross-reactivity of mouse anti-EcA, anti-PaA, and anti-OEP antibody with antigens of other gram-negative species*

Source of antigen used for challenge in the 48-h mouse PCA[a]	Reaginic antibody titer (48-h PCA) of antiserum induced by:		
	EcA[b]	PaA[b]	OEP[c]
E. coli O127 (EcA)	160	<5	<5
S. minnesota SF1111	160	<5	NT[d]
S. minnesota SF1167 (Re)	160	<5	NT
E. aerogenes	80	<5	NT
K. pneumoniae	80	<5	NT
P. mirabilis	80	<5	NT
P. aeruginosa PL509 (PaA)	<5	320	160
P. aeruginosa, type IV	NT	320	NT
P. aeruginosa, type V	NT	320	NT
P. aeruginosa, type VI	NT	320	NT
P. aeruginosa, type VII	NT	320	NT
OEP[e]	<5	80	640

[a] The PCA challenges were made by injecting 500 μg of the appropriate antigen dissolved in 0.25% Evans blue dye.

[b] The high-titered anti-EcA and anti-PaA sera were induced in CBA/J mice after i.p. immunization with 100 μg of EcA or PaA mixed with 8.6 mg of $Al(OH)_3$ gel.

[c] Anti-OEP IgE was induced in CBA/J mice using 50 μg of OEP mixed with 8.6 mg of $Al(OH)_3$ gel.

[d] NT, Not tested.

[e] The OEP challenge was made using 50 μg of antigen.

bacteria. The results of these experiments are shown in Table 5. In the control serum mixture (no antigen), the test conditions resulted in a twofold drop in the anti-EcA titer, i.e., from 1:160 to 1:80. When the reaginic antiserum was mixed with the homologous antigen (EcA, 10 μg), the titer was reduced to 1:40 and, when the concentration of EcA was increased to 50 or 100 μg, the antibody titer was reduced greater than eightfold to an undetectable level (<1:10). The homologous whole bacteria, both the heat-killed and the acetone-dried cells, significantly reduced the anti-EcA titer to 1:20 and <1:10, respectively. *E. coli* O127 LPS (100 μg) failed to neutralize the reagins, whereas even a heterologous bacterial species (*S. minnesota*, SF1111, acetone-dried cells) significantly reduced the PCA titer. The results obtained were specific for *Enterobacteriaceae* and were not due to a nonspecific adsorption of the antibody, as evidenced by the failure of *P. aeruginosa* PL509 cells, PaA and OEP, to neutralize the anti-EcA reaginic antibodies.

Results of adsorption experiments for anti-PaA antibody are presented in Table 6. Significant reduction (i.e., fourfold) in the titer of reaginic antibodies was obtained only when *P. aeruginosa* bacteria or antigenic extracts (PaA and OEP) were used in the adsorption mix-

TABLE 5. *Adsorption of anti-EcA reaginic antibodies by antigenic extracts and whole bacteria*

Antigen used in adsorption	Concn of antigen in the adsorption mixture	Reaginic antibody titer (48 h PCA)[a]
None		80
EcA	10 μg	40
	50 μg	<10
	100 μg	<10
E. coli O127 acetone-dried cells	10 mg	<10
E. coli O127 washed, heat-killed cells	5 % (vol/vol)	20
E. coli O127 LPS	100 μg	80
S. minnesota SF1111 acetone-dried cells	10 mg	20
P. aeruginosa PL509 acetone-dried cells	10 mg	80
PaA	10 μg	80
	50 μg	80
OEP	20 μg	80

[a] High-titered anti-EcA serum (diluted 1:5 in PBS and then mixed 1:1 with antigen) was the same as that used in the experiments shown in Table 4. A fourfold reduction in titer (from 1:80 to 1:20) was considered to be significant.

TABLE 6. *Adsorption of anti-PaA reaginic antibodies by antigenic extracts and whole bacteria*

Antigen used in adsorption	Concn of antigen in the adsorption mixture	Reaginic antibody titer (48 h PCA)[a]
None		320
PaA	10 μg	160
	50 μg	160
	100 μg	80
P. aeruginosa PL509 acetone-dried cells	10 mg	20
P. aeruginosa PL509 washed, heat-killed cells	5 % (vol/vol)	80
OEP	20 μg	80
	50 μg	160
P. aeruginosa LPS (Pseudogen)	100 μg	320
E. coli O127 acetone-dried cells	10 mg	320
EcA	10 μg	320
	50 μg	320

[a] The high titered anti-PaA serum used (diluted 1:5 in PBS and then mixed 1:1 with antigen) was the same as that used in the experiments depicted in Table 4. A fourfold reduction in titer (from 1:320 to 1:80) was considered to be significant.

tures. Neither *E. coli* nor EcA antigenic extract reduced the anti-PaA titer. A mixture of LPS isolated from the seven *P. aeruginosa* serotypes (Pseudogen) failed to adsorb or neutralize the anti-PaA reaginic antibody.

Trypsinization experiments. To determine whether or not the protein components of the extracts were responsible for the IgE-like antibody activity measured, an attempt was made to destroy the antigen(s) by incubating PaA and EcA with trypsin. The results (Table 7) show that the antibody specificity of the reagins induced by immunization of mice with antigen extracts was directed toward trypsin-sensitive antigenic determinants.

Heat lability of the antigens. In additional experiments, the heat lability of the antigen(s) present in EcA and PaA was determined. EcA and PaA, at concentrations of 1 mg/ml in PBS, pH 7.2, were heated in a boiling water bath (100°C) for 30 min. The results of the experiment are shown in Table 8. The antigen(s) in EcA responsible for inducing reaginic antibodies are heat-labile, since the heated EcA antigen mixture lost its antigenicity for the anti-EcA reaginic serum. Heating the PaA antigens resulted in a fourfold drop in antigenicity (reagin titer was 1:80 as opposed to 1:320 for the unheated antigen), indicating that some of the PaA antigens were also heat labile.

TABLE 7. *Trypsin sensitivity of the antigenic extracts*

Antigen(s) used in PCA challenge[a]	Reaginic antibody titer	
	Anti-EcA[b]	Anti-PaA[b]
500 μg of EcA, untreated	80	NT[c]
500 μg of EcA, trypsin-treated	<5	NT
500 μg of PaA, untreated	NT	320
500 μg of PaA, trypsin-treated	NT	<5

[a] The antigenic extracts, with and without trypsin, were incubated for 20 h at 37°C before use as challenge of HRS/J mice sensitized 48 h previously by i.c. injection of dilutions of the appropriate antiserum. A fourfold or greater reduction in titer was considered significant.

[b] Anti-EcA and anti-PaA sera were the same as those used in experiments described in Tables 5 and 6, respectively.

[c] NT, Not tested.

TABLE 8. *Heat lability of the antigenic extracts*

Antigen used in PCA challenge[a]	Reaginic antibody titer (48-h PCA)	
	Anti-EcA[b]	Anti-PaA[b]
500 μg of EcA, untreated	160	NT[c]
500 μg of EcA, heated	<5	NT
500 μg of PaA, untreated	NT	320
500 μg of PaA, heated	NT	80

[a] The antigenic extracts were placed in a boiling water bath (100°C) for 30 min before their use as antigens in the PCA challenge of HRS/J mice sensitized 48 h previously with i.c. injections of dilutions of the appropriate antiserum. A fourfold reduction in titer was considered significant.

[b] Anti-EcA and anti-PaA sera were the same as those used in experiments described in Tables 5 and 6, respectively.

[c] NT, Not tested.

DISCUSSION

The results of our experiments show that there exist antigens at or near the surface of many gram-negative bacterial species which are capable of eliciting the production of reaginic antibodies in mice when injected alone, as in the case of PaA, or in the presence of exogenously added homologous LPS, as in the case of EcA. The antigenic determinants to which the reaginic antibodies are directed are not found on the purified homologous LPS and seem to be protein in nature, as indicated by their sensitivity to trypsin and their heat lability. In this respect, the antigens that we isolated differ from the common enterobacterial antigen originally described by Kunin et al. (24) and recently the subject of investigations by McCabe (28), McCabe and Greely (29), Johns et al. (20), Mayer et al. (32), and Whang et al. (43). All of these investigators described their enterobacterial antigens to be polysaccharide in nature, sharing some determinants with the somatic antigen. The adsorption experiments reported here support the idea that at least a portion of the antigenic determinants responsible for the specificity of the reaginic antibody induced in mice was present at or near the cell surface. Both washed, heat-killed (70°C, 1 h) and acetone-dried homologous bacteria effectively reduced the titer of anti-EcA or anti-PaA antibodies present in the sera. In the case of the *P. aeruginosa* antigenic extract, the cross-reaction of anti-PaA reagins with a purified component of the outer membrane of *P. aeruginosa*, OEP, confirmed the identity of at least part of the PaA with a cell membrane component.

There are numerous reports of the isolation of protein antigens from gram-negative bacteria. Barber et al. (5) reported the isolation of protein antigens from *E. coli* O119. The antibody induced in rabbits to the protein antigens was found to react in precipitin tests with antigens isolated from several *E. coli* and *Salmonella* strains. The same group of investigators (4) reported the identification of a protein antigen from *Salmonella paratyphi* A which cross-

reacted serologically with antigens from *Salmonella typhi* and *Salmonella typhimurium*. Kaijser (21) also identified an antigenic substance, protein in nature, which was common to many *E. coli* and *Proteus* species, as well as to two pathogenic *Pseudomonas* species tested. The common antigen, designated high mobility antigen, also reacted with antimeningococcal serum. Homma and Suzuki have reported that the cell envelope protein antigen (OEP) isolated from *P. aeruginosa* (17) is common to all known *P. aeruginosa* serotypes and that both active and passive immunization with OEP protected mice from infection with *P. aeruginosa* of any serotype (1). Our results confirm the observation that OEP is an antigen common to *P. aeruginosa* strains and extend these observations to show that this protein antigen is not present in *E. coli* O127.

Our experiments were designed to detect the production of reaginic antibody in mice to antigenic extracts of gram-negative bacteria in the hope of developing a test for assessing the allergenic potential of bacteria and/or isolated bacterial antigens. We succeeded in showing that mild treatment of gram-negative bacteria allowed the isolation of antigens capable of inducing reaginic antibody in mice. No exogenous adjuvant was required to induce the reaginic response to *P. aeruginosa* antigens, as shown when heat-killed bacteria or the PaA, alone, were used to immunize mice. The failure of the *E. coli* antigenic extract to induce reaginic antibodies when injected alone could be explained on the basis of the low concentration (0.04 to 2%) of endotoxin present in EcA as compared with the concentration (10 to 33%) present in PaA. When homologous LPS was added to the EcA to a concentration of 10%, a reaginic antibody response was induced. Heat-killed *E. coli* injected alone failed to induce IgE-like antibodies to EcA. This might be related to the phenomenon described by Suzuki et al. (37), who reported that high concentrations of LPS inhibited the antibody response to common enterobacterial antigen. Our results do show that extraordinary means are not required to induce reaginic antibody to gram-negative antigens in mice.

There are several reports in the literature that implicate a role for gram-negative bacteria in the mechanism of asthma associated with acute respiratory infection (12, 23) and with chronic respiratory disease (7, 31). Hampton et al. (15) demonstrated a positive correlation of various parameters of immediate hypersensitivity induced by antigens extracted from *Neisseria catarrhalis* with the clinical history of the patients. Gordon et al. (12) described a patient with pulmonary allergic disease in whom the pathogenesis of the allergic pulmonary disease was found to be related to immediate hypersensitivity to a *P. aeruginosa* species isolated from the patient's sputum. There are reports in the literature that describe the presence of bacterial specific antibody in respiratory secretions in humans (7, 12) and in animals (11, 33). Reynolds et al. (33) showed that both intranasal and parenteral methods of immunization of rabbits with *P. aeruginosa* LPS effectively induced detectable amounts of respiratory antibodies. Local antibody production in the respiratory tract has been demonstrated by several groups of investigators. Kaltreider and Salmon (22) isolated from the normal canine lung lymphocytes which were capable of de novo synthesis of IgG in vitro. Immunoglobulin-containing cells were detected in the lungs of hamsters infected with *Mycoplasma pneumoniae* by Fernald et al. (11). Waldman and Henney (41) found that local administration of antigen resulted in local antibody production in the upper respiratory tract of guinea pigs. Tada and Ishizaka (38) showed that IgE-forming cells were predominant in the respiratory mucosa of humans and monkeys. Because the ratio of IgE: IgG in sputum and nasal washings was greater than that of serum, Ishizaka and Newcombe (18) and Waldman et al. (42) concluded that IgE is a secretory immunoglobulin and that local antibody production in the lungs contributed to the concentrations of antibodies found there. Deuschl and Johansson (9) calculated that 84% of the IgA and 73% of the IgE found in tracheo-bronchial secretions were produced locally in humans. The reports cited above, taken together with the evidence we have presented, which shows that under experimental conditions antigens of gram-negative bacteria induced reaginic antibody production in mice, suggest that the potential local production of IgE antibody to gram-negative bacteria in the respiratory tract warrants further investigation.

Gram-negative bacilli are responsible for most urinary tract infections. It is interesting to note that IgE antibody is found in human urine (40). Based on the size of the IgE molecule (200,000, molecular weight) and the clearance rates of known proteins from urine by the kidneys, Turner et al. concluded that 90 to 98% of the IgE found in the urine is locally produced (40). Local IgG, IgA, and IgM antibody production has been demonstrated in experimental pyelonephritis induced by *E. coli* in rabbits (35); however, no one has yet examined experimental local production of IgE. The antigenic specificity of human urinary IgE is unknown,

but it is interesting to speculate that local production of IgE specific for gram-negative antigens might occur and that this IgE might play some role in the pathogenesis of urinary tract infections just as bacterial-specific IgE may play a role in the pathogenesis of respiratory diseases.

We are in the process of further characterizing the antigens from *E. coli* and from *P. aeruginosa*. The ability of these antigens to induce protective antibodies, whether specific or cross-protecting, will also be examined.

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