Old Herborn University Seminar Monograph 17:
Possibilities for active and passive vaccination against opportunistic infections.
Editors: Peter J. Heidt, Richard I. Walker, Volker Rusch, and Dirk van der Waaij.
Herborn Litterae, Herborn-Dill, Germany: 31-37 (2004).

O ANTIGEN SEROEPIDEMIOLOGY OF *KLEBSIELLA* CLINICAL ISOLATES AND IMPLICATIONS FOR IMMUNOPROPHYLAXIS OF *KLEBSIELLA* INFECTIONS*

M. TRAUTMANN[1], T.K. HELD[2], and A.S. CROSS[3]

[1]Institute of Hospital Hygiene, Klinikum Stuttgart, Stuttgart, Germany, [2]Department of Haematology and Oncology, Charité, Klinikum Berlin-Buch, Germany, and [3]Division of Infectious Diseases, Department of Medicine, University of Maryland, Baltimore, Maryland, USA

SUMMARY

Prevention of *Klebsiella* infections by passive immunotherapy has received more attention during the last decade. Both K antigen- and O antigen-specific antisera and monoclonal antibodies (mAbs) have been studied with respect to phagocytosis-enhancing and *in vivo* protective capacities. Our own work has focussed on the generation of O serogroup-specific rabbit antisera and O antigen specific murine antibodies. O-specific rabbit sera were absorbed extensively with heterologous O antigen strains in order to obtain highly specific typing reagents. Using these for typing a collection of 378 clinical strains, we found that 82% of them belonged to one of the 4 serogroups O1, O2ab, O3 and O5. Phagocytosis experiments using antisera and mAbs showed that O antigen specific antibodies were able to opsonise non-encapsulated strains, while fully encapsulated bacteria were rather resistant against the opsonising effect. Nevertheless, *in vivo* experiments demonstrated a prophylactic effect on both *Klebsiella* septicaemia and pneumonia in a mouse model of lethal infection. Given the limited number of O serogroups, O antigen-specific antibodies may be suited to supplement K antigen-specific hyperimmune globulins for passive immunoprophylaxis of *Klebsiella* infections.

INTRODUCTION

Klebsiella spp., in particular *Klebsiella* (*K.*) *pneumoniae* and *Klebsiella oxytoca*, are important pathogens causing a variety of nosocomial infections (*Hansen* et al., 1997). In particular, *Klebsiella* ventilator-associated pneumonia in the Intensive Care Unit (ICU) setting carries a high mortality of up to 50% (*Carpenter*, 1990). Given the frequent occurrence of highly antibiotic-resistant *Klebsiella* strains, passive immunoprophylaxis of *Klebsiella* infections has received increasing attention in recent years. It has been shown by us and other groups, that capsule (K antigen) -specific antibodies are opsonic for *Klebsiella* organisms and protect against disseminating *Klebsiella* infection in

*: Reprinted with permission from: Vaccine 22, 818-821 (2004). All references should be made to the original article.

Table 1: *Klebsiella* O antigen serogroup reference strains

Strain designation	Antigen formula	Comment
Friedländer 201	O1:K-	contains O2a
7380	O2ab:K-	-
5053	O2ac:K-	-
390	O3:K11	-
1702	O4:K42	-
4425/51	O5:K57	-
NCTC 8172	O6:K64	serologically identical with O1
264 (1)	O7:K67	-
889	O8:K69	serologically identical with O1*
1205	O9:K72	contains O2ab
337	O10:K73	no *Klebsiella***
378	O11:K78	contains O4
708	O12:K80	-

* The O8 antigen was shown chemically to be distinguished from O1 by partial O-acetylation of the polysaccharide backbone structure (*Kelly* et al., 1993). However, the two antigens are not separable by means of conventional serology.

** This strain, originally described as a novel *Klebsiella* serotype, was later shown to belong to the genus *Enterobacter* because it was motile. Both the O10 and K73 antigens were removed from the list of recognised *Klebsiella* antigens. Adapted from *Trautmann* et al., 1997.

animal models. In humans, a large clinical trial performed by the group of *Donta* and colleagues (1996) has shown that pre-treatment of ICU patients with high-titred human immunoglobulins containing antibodies specific for various *Pseudomonas* O serotypes and *Klebsiella* K serotypes prevented a significant proportion of ICU acquired infections compared to a control group treated with non-specific IgG. However, for effective prophylaxis of *Klebsiella* infections, a total of 77 recognised K antigen serotypes must be taken into account. The currently available *Klebsiella* K antigen vaccine contains no more than 24 capsular types, and the immune response against individual antigens in this vaccine is variable (*Cross* and *Cryz*, 1990). Therefore, a vaccine consisting of fewer components might be desirable.

Like other Gram-negative bacteria, *Klebsiella* also possesses a somatic or O antigen. However, except for the recent decade, little work had been done on the *Klebsiella* O antigens. Several obstacles prevented the generation of highly specific O antigen typing sera and the elucidation of the sero-epidemiology of the O antigens. Firstly, O antigen-specific sera, even when produced against less encapsulated mutants, are in reality OK sera because they always contain significant amounts of K specific antibodies. Consequently, the presence of K specific antibodies in these sera can significantly confound the results of O serotyping. Secondly, simple and quick typing methods such as agglutination cannot be used because O antibodies get "buried" within the large capsule layer of most *Klebsiella* strains.

In our own work, we focussed on the development of a reliable typing method to elucidate the sero-epidemiology of the O antigens in clinical material. Also, we produced monoclonal antibodies (mAbs) specific for different epitopes of the O1 antigen in order to test their opsonising and protective effects.

MATERIALS AND METHODS

O antigen reference strains

These strains, which are listed in Table 1, were obtained from the Statens Serum Institute, Copenhagen, Denmark.

Production of rabbit antisera and mAbs against Klebsiella O antigens

Immune sera were produced by repeated intramuscular immunisations of rabbits with boiled *Klebsiella* organisms as described (*Trautmann* et al., 1996). Antisera were raised preferably against capsule-less mutants or O antigen-identical but K antigen-heterologous strains in order to avoid a confounding effect of anti-capsular antibodies on typing results. Murine mAbs were produced by conventional immunisation schedules as described (*Trautmann* et al., 1994).

Preparation of O antigens (lipopolysaccharides)

We used the hot phenol-water method as described (*Trautmann* et al., 1996).

Clinical *Klebsiella* isolates

During a 10-year period, clinical *Klebsiella* isolates from two University hospitals (Charité Virchow Klinikum, Berlin, Germany, and University Hospital of Ulm University, Ulm, Germany) were collected and frozen. Species identification was performed by determination of the biochemical reaction profile (API 20E). Isolates recovered from any body site during routine clinical diagnostics were accepted, but only primary isolates from each patient were retained. The origin of each isolate and whether it was associated with colonisation, non-invasive or invasive infection was documented.

O antigen typing

A competitive enzyme-linked immunosorbent assay (ELISA) method was using for typing. In short, the strain to be tested was grown freshly on agar plates, harvested, and boiled to release the O antigen from the outer cell layer. Bacterial cellular debris was removed by centrifugation, and the clear supernatant was added in a 1:1 ratio to an O antigen-specific rabbit antiserum. After repeated vortexing, the mixture was transferred to ELISA wells coated with the homologous O antigen lipopolysaccharide. After incubation, the mixture was washed off, and any remaining O antigen specific rabbit antibodies that had bound to the plates were detected by anti-rabbit-IgG alkaline phosphatase conjugate. In case of a positive reaction, it could be concluded that the O antigen of the test strain did not correspond to the antibody in the typing serum. Conversely, if the reaction remained negative, O antigen identity between test strain and the typing serum could be assumed. All isolates were typed with all O specific antibodies and mAbs available (*Trautmann* et al., 1996).

Opsonophagocytic assay

This was performed by means of a microtitre plate phagocytosis assay, using Ficoll-Paque-purified human neutrophils and fresh human serum as a source of complement (*Held* et al., 2000).

In vivo protection studies

Groups of 10 mice were pre-treated intraperitoneally with mAb Ru-O1 in ascending doses. Four hours later, the animals were challenged i.p. with an estimated dose of 50 organisms of *Klebsiella pneumoniae* Caroli, a fully encapsulated and highly virulent strain (serotype O1:K2). This dose corresponded to approximately 50x the lethal dose 50% (LD50) as determined previously. Mortality was recorded for 4 subsequent days (*Rukavina* et al., 1997).

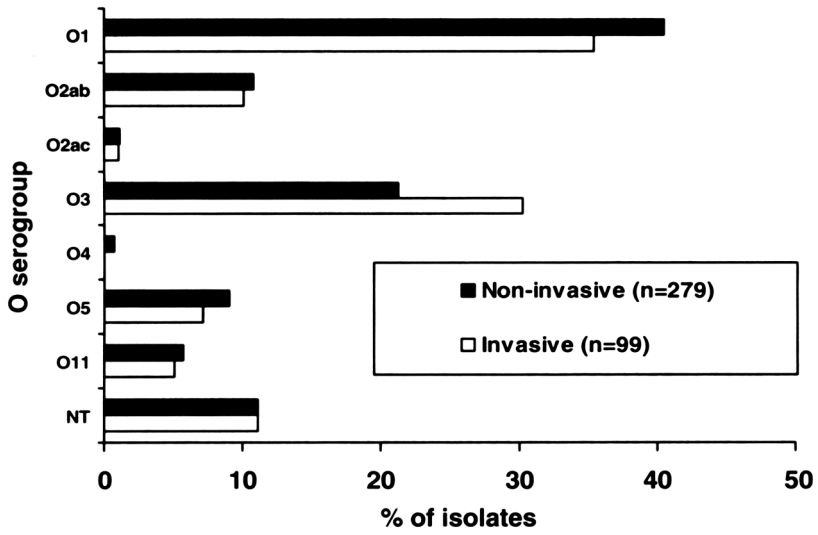

Figure 1: Relative distribution of isolates from invasive versus non-invasive infections.

RESULTS

Re-examination of O antigen reference strains

We found that the O8 antigen reference strain contained an O antigen indistinguishable by polyclonal and monoclonal serology from the O1 antigen. Therefore, we proposed to define this serogroup as a common O serogroup, although chemically, and additional O acetylation has been detected in the O8 type strain (*Kelly* et al., 1993). We also found that both of these strains contained the O2a partial antigen defined by strain 7380 (Table 1). Furthermore, we detected the O2a partial antigen in strain 1205 (O9), and the O4 antigen as a partial antigen in strain 378 (O11) (Table 1).

O antigen sero-epidemiology

A total of 378 clinical isolates were collected, 290 of which belonged to the species *K. pneumoniae* spp. *pneumoniae*, and 86 to the species *K. oxytoca*. Two isolates were identified as *K. ornithinolytica*. Ninety-nine strains were judged to have caused invasive infections because they were recovered from blood cultures (n=79), from open lung biopsies (n=7), or from the abdominal cavity during septic surgery (n=13). Typing results of invasive versus non-invasive isolates are summarised in Figure 1. Only 4 O antigens (O1, O2ab, O3, and O5) accounted for 82% of all clinical isolates, with no relevant differences between invasive and non-invasive strains (*Trautmann* et al., 1997).

O antigen specific mAbs

We raised 3 mAbs which we designated Ru-O1, IV/4-5 and V/9-5. MAb Ru-O1 reacted specifically with a high-molecular weight component of the lipopolysaccharide of serogroup O1 (and O8) strains, mAb IV/4-5 reacted with the O2a antigen, which represents a medium-weight component present in the O2ab, O2ac, and other O2a-containing strains such as O1 and O8. The broadly cross-reactive mAb V/9-5 recognises both low and high-molecular weight LPS components of all *Klebsiella* O serogroup reference strains ex-

Table 2: Opsonophagocytic effect of *Klebsiella* O antigen-specific mAbs for encapsulated and non-encapsulated *Klebsiella* strains

Strain designation	% Phagocytosis in the presence of mAb			
	Ru-O1	IV/4-5	V/9-5	K antibody
Klebsiella Caroli (O1:K2)	0	0	0	97.3 ± 2.2
Klebsiella Caroli decapsulated mutant (O1:K-)	94.4 ± 0.9	3.5 ± 1.8	21.7 ± 5.0	n.d.
strain 37 (O1:K7)	48.9 ± 4.8	38.6 ± 13.7	12.8 ± 6.4	97.0 ± 1.8
strain 37 decapsulated mutant (O1:K-)	93.5 ± 3.0	11.6 ± 6.6	59.9 ± 4.8	n.d.
strain 151 (O1:K21)	31.0 ± 8.03	3.4 ± 0.3	12.4 ± 3.7	94.3 ± 3.0
strain 151 decapsulated mutant (O1:K-)	93.9 ±1.5	54.1 ± 4.4	69.1 ± 4.8	n.d.

Values are % phagocytosis ± 1 standard deviation (3-4 separate experiments). Human neutrophils were used at a predetermined optimum ratio of bacteria to cells. The concentration of complement was 10%, and the final concentration of the mAbs was 5 μg/ml. Specific K antibody for K2 was mAb III/5-1, and polyclonal anticapsular rabbit sera raised against K7 and K21 were used in the respective experiments.

cept O7, and most of the clinical strains tested. In opsonophagocytosis experiments, a K2 antigen-specific anti-capsular mAb, III/5-1 (mouse IgM), was used as a positive control (*Trautmann* et al., 1988).

Opsonophagocytic tests

A variety of *Klebsiella* strains expressing and not expressing capsular antigens were tested in these experiments. We found that mAb Ru-O1 was the most active antibody in terms of a promotion of phagocytosis, however, even this antibody did not opsonise a fully encapsulated *Klebsiella* O1 strain, *K. pneumoniae* Caroli (Table 2). Thus, it was concluded that the capsule significantly hampered the access and functional activity of O antigen-specific antibodies (*Held* et al., 2000). Capsule-specific antibodies were highly opsonic for their homologous strains in these experiments (Table 2).

In vivo protection experiments

Although not opsonic for encapsulated *K. pneumoniae* strain Caroli, mAb Ru-O1 exerted significant protection against lethal infection with this strain. Doses necessary to provide protection were higher than those needed for a K antigen specific antibody tested for comparison (Table 3).

DISCUSSION

Our work on the *Klebsiella* O antigen serogroups has shown that the O antigen epidemiology of this genus is by far less diverse than that of other *Enterobacteriaceae*. For instance, in *E. coli*, more than 150 O antigen serogroups have been described, a significant proportion of which are found in clinical material. We found that only nine O antigen serogroups in *Klebsiella* can be accepted as truly separable groups, namely O1, O2, O3, O4, O5, O7, O9, O11 and O12. The previously described O and O8 antigens were

Table 3: Protective effect of mAb Ru-O1 (anti-O1) and mAb III/5-1 (anti-K2) in animals challenged with *Klebsiella* Caroli (O1:K2)

	No. of animals surviving at day 7/no. challenged, after pre-treatment with mAb	
Dose of mAb (μg/g)	III/5-1	Ru-O1
0.25	5/5	n.d.
0.5	5/5	n.d.
1.0	10/10	0/10
10.0	10/10	0/10
20.0	10/10	1/10
40.0	n.d.	7/10
200.0	n.d.	6/10

Adapted from *Rukavina* et al., 1997

found to be identical with O1, and several type strains were found to contain partial antigens of other strains. For instance, the O2a partial antigen was found in the type strain of O9, and the O4 partial antigen in the type strain of O11. The O2 serogroup is heterogeneous with various partial antigens that were not fully elucidated and that await their further clarification by mAb technology. We had mAbs at hand against the O2ab and O2ac partial antigens. While the former was present in all O1 and O2ab strains (i.e., in approximately 50% of clinical strains), the latter was found in only 4 out of 378 strains (1.1%). Our data are in nearly complete accordance with those obtained by *Hansen* et al. (1999) 2 years later, using a similar ELISA inhibition technique. These authors studied a total of 638 *Klebsiella* isolates from Denmark, Spain and the United States and found a virtually identical distribution of O serotypes in their clinical material. In their study, 78.9 % of strains belonged to serogroups O1, O2, O3 and O5.

Our studies with O antigen specific mAbs showed that these mAbs were able to bind to their target epitopes on whole bacteria (data not shown), but the capsule significantly hampered their opsonising effect. Nevertheless, our *in vivo* experiments with the most active of the antibodies, mAb Ru-O1, showed that protection may be achieved in spite of the relative lack of opsonic activity for encapsulated strains. We speculate that two mechanisms may account for this protective effect: Firstly, capsular antigen may be shed from growing bacteria *in vivo*, thereby exposing the O antigen layer for specific reaction with mAb, and secondly, soluble O antigen may contribute to pathogenicity by triggering pathophysiologic reactions such as disseminated intravascular coagulation or pro-inflammatory mediator release. It is possible, though not proven, that circulating O antigen specific mAb neutralises these effects, thereby contributing to protection. This mechanism may also explain why relatively large doses of O-specific antibody were needed for protection, compared to K antigen-specific antibodies (*Held* et al., 2000). Further studies will have to be done to clarify the protective mechanisms and study a possible synergism with K antigen specific antibodies before a clinical role of such antibodies can be defined.

LITERATURE

Carpenter, L.S.: *Klebsiella* pulmonary infections: Occurrence at one medical center and review. Rev. Infect. Dis. 5, 629-638 (1990).

Cross, A.S. and Cryz, S.J.: Vaccines against *Klebsiella* and *Pseudomonas* infections. In: New generation vaccines (Eds.: Woodrow, G.C. and Levine, M.M.). Marcel Dekker, New York, N.Y., 699-713 (1990).

Donta, S.T., Peduzzi, P., Cross, A.S., Sadoof, J., Haakenson, C., Cryz, S.J., Kauffman C., Bradley, S., Gafford, G., Elliston, D., Beam, T.R., John, J.R., Ribner, B., Cantey, R., Wels, C.H., Ellison, R.T., Young, E.J., Hamill, R.J., Leaf, H., Schein, R.M., Mulligan, M., Johnson, C., Griffiss, J.M., Slagle, D. and The Federal Hyperimmune Immunoglobulin Trial Study Group: Immunoprophylaxis against *Klebsiella* and *Pseudomonas aeruginosa* infections. J. Infect. Dis. 174, 537-543 (1996).

Hansen, D.S., Gottschau, A., and Kolmos, H.J.: Epidemiology of *Klebsiella* bacteraemia: a case-control study using *Escherichia coli* bacteraemia as control. J. Hosp. Infect. 37, 199-132 (1997).

Hansen, D.S., Mestre, F., Alberti, S., Hernandez-Alles, S., Alvarez, D., Domenech-Sanchez, A., Gil, J., Merino, S., Tomas, J.M., and Benedi, V.J.: *Klebsiella pneumoniae* lipopolysaccharide O typing: revision of prototype strains and O-group distribution among clinical isolates from different sources and countries. J. Clin. Microbiol. 37, 56-62 (1999).

Held, T.K., Jendrike, N.R.M., Rukavina, T., Podschun, R., and Trautmann, M.: Binding to and opsonophagocytic activity of O-antigen-specific monoclonal antibodies against encapsulated and nonencapsulated *Klebsiella pneumoniae* serotype O1 strains. Infect. Immun. 68, 2402-2409 (2000).

Kelly, R.F., Severn, W.B., Richards, J.C., Perry, M.B., MacLean, L.L., Tomas, J.M., Merino, S., and Whitfield, C.: Structural variation in the O specific polysaccharides of *Klebsiella pneumoniae* serotype O1 and O8 lipopolysaccharide: evidence for clonal diversity of *rfb* genes. Mol. Microbiol. 10, 615-625 (1993).

Rukavina, T., Ticac, B., Susa, M., Jendrike, N., Jonjic, S., Lucin, P., Marre, R., Doric, M., and Trautmann, M.: Protective effect of antilipopolysaccharide antibody in experimental *Klebsiella* infection. Infect. Immun. 65, 1754-1760 (1997).

Trautmann, M., Vogt, K., Hammack, C., and Cross, A.S.: A murine monoclonal antibody defines a unique epitope shared by *Klebsiella* lipopolysaccharides. Infect. Immun. 62, 1282-1288 (1994).

Trautmann, M., Cross, A.S., Reich, G., Held, T.K., Podschun, R., and Marre, R.: Evaluation of a competitive ELISA method for the determination of *Klebsiella* O antigens. J. Med. Microbiol. 44, 44-51 (1996).

Trautmann, M., Ruhnke, M., Rukavina, T., Held, T.K., Cross, A.S., Marre, R., and Whitfield, C.: O-antigen seroepidemiology of *Klebsiella* clinical isolates and implications for immunoprophylaxis of *Klebsiella* infections. Clin. Diagn. Lab. Immunol. 4, 550-555 (1997).

Trautmann, M., Cryz, S.J., Sadoff, J.C., and Cross, A.S.: A murine monoclonal antibody against *Klebsiella* capsular polysaccharide is opsonic *in vitro* and protects against experimental *Klebsiella pneumoniae* infection. Microb. Pathog. 5, 177-187 (1988).