Role of Antibodies against Outer-membrane Proteins in Murine Resistance to Infection with Encapsulated *Klebsiella pneumoniae*

By BAZIR AKE SERUSHAGO,¹* MASAO MITSUYAMA,²
TOSHIYA HANDA,¹ TETSUYA KOGA¹ AND KIKUO NOMOTO¹

¹Department of Immunology, Medical Institute of Bioregulation, Kyushu University 69,
3-1-1 Madashi, Higashi-Ku, Fukuoka 812, Japan

²Department of Bacteriology, Niigata University School of Medicine, Niigata 925, Japan

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In the assessment of immunity to the encapsulated virulent strain of *Klebsiella pneumoniae* and its avirulent mutant defective for capsular polysaccharide (CPS), killed bacterial vaccine of both strains could protect mice equally against challenge with $100 \times \text{LD}_{50}$ of encapsulated wild strain. Antisera to each strain conferred the same level of protection on naive mice upon transfer; the protective anti-mutant serum was highly capable of opsonizing the encapsulated bacteria. In addition to the common antigenic components shared by both strains, the wild strain had antigen(s) unrelated to the mutant since the protective capacity of the anti-wild serum was not affected by preabsorption with the mutant strain; the protection conferred by the anti-mutant serum was mediated by antibodies against non-capsular antigens since the antiserum did not contain antibodies against purified CPS detectable by ELISA. As possible candidates among the non-capsular antigens, outer-membrane proteins (OMPs) extracted from the mutant strain were examined for their immunogenicity. Immunoblotting of the protein-containing fraction and ELISA using LPS-free OMP suggested that a number of proteins were involved in the immune response evoked by *K. pneumoniae*. Furthermore, mice immunized with OMP or anti-OMP serum could overcome a lethal challenge with the wild strain. These results indicated that OMPs of *K. pneumoniae* are implicated as the protective antigens and may pave the way for the development of non-capsular, proteinaceous vaccines.

INTRODUCTION

Klebsiella pneumoniae causes high morbidity and mortality especially in neonates, and in elderly and immunocompromised hosts (Liu *et al.*, 1986; Montgomerie, 1979). With growing limitations in the use of antibiotics, due to the generation of drug resistant strains (Denoya *et al.*, 1986) and the increased cost of antibiotic production, the possibility of controlling infection by prophylactic immunization is being assessed. Surface antigens that are major or minor determinants of pathogenicity have been sought, since they may be candidates for new or improved vaccines. Several factors contributing to the virulence of *K. pneumoniae* have been widely reported (Highsmith & Jarvis, 1985; Williams *et al.*, 1986). With respect to the critical role of capsular polysaccharide (CPS) in the outcome of severe infections with *K. pneumoniae* (Cryz *et al.*, 1984a; Domenico *et al.*, 1982; Simoons-Smith *et al.*, 1986), attempts at prophylactic prevention have used either inactivated encapsulated bacteria (Cooper *et al.*, 1983; Jones & Roe, 1984), ribosomal preparations (Fournier *et al.*, 1981; Riottot *et al.*, 1979, 1981) or purified CPS (Cryz *et al.*, 1984b, 1985) as vaccines. In general it has been accepted that CPS is not only the major virulence factor but also the major protective antigen. Several reports showing that

Abbreviations: CPS, capsular polysaccharide; IFA, incomplete Freund's adjuvant; i.p., intraperitoneally; OMP, outer-membrane protein; PEC, peritoneal exudate cells; RT, room temperature.

repeated stimulation with CPS of *K. pneumoniae* induced an antibody response to syngeneic tissue extracts (Nakashima & Kato, 1975), and that the breakdown of tolerance might result in autoimmune arthritis (Kurbeski *et al.*, 1983; Trull *et al.*, 1983), have drawn attention to the need to search for other potential immunogens of this bacterium. Although Williams *et al.* (1983) reported that antisera raised against non-capsular antigens of *K. aerogenes* (= *K. pneumoniae*) could enhance the *in vitro* phagocytosis of encapsulated bacteria, little is known about the role of surface antigen(s) other than CPS in immunity to encapsulated *K. pneumoniae*.

In the present study, we examined the ability of antisera against non-capsular antigens of *K. pneumoniae* to confer protection on naive murine hosts against the encapsulated wild strain and its CPS-defective mutant.

METHODS

Animals. Female mice of an outbred ddY strain were purchased from Shizuoka Laboratory Animal Center (SLC, Hamamatsu, Japan) and were used at 6–7 weeks of age. They were maintained in specific pathogen-free conditions.

Bacteria and growth conditions. Two variants of *Klebsiella pneumoniae*, maintained in our laboratory, were used. The wild-type, strain Ful1, was originally a clinical isolate. Ful1-m21, a variant which lacked the ability to form a capsule, was a mutant obtained by UV irradiation of Ful1 (Osada *et al.*, 1982). Ful1 and Ful1-m21 are K⁺1 and K⁻ capsular serotypes, respectively. Further characteristics of the strains are described in Results. Bacteria (3 ml) cultured overnight at 37 °C in tryptic soya broth (Difco) were transferred to 60 ml fresh broth in test-tubes and cultured for an additional 4–6 h at 37 °C. After three washes with phosphate-buffered saline (PBS), bacteria were suspended in PBS supplemented with 10% (v/v) glycerol and stored at -70 °C, before use for the challenge of mice. Formalin-killed bacteria were used for active immunization. The concentration of the bacterial suspension was calculated by colony counts on nutrient agar plates (Eiken Chemical Co., Tokyo, Japan) and expressed as colony-forming units (c.f.u.).

Extraction of surface components. (a) *Purification of CPS.* CPS was purified using a modification of methods previously described (Batshon *et al.*, 1963; Cryz *et al.*, 1984b). Supernatant collected from 3 d shaking cultures of the wild strain in heart infusion broth (Difco) was treated overnight with 2 vols 95% (v/v) ethanol at 4 °C. The resulting precipitate was collected by centrifugation at 8500 g for 30 min and dissolved in water. Water-insoluble material was removed by additional centrifugation. The supernatant was then dialysed against water for 12 h at 4 °C. Proteins were removed by shaking with 2 vols chloroform/*n*-butanol (5:1, v/v). After centrifugation at 1000 g for 10 min, the upper phase (avoiding the insoluble material at the interphase) was collected and re-extracted a further four times. The upper phase was then dialysed against water, any precipitate was removed by ultracentrifugation at 100000 g for 16 h and the CPS obtained by precipitation with 95% ethanol. Contaminating lipopolysaccharide (LPS) was deacylated with NaOH/ethanol solution, since this alkali treatment does not affect the antigenicity of CPS (Cryz *et al.*, 1985). CPS (5 mg) was placed in 2 ml 0.1 M-NaOH in 95% ethanol and incubated at 37 °C for 30 min. The solution was neutralized with 1 M-acetic acid, and CPS was removed by centrifugation. The preparation contained more than 75% carbohydrate and less than 1% protein as quantified by the phenol/sulphuric acid method (Dubois *et al.*, 1956) and that of Lowry, respectively.

(b) *OMP preparation.* Bacteria collected from 2 l of culture were washed with 0.03 M-Tris/HCl buffer, pH 8.1, and resuspended in 20 ml of the same buffer containing 20% (w/v) sucrose. The bacterial suspension was treated with 0.1 vol. freshly prepared lysozyme (Sigma; 1 mg ml⁻¹) in 0.1 M-EDTA, pH 7.5. After incubation for 30 min on ice, the periplasmic fraction was removed by centrifugation at 15000 g for 15 min. Spheroplasts were suspended in 20% (w/v) sucrose in 3 mM-EDTA, pH 7.5, and subsequently ultrasonicated. To remove intact spheroplasts, the lysate was centrifuged at 1000 g for 15 min at 4 °C. Supernatant was collected and overlaid onto a two-step gradient consisting of 15% and 70% (w/v) sucrose in 3 mM-EDTA. After ultracentrifugation at 145000 g for 2 h a crude membrane fraction settled at the interphase between the upper phase and the 70% cushion. The crude preparation was subjected to another two-step sucrose gradient of 53 and 70% (w/v) sucrose in 3 mM-EDTA. The OMP fraction was collected in the 53% sucrose layer just above the 70% cushion as reported by Hansen *et al.* (1981) and Yamamoto *et al.* (1975). Contaminating LPS was removed using Detoxi-Gel, a gel with specific affinity for endotoxins, according to the manufacturer's instructions (Pierce). To ascertain the effectiveness of the procedure, LPS content in the OMP preparation was quantitatively measured before and after passage through the column using an endotoxin-specific chromogenic kit test (Seikagaku Kogyo Co., Tokyo, Japan) (Ikeda *et al.*, 1987; Obayashi *et al.*, 1985, 1986). An initial value of contaminating LPS of 99.7 mg ml⁻¹ could be reduced to as little as 0.380 ng ml⁻¹ after column passages. In all experiments, OMP preparations were pretreated with Detoxi-Gel to remove the contaminating LPS. Protein concentration was determined by the Lowry method.

Immunization. In immunization of mice with whole bacterial cells, formalin-killed bacteria were used in all

experiments because of the high virulence of the wild strain. A sample of cells derived from the same batch that was used for challenge was treated overnight with formalin at a final concentration of 1% (v/v). The cells were subsequently washed and suspended in PBS supplemented with 10% (v/v) glycerol before storage at -70°C . For active immunization, mice were intravenously injected with 10^8 c.f.u. of either the wild or mutant strain and the same dose of booster was given 10 d later. In experiments performed with OMPs extracted from mutant cells, mice were subcutaneously primed with $10\text{ }\mu\text{g}$ of OMP in incomplete Freund's adjuvant (IFA) followed by an intravenous injection with $5\text{ }\mu\text{g}$ of OMP 10 d later. Immune sera were collected 5 d after the final booster.

Passive immunization was done by transferring 1 ml of antisera intraperitoneally (i.p.) to naive mice. Non-immune mouse sera were obtained from age-matched, untreated mice for control experiments.

Virulence of the strains. Groups of 10 mice were challenged i.p. with a variety of dilutions of each strain and the mortality was recorded for 7 d. The 50 lethal dose (LD_{50}) was calculated by the method of Reed & Muench (1938).

Protection experiments. A $100 \times \text{LD}_{50}$ dose (10^4 bacteria) of the encapsulated strain was used to assess the effect of active and passive immunization on murine resistance. Groups of 5 to 8 mice, actively immunized with killed bacteria of either the wild or the mutant strain, were challenged with encapsulated bacteria 10 d after priming. In the passive immunization system, 1 ml of twofold serially diluted antiserum was injected i.p. into naive mice and challenge was carried out 4 h later. Survival of mice was recorded for 1 week after the challenge and expressed as percentage survival. Death of mice was usually observed within 3–4 d of the challenge infection.

Absorption of antisera. Immune sera were absorbed with formalin-killed bacteria by conventional methods. In brief, bacteria grown in tryptic soya broth were washed and suspended to $200\text{ mg (wet weight) ml}^{-1}$ in PBS. The bacterial suspension was incubated overnight in 1% (v/v) formalin at 4°C . After several washes, the cell pellet was suspended in antiserum to give a concentration of 200 mg ml^{-1} . The mixtures incubated at room temperature for 2 h were subjected to an overnight rotating incubation at 4°C . After centrifugation at 17400 g for 25 min, supernatants were collected and used either immediately or stored at -20°C .

Antibody titration by ELISA. Antibodies in immune sera were assessed by an indirect ELISA (Engevall & Perlman, 1972), using glutaraldehyde-fixed whole bacteria or OMP fraction as solid-phase antigens (Black *et al.*, 1985). Formalin-killed wild strain ($50\text{ }\mu\text{l}$) suspended in PBS to give an OD_{440} value of $0.9 (10^8\text{ ml}^{-1})$, were placed in PVC microplate wells. The wells were dried overnight at 37°C and fixed with 0.2% glutaraldehyde. In other experiments, plates were coated overnight at 4°C with $50\text{ }\mu\text{l}$ OMP ($2.5\text{ }\mu\text{g ml}^{-1}$). Test sera, serially diluted in 0.05% (v/v) PBS/Tween 20, were added to individual wells precoated with 0.5% (w/v) bovine serum albumin in coating buffer. Microplates were incubated in a humidified chamber at room temperature (RT) for 90 min followed by repeated washes with PBS/Tween 20. Peroxidase-conjugated goat anti-mouse IgG antibodies (Tago, Burlingame, California, USA) diluted 1:800 in PBS/Tween 20 were added to the wells and incubated for a further 90 min in a humidified chamber. After washing as described above, $50\text{ }\mu\text{l}$ of freshly prepared substrate solution consisting of *o*-phenylenediamine (0.4 mg ml^{-1}) and 0.03% (v/v) H_2O_2 in citrate buffer was added to the wells. The reaction was terminated by adding 2.5 M- H_2SO_4 and absorbances were read at 492 nm using an EAR 400 FW (SLT-Lab Instruments, Austria). Titres were expressed as the reciprocal of the highest dilution of sera yielding an A_{492} value of 1.78.

Specific antibodies for CPS in immune sera were measured using the poly-L-lysine precoat technique (Leinonen & Frasch, 1982) with modification as follows. PVC microplates, precoated by an incubation for 5 h at RT with $50\text{ }\mu\text{l}$ poly-L-lysine solution ($M_r 150\,000\text{--}300\,000$) in PBS ($10\text{ }\mu\text{g ml}^{-1}$), were washed twice with PBS/Tween 20. CPS solution ($50\text{ }\mu\text{l}$) in PBS ($10\text{ }\mu\text{g ml}^{-1}$) was added to the wells, which were subsequently incubated overnight at RT. Sample sera were added to the wells after they had been washed and coated with bovine serum albumin. After incubation in a humidified chamber at RT for 90 min, the wells were washed and filled with $50\text{ }\mu\text{l}$ phosphatase-conjugated goat anti-mouse IgG antibody (Tago) diluted 1:800 in PBS/Tween 20, and the microplates were again incubated for 90 min in a humidified chamber at RT. To each well $50\text{ }\mu\text{l}$ substrate solution composed of *p*-nitrophenyl phosphate in diethanolamine buffer (5 mg ml^{-1}) and 0.3 mM- MgCl_2 was added and, after an incubation for 20 min, 2 M- NaOH was added. Titres were expressed as the reciprocal of the highest dilution of sera yielding an A_{405} of 0.5.

Opsonization assay. *In vitro* phagocytosis of the wild strain by peritoneal exudate cells (PEC) was examined. PEC were induced by i.p. injection of 2 ml of 2% (w/v) casein and collected by washing the peritoneal cavity of the mice with Hanks' balanced salt solution 4 h after injection. PEC (5×10^6 cells), washed and suspended in 1 ml Hanks' balanced salt solution, were incubated with $100\text{ }\mu\text{l}$ of relevant bacterial strain (10^7) in the presence of $100\text{ }\mu\text{l}$ of non-complement depleted test sera for 15 min at 37°C . The mixtures were washed three times and pelleted cells were lysed with distilled water. The number of phagocytosed bacteria was determined after plating serially diluted samples on nutrient agar for 12 h at 37°C .

SDS-PAGE and Western blotting. Whole lysates of bacteria or the OMP preparation adjusted to $200\text{ }\mu\text{g protein ml}^{-1}$ were boiled for 3 min in sample buffer [60 mM-Tris/HCl buffer containing 10% (w/v) SDS and 5% (w/v) 2-mercaptoethanol]. SDS-PAGE was performed at a constant current in a 12.5% (w/v) separating gel (Laemmli, 1970). After electrophoresis, the proteins were electrophoretically transferred to nitrocellulose paper (Schleicher &

Schuell) under constant voltage at 30 V (Towbin *et al.*, 1979) using a Trans-Blott system (Bio-Rad). The nitrocellulose sheet was cut into strips and each strip was incubated in immune serum diluted 1:70 in PBS/Tween 20 for 90 min on a rocking platform. After washing, the strips were incubated in peroxidase-conjugated goat anti-mouse IgG antibody (Tago) diluted 1:800 in PBS/Tween 20. Repeatedly washed paper strips were soaked in a substrate solution consisting of 0.04% (w/v) 4-chloro-1-naphthol and 0.01% H_2O_2 in 50 mM-Tris/HCl, pH 7.5. The reaction was stopped with distilled water a few minutes after visualization of the bands.

RESULTS

Difference between wild and mutant strains of K. pneumoniae

After overnight culture of the wild strain Ful on nutrient agar, large and highly mucoid colonies were formed, while colonies of the mutant strain, Ful-m21, were small and less mucoid. Biochemical examination of the metabolic activity of both strains showed no difference. Staining by the Hiss method (Lennep *et al.*, 1974) showed the presence of a capsule in the wild strain. Electron micrographs revealed a thick capsular substance surrounding the wild strain whereas the mutant failed to form such a structure (not shown). Serological typing showed that both Ful and Ful-m21 possessed identical O antigens (O1) but were K^+ and K^- , respectively. The virulence of each strain was determined by LD_{50} in ddY mice. The wild strain was found to be extremely virulent as shown by a difference of over 10^6 -fold in the LD_{50} value between Ful ($\text{LD}_{50} < 100$ c.f.u.) and its counterpart Ful-m21 ($\text{LD}_{50} > 10^8$ c.f.u.).

Effect of immunization with killed bacteria on protection against infection with the wild strain

Mice immunized with formalin-killed cells of the wild or mutant strain were challenged 10 d later with $100 \times \text{LD}_{50}$ of the wild strain. As expected, immunization with the wild strain provided 100% protection. Surprisingly, even the avirulent mutant, defective in capsule formation, induced full protection against the lethal challenge with encapsulated bacteria. To determine whether the protection was mediated by antibodies, naive mice were passively immunized by transferring 1 ml of serially diluted sera from immune mice. Mice given anti-mutant sera completely overcame the challenge of infection with a lethal dose of the live wild strain as did their counterparts treated with anti-wild serum. All mice that received non-immune mouse serum died 3–4 d after the challenge. Up to 32-fold dilution of either antiserum gave 100% protection (data not shown).

Opsonizing effect of anti-mutant serum on in vitro phagocytosis of the wild strain

Since antibodies directed against the mutant strain defective in capsule formation were able to confer protection against the encapsulated strain, we examined the opsonizing capacity of anti-mutant serum on the wild strain in an *in vitro* phagocytosis system (Table 1). The wild strain was highly resistant to phagocytosis by PEC in the absence of opsonization compared to the mutant strain. Uptake of the wild bacteria by casein-induced PEC was increased by almost 10-fold in the presence of anti-mutant or anti-wild serum (Table 1). There was no significant difference in the opsonizing capacity on the wild strain between these two immune sera.

Effect of absorption of antisera with inactivated bacteria on protective activity and antibody titres

Experiments were designed to determine whether the wild and mutant strains have the same or different antigenic features. As shown in Table 2, absorption with killed mutant cells could not remove the protective ability of anti-wild serum. In contrast, absorption of anti-mutant serum with the wild strain abolished the ability of the serum to confer protection on naive hosts. This observation strongly suggested that both strains shared the common protective antigen(s) while unrelated antigen(s) was present only in the wild strain. In view of the electron micrograph data, it could be postulated that the common antigens, responsible for protection induced by the mutant, are of non-capsular nature. In order to verify this possibility, antibodies specific for CPS were assessed by ELISA. Equal levels of antibody titre, as high as 1024, were detected in both antisera when individually tested against whole inactivated wild strain as fixed antigens (Table

Table 1. *Opsonizing effect of immune sera on in vitro phagocytosis of the wild strain*

Casein-induced PEC (5×10^6) were incubated with 0.1 ml of the appropriate bacterial strain (10^7 cells) in the presence of 0.1 ml of test serum.

Serum used	$10^{-3} \times$ No. of <i>K. pneumoniae</i> phagocytosed by PEC*	
	Wild strain	Mutant strain
None	11 ± 2	288 ± 26
Non-immune	30 ± 7	483 ± 124
Anti-wild	373 ± 7	600 ± 3
Anti-mutant	709 ± 112	630 ± 72

* Number of bacteria taken up by effector cells. Mean $\pm$ SD of three plates.

Table 2. *Correlation of protective activity with antibody titres in sera as measured by ELISA using whole wild K. pneumoniae or its purified CPS*

Serum*		Antibody titre against:		Passive protection (%)†
Antibody to:	Absorbed with:	Whole bacteria	CPS	
NMS‡	—	<16	<16	0
Wild	—	1024	1024	100
Wild	Wild	<16	<16	0
Wild	Mutant	512	512	100
Mutant	—	1024	64	100
Mutant	Wild	<16	<16	0
Mutant	Mutant	<16	<16	0

* Antisera were used for ELISA and passive protection before or after absorption with the appropriate bacterial strain.

† Percentage survival of groups of at least five mice passively immunized with 32-fold diluted antiserum.

‡ Non-immune mouse serum.

2). The antibody titre of anti-mutant serum against purified CPS was only 64, as against 1024 found for the anti-wild serum. Although this titre is somewhat higher than that in non-immune mouse serum, it is unlikely that it represents CPS-specific antibodies. The detection of a low level of anti-CPS antibody might rather be attributable to the presence of a certain amount of LPS in the CPS preparation. As reported by Cryz *et al.* (1985), although the technique used here removes the majority of LPS, trace amounts may remain. ELISA quantification with purified CPS of the mutant-absorbed parental antiserum is good evidence for the absence of capsular antigens on the mutant strain. The anti-wild serum, which was protective even after absorption with mutant cells, exhibited a high level of antibodies against CPS. Likewise, anti-mutant serum contained antibodies raised against non-capsular antigens. With respect to the importance of these non-capsular antigens in the induction of resistance against encapsulated bacteria, further experiments were performed to examine whether OMPs are involved or not.

Assessment of immunogenicity of OMPs

An OMP preparation obtained from the mutant strain was examined for antigenicity. In order to eliminate contaminating LPS and to ascertain the protein nature of antigens used in these assays, the preparation was passed through a gel column (Detoxi-Gel) and/or subsequently digested with proteinase K, a non-specific proteolytic enzyme. The preparation completely lost its antigenicity in ELISA when treated with proteinase K after passage through Detoxi-Gel.

Fig. 1. Lanes B and C, SDS-PAGE of whole lysates of wild and mutant bacteria, respectively. Lane A, molecular mass markers. Coomassie blue staining was used to detect protein bands.

However, an OMP preparation treated with proteinase K without passage through a Detoxi-Gel column retained some antigenicity (data not shown), implying that column passage was necessary to eliminate contaminating LPS. Since the column should drastically reduce LPS contamination, to a level undetectable by ELISA (0.38 ng ml^{-1}), Detoxi-Gel-passed OMP was regarded as an endotoxin-free preparation and was used in subsequent experiments. Titres of antibodies as high as 1024 and 4096 were detected against Detoxi-Gel-passed OMP in anti-wild and anti-mutant serum by ELISA, respectively. In addition, the LPS-free OMP, used as a vaccine, could elicit a high titre of specific antibodies (1024) in host mice. Whole lysates of wild and mutant cells were analysed by SDS-PAGE followed by staining with Coomassie blue. The profile of protein bands of the two strains was similar (Fig. 1). Using immunoblotting, we compared the profile of antigenic polypeptides recognized by antibodies in the anti-mutant and anti-wild serum. Whole ultrasonicated mutant cells or OMP preparations were resolved by SDS-PAGE followed by Western blotting. The homologous (anti-mutant) and heterologous (anti-wild) antisera contained antibodies directed against the same peptides in the whole mutant lysates (Fig. 2 B, C). The pattern of binding to polypeptides in the OMP extract was also similar in anti-mutant and anti-wild serum (Fig. 2 D, E). Again, digestion of the OMP with proteinase K abrogated the antigenicity recognizable by both antisera (Fig. 2 F, G).

In vivo protection by OMP vaccine

The results of ELISA and immunoblot analysis implied that OMPs take part in the host immune responses evoked by *K. pneumoniae* and this prompted us to investigate the protection conferred by use of OMP as vaccine. Table 3 shows that immunization of mice with OMP in IFA provided full protection against a lethal challenge with the wild strain ($100 \times \text{LD}_{50}$). To determine whether antibodies raised against OMP could mount passive protection, a pool of

Fig. 2. Immunoblot analysis of proteins present in whole cells of the mutant strain and the outer-membrane-containing fraction prepared from these cells. Antigenic proteins in crude lysates of the mutant strain were reacted with anti-mutant (B) and anti-wild (C) antisera. OMPs were used as antigens to detect specific antibodies in the homologous (anti-mutant, D) and heterologous (anti-wild, E) antisera. Lanes F and G represent the former and the latter antisera tested against proteinase-K-treated OMPs. Lane A, molecular mass markers.

Table 3. *Effect of immunization with OMPs of the mutant strain, or with antiserum prepared against OMPs, on murine resistance to infection with wild K. pneumoniae*

Immunization	Survival*
Actively with:†	
None	0/5 (0)
OMP	7/7 (100)
Proteinase K-digested OMP	0/6 (0)
Passively with:‡	
Anti-OMP serum, 1:2	5/5 (100)
1:4	6/7 (86)
1:8	3/7 (42)
1:16	3/10 (30)
Anti-mutant serum, 1:32	5/5 (100)
Anti-immune serum, 1:4	0/5 (0)

* Number of survivors/total number of mice after observation for 7 d. Percentage survival is shown in parentheses.

† Mice were immunized with OMP preparations in IFA and challenged with $100 \times LD_{50}$ wild strain 10 d later.

‡ Serially diluted serum was transferred i.p. to naive mice and they were infected with a lethal dose of wild bacteria 4 h later.

antisera against OMP was i.p. transferred into naive mice before the lethal challenge. These mice survived better than control animals that received non-immune mouse serum. However, high dilutions of the anti-OMP sera were less protective than similar dilutions of antisera prepared against whole mutant cells. The difference in protection between these two antisera correlated with that in ELISA titres against fixed OMP antigens.

DISCUSSION

CPS appears to be the critical factor in the outcome of severe infections with *K. pneumoniae*. The large capsule surrounding the cell protects *K. pneumoniae* from phagocytosis and is therefore largely responsible for the escape from host defence mechanisms and hence the capacity to cause diseases (Cryz *et al.*, 1984a; Domenico *et al.*, 1982; Simoons-Smith *et al.*, 1986). To combat this

bacterial escape mechanism, the host elaborates specific antibodies capable of enhancing phagocytosis of the bacteria by effector cells (Fukutome *et al.*, 1980). Considerable attention has been focused upon the antibodies raised against the capsular matrix in protection against *K. pneumoniae*. Thus far, trials on immunological therapy have been based mainly on CPS-specific antibodies using purified CPS or inactivated encapsulated bacteria (Cryz *et al.*, 1985; Jones & Roe, 1984).

The results presented here confirm the relationship between the presence of CPS and the virulence of *K. pneumoniae*. The encapsulated wild strain was extremely virulent as determined by its LD₅₀ value in mice, whereas the capsule-defective mutant could be regarded as avirulent, since its LD₅₀ value was over 10⁸ c.f.u. Accordingly, it was expected that protective immunity would be generated only by vaccination with wild strain, but not with the mutant strain. Contrary to such expectation, the experimental results showed that both strains equally induced protection against challenge with encapsulated virulent *K. pneumoniae*. Passive immunization using diluted antisera revealed that there was no difference in the protective titre between anti-wild and anti-mutant sera from immune mice.

Absorption experiments and ELISA showed that the protective anti-wild serum contained antibodies to both capsular and non-capsular antigens, while anti-mutant serum only contained the latter. Anti-mutant serum containing no anti-capsular antibody was highly effective in the opsonization of encapsulated bacteria in an *in vitro* phagocytosis by polymorphonuclear-leucocyte-rich PEC. This observation is consistent with the enhancement of *in vitro* phagocytosis of encapsulated *K. aerogenes* (= *K. pneumoniae*) by antiserum against unencapsulated variants (Williams *et al.*, 1983). Possible candidates for these somatic antigens include both LPS and proteins present in the outer membrane of this Gram-negative bacterium. In view of the controversial reports on the protective capacity of anti-LPS antibodies, particularly those showing their failure to prevent infection with Gram-negative bacteria including *Haemophilus influenzae* type b (Shenep *et al.*, 1983) and *K. pneumoniae* (Cooper & Rowley, 1982), we investigated the role of OMPs in protection against encapsulated *K. pneumoniae*. OMPs extracted from the mutant strain contained antigenic polypeptides recognized by antibodies prepared against whole wild or mutant cells. In addition, the antigenicity of the protein components was revealed using Detoxi-Gel-passed-OMP for ELISA assessment. High levels of specific antibodies were detected in both anti-wild and anti-mutant sera, as well as in antiserum elicited by OMP. Active immunization with LPS-free OMP fully protected mice from the lethal infection. Furthermore, antiserum prepared against OMP was protective although a higher concentration was required for protection by passive immunization. It is conceivable that a number of OMPs are responsible for the induction of protective immunity, since digestion of the OMP preparation with proteinase K completely removed its activity. This observation is consistent with the results obtained from experimental bacteraemia with *H. influenzae* type b in rats which showed that several different OMPs could induce protective antibodies against the bacterium (Hansen *et al.*, 1981; Kimura *et al.*, 1985; Shenep *et al.*, 1983). Cooper & Rowley (1982) reported a heat-labile antigen that could induce antibody-mediated protection against infection with encapsulated *K. pneumoniae* in mice, although the exact nature of the antigen was not elucidated.

Our results present strong evidence of the immunogenic capacity of OMPs to induce protective antibodies against an encapsulated strain of *K. pneumoniae*. They also support further studies aimed at determining the structure of the immunogenic proteins that may pave the way for vaccine development.

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