Preparation and Quantitative Determination of Antibodies Against Major Outer Membrane Proteins of *Escherichia coli* O26 K60

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Antisera against isolated outer membrane (OM) proteins I and II* of *Escherichia coli* O26 K60 were elicited in rabbits. Antisera obtained after intramuscular administration with Freund's complete adjuvant showed high titres of specific antibodies. Intravenous administration of the same preparations yielded a considerable antibody response against bacterial lipopolysaccharide, a minor contaminant of the protein preparations. Antibody titres against OM proteins I and II*, lipopolysaccharide and murein-lipoprotein were determined by the enzyme-linked immunosorbent assay (ELISA) in these sera, and in antisera elicited against whole formaldehyde-fixed bacteria or isolated OM. Comparison of ELISA with single radial immunodiffusion and interfacial immunoprecipitation tests revealed that ELISA was not only the most uniformly applicable, but also the most specific and the most convenient method. In double diffusion tests no cross-reactivity between proteins I and II* was seen. Antibodies against proteins I and II*, lipopolysaccharide and lipoprotein could be specifically absorbed from the sera with the appropriate antigen preparations. Absorption experiments with intact *E. coli* O26 K60, Tris/EDTA-sheared bacteria and isolated OM revealed that antibodies against protein I were hardly absorbed at all probably because the antibody, evoked against denatured protein I, did not react with the protein in its native configuration. Antibodies against protein II* and lipoprotein were absorbed by intact as well as by sheared bacteria, but to a much greater extent by isolated OM, which indicates that these OM components are accessible from the outside, but that they are situated relatively deep in the OM structure.

INTRODUCTION

The outer membrane (OM) of *Escherichia coli* contains a number of major proteins in the 27 000 to 41 000 molecular weight range, designated Ia, Ib (Schmitges & Henning, 1976) and II* (Garten *et al.*, 1975) or b, c and d (Lugtenberg *et al.*, 1975). Another major OM constituent is the murein-bound or free lipoprotein (Braun & Rehn, 1969). The function of OM proteins in the immunology of Gram-negative bacteria is not yet fully understood. Their immunological investigation requires the purification of these antigens and the development of reliable techniques to determine antibodies against them. Braun *et al.* (1976) determined antibodies against the lipoprotein by passive haemagglutination. The enzyme-linked immunosorbent assay was applied by Sippel *et al.* (1978) for the detection of antibodies against cell envelope proteins of *Neisseria meningitidis* and *Salmonella typhi*. Studying phage reception by OM proteins, Diedrich *et al.* (1977) and Chai & Foulds (1979) used antibodies against the isolated proteins, the specificity of which

was determined by double diffusion in agarose. Other methods applied for the detection of antibodies against OM proteins are immunoprecipitation (Inouye *et al.*, 1976; Dankert & Hofstra, 1978) and crossed immunoelectrophoresis (Van Tol *et al.*, 1979).

In this study we report the quantitative determination of antibodies against OM proteins I and II*, lipopolysaccharide and murein-lipoprotein of *E. coli* O26 K60 by single radial immunodiffusion (SRID) and enzyme-linked immunosorbent assay (ELISA). Purified protein preparations were administered to rabbits intravenously and intramuscularly to study the effect of the route of administration of these antigens on the immune response. The specificity of the antisera was investigated by double diffusion and interfacial immunoprecipitation. The affinity of the antibodies for the antigens in their native situation was studied by absorption experiments with intact or Tris/EDTA-sheared *E. coli* O26 K60 or isolated OM.

METHODS

Bacterial strains and growth conditions. *Escherichia coli* serotype O26 K60 was maintained and grown as described previously (Dankert & Hofstra, 1978). *Escherichia coli* JE5513, a lipoprotein-negative mutant strain derived from JE5512 (Hirota *et al.*, 1977), was generously donated by Dr B. Lugtenberg, Utrecht, The Netherlands. It was maintained and grown in the same way as *E. coli* O26 K60.

Extraction of membrane proteins. Outer membranes (OM) of *E. coli* O26 K60 were prepared as described by Dankert & Hofstra (1978). The major OM proteins were extracted by a procedure based on the method of Reithmeier & Bragg (1974) for protein II* and of Rosenbusch (1974) for protein I. Protein II* was extracted from 200 mg OM in 50 ml 0.05 M-Tris/HCl buffer (pH 7.8) containing 0.5 % (w/v) sodium dodecyl sulphate (SDS). This suspension was incubated for 1 h at 37 °C. After ultracentrifugation (100000 g, 45 min, 15 °C; Hereaus, Osterode, F.R.G.) the supernatant was concentrated to 10 ml by ultrafiltration (PM10; Amicon, Lexington, U.S.A.) and precipitated with acetone (90 %, v/v) at -20 °C for 18 h. The precipitate was washed twice with ice-cold acetone (90 %, v/v) and freeze-dried. The sediment of the first ultracentrifugation was re-extracted twice with the same volume of 0.05 M-Tris/HCl (pH 7.8) containing 1.0 % (w/v) SDS (1 h, 37 °C) to remove the rest of the protein II*. The supernatants of these extractions were discarded. Protein I was extracted from the remaining pellet with the same volume of 0.05 M-Tris/HCl (pH 7.8) containing 2.0 % (w/v) SDS (5 min, 100 °C), followed by ultracentrifugation. The supernatant was concentrated, precipitated, washed and lyophilized as described above. The final pellet containing murein-lipoprotein (Braun & Rehn, 1969) was re-extracted with 4 % (w/v) SDS (1 h, 100 °C) to remove any remaining protein I, washed twice with distilled water and freeze-dried.

Isolation of lipopolysaccharide (LPS). LPS of *E. coli* O26 K60 was isolated according to Westphal *et al.* (1952) as described previously (Hofstra & Dankert, 1979).

Isolation of lipoprotein-free murein. Outer membranes of *E. coli* JE5513, prepared as described for *E. coli* O26 K60, were boiled in 0.05 M-Tris/HCl (pH 7.8) containing 4 % (w/v) SDS for 1 h, followed by ultracentrifugation. The pellet was re-extracted, washed twice with distilled water and freeze-dried.

Column chromatography. The extracted OM protein fractions were purified by chromatography on a 5 × 60 cm column with water jacket (LKB, Bromma, Sweden) filled with Biogel P-150 (Bio-Rad, Richmond, U.S.A.). The elution buffer was 0.01 M-Tris/HCl (pH 7.8) containing 0.25 % (w/v) SDS. The elution rate was 25 ml h⁻¹ at 28 °C or 50 ml h⁻¹ at 56 °C. Samples (5 ml) were collected with an LKB sample collector. Absorptions were followed at 206 and 280 nm (Uvicord III, LKB). Crude protein II* was solubilized at room temperature in elution buffer (10 mg ml⁻¹), applied to the column and chromatographed at 28 °C. Fractions containing the peak of protein II* were pooled, concentrated to 10 ml by ultrafiltration and precipitated with ice-cold acetone. Crude protein I was solubilized at 100 °C in elution buffer (5 to 10 mg ml⁻¹) and chromatographed at 56 °C. Fractions containing protein I were pooled, concentrated and precipitated with ice-cold acetone. Chromatography of the fractions containing protein I or II* was repeated three times.

Preparation of antisera. Antisera against formaldehyde-fixed bacteria and isolated OM were prepared as reported previously (Dankert & Hofstra, 1978). Antisera against the isolated OM proteins were elicited by intravenous (i.v.) or intramuscular (i.m.) administration of the antigens. Two pairs of rabbits received four i.v. injections with increasing doses (0.5, 1.0, 1.5 and 2.0 ml) of protein I or II* [1 mg ml⁻¹ in 0.9 % (w/v) NaCl] at 5 d intervals. Two other pairs received 5 mg protein I or II*, respectively, suspended in 0.5 ml 0.9 % (w/v) NaCl and the same amount of Freund's complete adjuvant. The antigens were administered (i.m.) in the upper hindleg region on days 0 and 28. Blood was collected from all rabbits 2 to 3 weeks after the last injection. Sera were obtained by centrifugation of the clotted blood and stored at -70 °C.

Absorption of antisera. To remove antibodies directed against *E. coli* O26 LPS, a 10 mg ml⁻¹ solution of LPS O26 in phosphate-buffered saline (PBS; pH 7.2) was added to the sera (Hofstra & Dankert, 1979). Specific absorption of antisera with the isolated OM proteins I or II*, murein-lipoprotein or lipoprotein-free murein was carried out by stepwise addition of one or more of these antigens, suspended in PBS to a concentration of 10 mg ml⁻¹. Each addition was followed by incubation (30 min, 37 °C) and centrifugation (10 min, 10000 g) to remove immune complexes. To test the adherence of antibodies to the outermost layer of *E. coli* O26 K60, 1 ml samples of the antisera, undiluted or diluted 1:20 and 1:80 with PBS, were subjected to ten successive incubations with approximately 2×10^8 bacteria, untreated or sheared in Tris/EDTA (Dankert & Hofstra, 1978). Each incubation (20 min, room temperature) was followed by centrifugation (10 min, 8000 g) to remove the bacteria. To test the adherence of antibodies to isolated OM of *E. coli* O26 K60, antisera were incubated with 5 mg freeze-dried OM (ml serum)⁻¹. After incubation for 1 h at room temperature the sera were centrifuged (20 min, 12000 g) to remove OM and absorbed antibodies.

Immunodiffusion. Double diffusion (Ouchterlony, 1958) was performed on glass slides in 0.5 % (w/v) agarose (Hoechst, Frankfurt, F.R.G.) in PBS (pH 7.2). LPS O26 was dissolved in PBS to a concentration of 1 mg ml⁻¹. Proteins I and II* were dispersed to the same concentration in PBS containing 0.2 % (w/v) SDS. Centre wells were filled with undiluted antisera (20 µl) or with LPS-absorbed sera (30 µl), and 30 µl of the antigen preparations were added to the other wells. After incubation (24 h, room temperature), plates were washed with PBS for 48 h at 4 °C. The agarose was covered with several sheets of filter paper, dried under mild pressure, stained with Amido Black [0.5 % (w/v) in 0.5 % (v/v) acetic acid] and destained with 0.5 % (v/v) acetic acid. Immunodiffusion patterns were photographed on Agfa Ortho 25 Professional document copying film.

Single radial immunodiffusion (SRID). SRID was carried out essentially as described by Vaerman *et al.* (1969). The antigens were dispersed in separate lots of PBS containing 2 % (w/v) SDS to a concentration of 1 mg ml⁻¹. LPS dissolved very well, protein I had to be solubilized by boiling for 5 min in this solvent, while protein II* was dispersed sonically to a clear solution. These antigen solutions were added to separate 20 ml quantities of 0.8 % (w/v) agarose (Hoechst) in PBS kept liquid at 50 °C, the final concentrations being 50 µg antigen ml⁻¹ and approximately 0.1 % (w/v) SDS. The agarose was poured into Petri dishes (940 cm²) to an antigen concentration of approximately 10 µg cm⁻². Wells (4 mm²) were cut out and filled with 20 µl of the undiluted antisera or 30 µl of LPS-absorbed sera. Plates were incubated for 36 h at room temperature and then washed with several changes of fresh PBS (72 h, 4 °C). Plates were dried and stained as described above. After staining the total area enclosed by the precipitation ring was calculated.

Enzyme-linked immunosorbent assay (ELISA). ELISA was carried out essentially according to Engvall & Perlmann (1972), using the conjugate and substrate system described by Ruitenberg *et al.* (1974). Antigens (LPS, protein I, protein II* or murein-lipoprotein) were dispersed in 0.1 M-Na₂CO₃ (pH 9.6) to a concentration of 30 µg ml⁻¹. Disposable polystyrene microtitre trays (M-24-AR; Greiner, Nürtingen, F.R.G.) were filled with antigen suspensions (100 µl per well) and incubated for 3 h at 37 °C. Non-attached antigen was removed by three washings with tap water. Dilution series of the rabbit antisera were made with PBS containing 1 % (w/v) bovine serum albumin (BSA) and 0.05 % (v/v) Tween 20 (PBS-BSA-Tween). Wells were filled with 100 µl of diluted serum. Trays were incubated for 2 h at 37 °C. After three washings with tap water, 100 µl of goat anti-rabbit IgG conjugated with horseradish peroxidase (Nordic, Tilburg, The Netherlands) diluted 1:1250 with PBS-BSA-Tween, was added to each well, followed by 2 h incubation at room temperature. Trays were washed as described above and 100 µl of peroxidase substrate solution was applied to each well. Peroxidase substrate was composed of 100 ml distilled water, 80 mg 5-amino-salicylic acid (Merck, Darmstadt, F.R.G.) and 16 µl H₂O₂ (30 %, v/v). The pH was adjusted to 6.0 with NaOH. The reaction was developed for 30 min at room temperature. The endpoint titre was determined visually, being the highest serum dilution giving a positive colour development. Antiserum dilution series applied to uncoated wells and PBS-BSA-Tween (not containing antiserum) added to antigen-coated wells were used as negative controls.

Interfacial immunoprecipitin test (IIP). This test was carried out essentially as reported by Dankert & Hofstra (1978). OM of *E. coli* O26 K60 were solubilized by boiling for 5 min in PBS containing 2 % (w/v) SDS, followed by dilution to 0.2 % (w/v) SDS, the final OM concentration being 1 mg ml⁻¹. This preparation was reacted with LPS-absorbed antisera against whole bacteria, isolated OM or purified OM proteins. Immunoprecipitates were analysed by SDS-polyacrylamide gel electrophoresis according to Laemmli (1970) on slab gels. Procedures for staining and destaining the gels have been described previously (Dankert & Hofstra, 1978).

Determinations. Protein was estimated by the Lowry method and 2-keto-3-deoxyoctonate by the method of Ellwood (1970). Endotoxin activity was estimated by the Limulus lysate test (Yin *et al.*, 1972) using Limulus amoebocyte preparation (Mallinckrodt, St Louis, U.S.A.) and related to a standard endotoxin reference (Sigma). Hexoses were determined by the anthrone method (Trevelyan & Harrison, 1952).

RESULTS

Chemical properties of the E. coli O26 K60 isolates

After repeated column chromatography proteins I and II* seemed pure in SDS-polyacrylamide gel electrophoresis (PAGE). Protein I gave two bands – a dense band, representing subcomponent Ib, and a very faint one of subcomponent Ia (designations after Schmitges & Henning, 1976). When gels were overloaded ($> 100 \mu\text{g}$ protein per slot) a minute contamination with protein II* was detectable in our protein I preparation. No 2-keto-3-deoxyoctonate (KDO) could be detected in protein I, while the activity found with the Limulus lysate test corresponded to less than 0.5% (w/w) endotoxin. Protein II* contained 0.3 to 0.5% (w/w) KDO while the Limulus lysate test indicated an endotoxin content of 0.5% (w/w). Carbohydrates were not detectable in protein I; the protein II* preparation contained 4 to 5% (w/w). The isolated LPS O26 (not free of nucleic acids) contained less than 1% (w/w) protein. Its carbohydrate content was 50 to 60% (w/w) and its KDO content was 10 to 12% (w/w). The murein-lipoprotein preparation showed no endotoxin activity in the Limulus lysate test. Analysis of this preparation by SDS-PAGE showed no protein bands.

Double immunodiffusion

Figure 1 shows the results of the double diffusion tests, using the antisera elicited by intravenous (i.v.) administration of whole formaldehyde-fixed *E. coli* O26 K60, its isolated OM and proteins I and II* or by intramuscular (i.m.) administration of the two OM protein preparations. The antisera obtained after i.v. injection of whole cells or isolated OM gave heavy precipitin lines with LPS O26 as well as with the LPS present in protein II* (Fig. 1a and c). LPS absorption resulted in their disappearance. Only a faint precipitin line with protein II* and an extremely faint line with protein I remained (Fig. 1b and d). The antisera obtained after i.v. administration of proteins I and II* showed precipitin lines with LPS and the LPS-contaminant in protein II* which disappeared after LPS absorption of the sera (Fig. 1e, f, g and h). After this absorption only anti-II* serum gave a faint line with its specific antigen (Fig. 1h). The antisera resulting from i.m. administration of the isolated proteins I and II* with Freund's complete adjuvant showed heavy precipitin lines with their specific antigens (Fig. 1i and k). The antiserum against protein I gave no precipitin line with LPS. Consequently, LPS absorption did not affect the pattern obtained with this serum (Fig. 1j). The antiserum against protein II* gave a weak line with LPS which could be removed by LPS absorption (Fig. 1l). Cross-reactivity between proteins I and II* was not observed.

Single radial immunodiffusion

In SRID experiments, LPS-absorbed antisera were applied to agarose plates containing proteins I or II*. The antisera against isolated OM or whole bacteria reacted only weakly with protein I and moderately with protein II* (Table 1). Antisera elicited by i.m. administration of proteins I and II* gave large precipitation rings when reacted with their specific antigens (132.3 and 147.8 mm² for protein I and protein II*, respectively). The antisera raised by i.v. administration of the proteins were less reactive. All antisera which had not been absorbed with LPS gave considerable precipitin areas when applied to LPS-containing plates.

Enzyme-linked immunosorbent assay

Antisera against formaldehyde-fixed *E. coli* O26 K60 or against isolated OM of this serotype contained high titres of antibodies against LPS O26, which were effectively removed by absorption (Table 2). Lower titres against proteins I and II* and against murein-lipoprotein were detected in these sera. The antibody titre against murein-lipo-

Fig. 1. Double diffusion patterns obtained with rabbit antisera against several preparations of *E. coli* O26 K60 and antigenic preparations of the same strain. Peripheral wells were filled with 30 μ l of solutions (1 mg ml⁻¹) of LPS (top and bottom wells), protein I (upper right and lower left) or protein II* (upper left and lower right) as indicated in (m). Centre wells were filled with 20 μ l of antisera against: formaldehyde-fixed cells of *E. coli* O26 K60 (a); isolated OM (c); protein I, administered intravenously (i.v.) (e); protein II*, i.v. (g); protein I, administered intramuscularly (i.m.) (i) and protein II*, i.m. (k). Centre wells of the right hand column (b to l) were filled with 30 μ l of the same sera after absorption with LPS of *E. coli* O26 K60. Precipitin lines were allowed to develop for 24 h at room temperature. Plates were washed with PBS (48 h, 4 °C), dried and stained with Amido Black.

Table 1. *Quantitative determination of antibodies by single radial immunodiffusion (SRID)*

Antisera against several preparations derived from *E. coli* O26 K60 were elicited by intravenous or intramuscular administration to rabbits. For SRID, antigens were solubilized in PBS (pH 7.2) containing 2% (w/v) SDS and added to 20 ml agarose (0.8%, w/v) in PBS in Petri dishes [final antigen concentration $10 \mu\text{g cm}^{-2}$ in 0.1% (w/v) SDS]. Wells (4 mm^2) were cut out and filled with $30 \mu\text{l}$ of LPS-absorbed antiserum if antibodies against proteins I or II* were to be determined and with $20 \mu\text{l}$ unabsorbed antiserum, when LPS-containing agarose plates were used. Precipitin haloes were allowed to develop for 36 h at room temperature. Plates were washed (72 h, 4°C), dried and stained with Amido Black. The total area (mm^2) enclosed by the precipitin halo was calculated.

Antiserum against:	Administration of antigen	Area of precipitin halo (mm^2)		
		Agarose containing:		
		Protein I	Protein II*	LPS
Formaldehyde-fixed bacteria	Intravenous	12.3	35.7	137.6
Outer membrane	Intravenous	18.6	37.2	118.8
Protein I	Intramuscular	132.3	21.6	49.0
Protein I	Intravenous	28.4	13.3	51.6
Protein II*	Intramuscular	24.2	147.8	84.3
Protein II*	Intravenous	24.1	49.0	100.7

Table 2. *Quantitative determination of antibodies by the enzyme-linked immunosorbent assay (ELISA)*

Antisera against several preparations of *E. coli* O26 K60 were elicited by intravenous or intramuscular administration to rabbits. ELISA was carried out in microtitre trays coated with suspensions of the antigens at $30 \mu\text{g ml}^{-1}$ in 0.1 M- Na_2CO_3 (pH 9.6). Sera were diluted with PBS (pH 7.2) containing 1% (w/v) bovine serum albumin and 0.05% (v/v) Tween 20. The second incubation was performed with horseradish peroxidase conjugated goat anti-rabbit IgG, diluted 1:1250. 5-Aminosalicylic acid was used as peroxidase substrate. Titres are given as the reciprocal of the highest dilution giving a positive colour reaction after 30 min at room temperature.

Antiserum against:	Administration of antigen	Reciprocal titre				Reciprocal titre after absorption with LPS			
		Protein I	Protein II*	LPS	Murein-lipoprotein	Protein I	Protein II*	LPS	Murein-lipoprotein
Formaldehyde-fixed bacteria	Intravenous	160	640	10000	160	160	320-640	< 20	160
Outer membrane	Intravenous	320	1280	5120	640	320	640	< 20	640
Protein I	Intramuscular	20000	320	160	2560	20000	160-320	< 20	2560
Protein I	Intravenous	640	640	320	160	640	320	< 20	160
Protein II*	Intramuscular	160	160000	320	< 20	160	160000	< 20	< 20
Protein II*	Intravenous	160	1280	2560	< 20	160	640-1280	< 20	< 20

protein was higher in the antiserum against isolated OM than in the serum against whole bacteria. The antisera elicited after i.m. administration of proteins I and II* showed high titres of antibodies against their appropriate antigens and low titres against LPS. A marked antibody content against murein-lipoprotein was only detected in the antiserum against protein I. The antisera formed after i.v. administration of proteins I and II* showed low titres against their appropriate antigens. In the anti-protein I serum a relatively high antibody titre against protein II* was found, though protein II* was only a minor contaminant of the protein I preparation. Antibodies against LPS were present in both sera; in that against protein II* the titre even exceeded that against the protein itself.

Table 3. *Specific absorption of antibodies from sera against outer membrane proteins of E. coli* O26 K60

Antisera against outer membrane proteins I and II* were elicited by intramuscular administration of the antigens to rabbits. Absorptions were carried out by stepwise addition of proteins I and II*, murein-lipoprotein of *E. coli* O26 K60 or lipoprotein-free murein of *E. coli* JE5513, suspended in PBS to 10 mg ml⁻¹. Absorptions were continued until visible immunoprecipitates were no longer formed. Immunoprecipitates were removed by centrifugation (10 min, 10000 g). ELISA was performed as described in the legend to Table 2.

Antiserum		Reciprocal titre		
Elicited against:	Absorbed with:	Protein I	Protein II*	Murein-lipoprotein
Protein I	—†	20 000	80	2 560
Protein I	Protein I†	160	80	2 560
Protein I	Murein-lipoprotein O26†	20 000	80	20
Protein I	Murein JE5513†	20 000	80	2 560
Protein I	Protein I + murein JE5513†	80–160	80	2 560
Protein I	Protein I + murein-lipoprotein O26†	80	80	20
Protein II*	—‡	80–160	160 000	< 20
Protein II*	Protein II*‡	80–160	160	< 20

† Antiserum against protein I was absorbed with lipopolysaccharide O26 and protein II* before the absorptions described here.

‡ Antiserum against protein II* was absorbed with lipopolysaccharide O26 and protein I before the absorptions described here.

The specificity of the antibodies was tested by selective absorptions with several isolates of *E. coli* O26 K60 or *E. coli* JE5513. Absorption of the antiserum against protein I with the same antigen suspended in PBS effectively removed the antibodies against protein I, but the titre against murein-lipoprotein remained unchanged (Table 3). Incubation of this antiserum with murein-lipoprotein of *E. coli* O26 K60 absorbed the antibodies against this complex, without affecting the titre against protein I. Absorption with lipoprotein-free murein isolated from *E. coli* JE5513 showed that no antibodies directed against murein were involved. All antibody activity was removed by a combined absorption with protein I and murein-lipoprotein. Antibodies against protein II* were removed from the antiserum against this antigen with protein II* suspended sonically in PBS.

Table 4 shows the results of absorption experiments carried out with whole cells of *E. coli* O26 K60, bacteria sheared in Tris/EDTA or isolated OM. Antibodies against protein I were not absorbed to untreated or sheared bacteria and insignificantly to isolated OM. Antibodies against lipoprotein were absorbed to untreated as well as to sheared cells but were removed more effectively by incubation with isolated OM. The antibody titre against protein II* decreased after absorption with whole or sheared bacteria, but absorption with isolated OM again gave the strongest reduction.

Interfacial immunoprecipitation

OM of *E. coli* O26 K60, solubilized by boiling in 2% (w/v) SDS, followed by dilution to 0.2% (w/v) SDS, was reacted in IIP tests with LPS-absorbed antisera. SDS-PAGE analysis of the immunoprecipitates revealed that those obtained with antisera against whole bacteria or isolated OM contained predominantly protein II* and much less protein I. The antiserum against protein I (i.m. administered) precipitated predominantly protein I, although a considerable quantity of protein II* was also detected. Pre-absorption of this antiserum with protein II* gave a decrease, though not a disappearance, of protein II* from the immunoprecipitates. Application of the antiserum against protein II* (i.m. administered) gave immunoprecipitates containing exclusively protein II*. The antisera,

Table 4. *Absorption of antibodies against outer membrane proteins to the proteins in their native situation*

Antisera against outer membrane proteins I and II* of *E. coli* O26 K60 were elicited by intramuscular administration to rabbits. Antisera, undiluted and diluted 1:20 or 1:80 with PBS (pH 7.2), were subjected to ten incubations with approximately 2×10^9 *E. coli* O26 K60 cells, untreated or sheared in Tris/EDTA, or to one incubation with 5 mg isolated OM (ml serum)⁻¹. ELISA was performed as described in the legend to Table 2.

Antiserum		Reciprocal titre after absorption in serum dilutions															
		Reciprocal titre				Undiluted				1 : 20 dilution				1 : 80 dilution			
		Protein I	Protein II*	Murein-lipo-protein	Absorbed with:†	Protein I	Protein II*	Murein-lipo-protein	Protein I	Protein II*	Murein-lipo-protein	Protein I	Protein II*	Murein-lipo-protein	Protein I	Protein II*	Murein-lipo-protein
Elicited against:																	
Protein I	—	20000		Whole bacteria			2560	20000			1280	20000			1280	20000	640
Protein I	Whole bacteria							20000			640	20000			640	20000	320
Protein I	Sheared bacteria							10000			160	10000			80-160	10000	80
Protein I	Outer membranes																
Protein II*	—		160000														
Protein II*	Whole bacteria									40000			40000			20000	
Protein II*	Sheared bacteria									20000			10000			10000	
Protein II*	Outer membranes									640			640			320	

† Antisera were absorbed with lipopolysaccharide O26 before the absorptions described here.

resulting from i.v. administration of protein I or II* both precipitated a small amount of protein II* and no protein I. Pre-immunization sera applied in IIP tests gave no immunoprecipitation.

DISCUSSION

Our results show that high titres of antibodies against the major OM proteins I and II* of *E. coli* O26 K60 were raised after intramuscular (i.m.) administration of the isolated proteins to rabbits. After intravenous (i.v.) administration of these preparations a very low antibody response to the proteins was found, while the titre against LPS was higher than expected. The significant presence of anti-LPS in these sera may be caused by the fact that the hydrophobic OM proteins acted as carriers for the contaminating LPS molecules. The hydrophilic polysaccharide O-chain of the LPS probably protruded from the protein-LPS complex, thus providing a highly immunogenic conformation for the O antigen. Apart from this passive carrier function, the OM proteins might well have played a more active part in antibody formation against LPS. LPS-associated protein is known to stimulate murine lymphocytes *in vitro* (Sultzer & Goodman, 1976; Betz & Morrison, 1977) and to evoke the proliferation of rabbit splenic lymphocytes and human peripheral blood lymphocytes (Goodman & Sultzer, 1979*a, b*), while protein-free LPS is inactive. The antibody response against the LPS in our protein preparations might indicate that protein II* and possibly protein I could be responsible for B-cell proliferation resulting in antibody synthesis, after priming with LPS by the first injections of the immunization series. This may also provide an explanation for the observation that i.v. administration of protein-free LPS did not evoke an anti-LPS response (results not shown). The low antibody response against the proteins after i.v. injection shows that these proteins are obviously poor immunogens after administration by this route, despite the particulate character of these hydrophobic antigens. The poor antibody response against the OM proteins after i.v. injection of whole bacteria or isolated OM may be due to inaccessibility as a result of LPS shielding the proteins (Hofstra *et al.*, 1979).

After i.m. administration with Freund's complete adjuvant, the antigen is released very slowly from the i.m. depot. This may have prevented the antibody synthesis against the small amount of LPS, while antibody formation against the proteins was apparently effectively stimulated.

A considerable antibody titre against murein-lipoprotein was detectable in our antiserum against isolated protein I (i.m. administered), though this protein seemed electrophoretically pure. The fact that absorption of this antiserum with the same protein I preparation did not affect the antibody titre against murein-lipoprotein illustrates that protein I was only minutely contaminated with lipoprotein. The tight linkage between lipoprotein and the peptidoglycan-bound OM protein I (DeMartini & Inouye, 1978) may prevent proper separation by column chromatography. Indeed, the antiserum against protein II* (not peptidoglycan-bound) was free from antibodies against lipoprotein, which excludes the presence of lipoprotein in this protein. In antiserum against OM the antibody titre against murein-lipoprotein was evidently higher than in the serum against whole cells. This corresponds to the findings of Braun *et al.* (1976) who found a relation between antibody formation against enterobacterial lipoprotein and the exposure of this antigen on several smooth and rough (Ra- to Re-) strains of various *Enterobacteriaceae* species.

The unusual character of hydrophobic OM proteins inhibits the application of the usual immunochemical methods (Salton & Owen, 1976). Their insolubility requires the use of SDS as a solubilizing agent in methods based upon diffusion in agarose. Without the use of SDS our results obtained in double diffusion tests were insignificant. Non-immunological precipitation of antisera with SDS (Palmer *et al.*, 1971; Cho & Feng, 1974) is contradicted by the specificity of our precipitin lines. SRID led to a satisfactory estimation of antibody

titres against OM proteins and LPS. However, without SDS, precipitation haloes were only seen with LPS. Attempts to detect antibodies against murein-lipoprotein in SRID with or without the use of SDS were unsuccessful. The ELISA system was the only method allowing standardization of the procedure irrespective of the chemical or physical nature of the antigens applied. Results of IIP experiments carried out with OM of *E. coli* O26 K60 boiled in SDS agreed with previous observations (Hofstra & Dankert, 1979). However, IIP appeared to be not only less convenient, but also less specific than the other methods applied. The antiserum against protein I (i.m. administered) indeed precipitated predominantly protein I but also some protein II*, even after pre-absorption of this antiserum with protein II*. Thus some non-specific precipitation of protein II* in IIP cannot be excluded. As long as the isolated OM proteins and their specific antisera were not available, IIP was the most useful method for the immunological investigation of these antigens.

Our experiments showed that antibodies against LPS, lipoprotein and proteins I and II* could be specifically absorbed with the appropriate antigens solubilized or suspended in PBS. Absorptions to intact or sheared bacteria or isolated OM showed that anti-protein I was hardly absorbed or not at all. This indicates that protein I is buried too deep in the structure of the OM to be reached by its antibody, or that the antibody elicited against heat-denatured protein I is not able to react with the antigen in its native configuration. Experiments carried out with an antiserum made against a new preparation of protein I, more resembling its native configuration, showed a significantly better absorption to whole bacteria or isolated OM (unpublished results). Heat denaturation of the antigen is probably the cause of the lack of absorption found in this study. Antibodies against protein II* and lipoprotein were absorbed by intact or sheared bacteria, but the absorption to isolated OM was much stronger. This implies that the accessibility of these antigens to their specific antibodies is interfered with by other wall components, possibly LPS (Hofstra *et al.*, 1979), or that these proteins are not located predominantly on the outer side of the OM. The localization on the OM has not yet been fully evaluated (Di Rienzo *et al.*, 1978). However, results of studies concerning phage reception by OM proteins (Datta *et al.*, 1977; Van Alphen *et al.*, 1977) and labelling by CNBr-activated dextran (Kamio & Nikaido, 1977) indicate their accessibility on the outer surface of the bacteria. Our findings are in agreement with those of these workers although a relatively deep location is suggested by the fact that antibodies were absorbed significantly better by isolated OM than by (sheared) bacteria.

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