Bull Yamaguchi Med Sch 34 : 53—59, 1987

Immunization Effects of *Klebsiella pneumoniae* Surface Antigens on Mice

Junko Uchiyama

Department of Pharmacy, Yamaguchi University Hospital, Ube Yamaguchi 755, Japan
(Received August 18, revised September 25, 1987)

Abstract The effects of immunization of mice with *Klebsiella pneumoniae* were examined. The protection against *Klebsiella pneumoniae* by the immunization with viable cells or dead cells could be replaced by the immunization with LPS, sodium deoxycholate extract of *K. pneumoniae* or the immunoaffinity-purified antigen (Kp-62 antigen) of *K. pneumoniae*. The levels of antibody to *K. pneumoniae* in the serum of mice immunized with *K. pneumoniae* or its antigens were determined by radioimmunoassay. The antibody titres were always high when the mice showed protection against *K. pneumoniae*.

Among the *K. pneumoniae* antigens enough to get defense, the immunoaffinity-purified antigen (Kp-62 antigen) might be the responsible antigen. This is supported by the observations that the antigen could induce enough immunity to *K. pneumoniae* infection, and antigens such as LPS or sodium deoxycholate extract which were also effective for immunization cross-reacted with Kp-62 monoclonal antibody to *K. pneumoniae*.

A strong antigenic effect of Kp-62 antigen was proved by the histological findings of local lymph nodes, after injection of this antigen to the foot pads of mice.

Key Words : *Klebsiella pneumoniae*, Monoclonal antibody, Defense antigen, Specific antibody, Mouse

Introduction

We have reported previously that human serum immunoglobulin (IgG) binds on the surface of clinically isolated strain of *Klebsiella pneumoniae, Klebsiella pneumoniae* 3296[1)-3)]. These results suggested that there exist antibodies in the normal human serum which react with *K. pneumoniae* 3296. Thus, I have interested in the defense and the specific antibodies to *K. pneumoniae* and the defense antigen of *K. pneumoniae*, This time, the immunization effects of the bacteria or bacterial surface antigens on mice were examined. To analyse the effects, I have studied the protection against *K. pneumoniae* after immunization with whole cells of *K. pneumoniae* or its surface antigens. I have also analysed changes of the local lymphoid tissue after administration of bacterial surface antigens into the foot pad of mouse. To analyse antigenic nature of the defense antigen of *K. pneumoniae* I have made and used monoclonal antibodies to the surface of *K. pneumoniae*.

Materials and Methods

Mice Female BALB/c mice of 8 to 9 weeks old, weighing 20-22 g were used. Mice were purchased

from Seiwa Experimental Animal, Japan.

Bacteria *K. Pneumoniae* 3296 (provided by the Central Research Laboratory of Shionogi Co Ltd, Japan) was used. Cells were grown on Heart infusion agar (Difco, USA) at 37℃ overnight.

Animal model To examine the protection against *K. pneumoniae* infection, BALB/c mice which were immunized previously by several ways, were challenged intraperitoneally with the minimum lethal dose of viable cells of *K. pneumoniae* 3296. The minimum lethal dose was determined by the preliminary experiments.

Bacterial antigens for immunization

(a) *Viable cells* : Freshly harvested cells were washed with saline and used as viable cells.

(b) *Dead cells* : The cultured cells were treated with 0.5% formaldehyde for overnight at 5℃, washed and resuspended in saline. The treated cells did not grow when cultivated. For each mouse 6.4×10^8 cells were injected.

(c) *Lipopolysaccharide* (*LPS*) : LPS prepared from *K. pneumoniae* No.1 follwing the method of Westphal[4], was kindly donated from Fujisawa Pharmaceutical Co. Ltd Osaka, Japan. LPS suspended in saline was used for immunization. For each mouse, 420 μg of LPS was injected.

(d) *Deoxycholate extract of bacterial membrane fraction* : *K. pneumoniae* 3296 suspended in phosphate-buffered saline (PBS) at the cell concentration of 5×10^9/ml was homogenised at 4℃ by Waring blendor for 10 min. Then, the homogenate of bacteria was solubilised with an equal volume of 8 % sodium deoxycholate (DOC) (Sigma, USA) on ice for one hour. *p*-Amidinophenyl methanesulfonyl fluoride hydrochloride (Wako Pure Chemical Industries Ltd, Japan) was added to inhibit proteolysis at the final concentration of 0.01 M. The solubilised homogenate was spun at 8,000 x g for 1 hour, and the supernatant was used as the DOC extract of the bacteria. For each mouse, 140 μg protein of DOC extract, which was dialysed against 70 mM phosphate buffer, pH 6.0 was used as the antigen.

(e) *Immunoaffinity-purified bacterial membrane antigen* (*Kp-62 antigen*) : Four monoclonal antibodies to *K. pneumoniae* 3296 were isolated following the method of Köhler and Milstein[5]. One of the monoclonal antibodies (Kp-62) was rather specific to *K. pneumoniae*. The immunoglobulin fraction was prepared from ascitic fluid of Kp-62-producing hybridoma by ammonium sulphate precipitation. The isotype of Kp-62 was IgGl. Kp-62-IgG thus obtained was coupled with cyanogen bromide-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) following the method described previously[6], and an immunoaffinity column was established. DOC extract was loaded on this column, and specifically bound antigen (Kp-62 antigen) was eluted by diethylamine buffer, pH 11.5. The molecular weight of the major antigen was about 60,000. This antigen, dialysed against 70mM phosphate buffer, pH 6.0, was used as Kp-62 antigen. Fifty microgram protein of Kp-62 antigen was used for each mouse.

Immunization or stimulation protocol

Each BALB/c mouse was injected intraperitoneally with viable bacteria of 1.4×10^8, 6.8×10^7, 5.5×10^7, 1.1×10^7, 2.2×10^6 or 4.3×10^5. After one week, defense of the mice was examined. With respect to dead cells, LPS, DOC extract and Kp-62 antigen, each antigen was mixed with an equal volume of Freund incomplete adjuvant (Difco, USA) and injected twice every two weeks subcutaneously. Two weeks after the final injection, the defense of mice was examined.

To investigate the effect of Kp-62 antigen on local lymph nodes, the antigen (100μg or 50μg protein) was injected into mouse foot pads. One or two weeks later, mice were sacrificed and popliteal lymph nodes were obtained. The lymph nodes were fixed in Carnoy fixatives, paraffin embedded, sectioned and stained with Hematoxylin-Eosin or Methyl green-pyronin.

Radioimmunoassay (*RIA*)

Blood samples were obtained from each immunized mouse and the levels of serum antibody to *K. pneumoniae* were examined by RIA. Polystylene beads (#30) were purchased from Sekisui Kagaku, Co. Ltd, Japan and coated with bacteria. Serially diluted serum was incubated with *K. pneumoniae*-coated beads for 1 hr at 4℃. Then, the beads were washed and incubated further for 1 hr at 4℃ with ^{125}I-labelled rabbit F(ab')$_2$ anti-mouse IgG. After washing, the radioactivity on the beads was counted in a gamma counter (Aloka, ARC-202).

To estimate the cross-reaction of antigens with Kp-62 monoclonal anti-*K. pneumoniae* antibody, Kp-62 was incubated with bacteria or bacterial antigens at 4℃ overnight. The adsorbed Kp-62 was incubated with *K. pneumoniae*-coated beads for 1 hr at 4℃.

After washing, beads were incubated with ^{125}I-labelled rabbit F(ab')$_2$ anti-mouse IgG for 1 hr at 4℃. After washing, the radioactivity on the beads was counted in a gamma counter.

Immunoblotting

DOC extract of *K. pneumoniae* was analysed by sodium dodecylsulfate-acrylamide gel electrophoresis (SDS-PAGE). Samples run on the gels were trans-blotted to cellulose acetate membranes (Bio-Rad, Richmond, USA), reacted with antibodies for 1 hr, washed and then stained with ABC Vectastain (Vector Laboratories Inc., USA).

Results

a) *Protection against K. pneumoniae after immunization*

The lethal dose of *K. pneumoniae* optimal for challenging mice was determined. All the mice challenged with 1.4×10^8, 6.8×10^7, 5.5×10^7, 1.1×10^7, 2.2×10^6 and 4.3×10^5 cells per mouse were alive. However, two of the three mice challenged with 2.7×10^8 bacteria died on the 1st day of injection, and none of the three mice challenged with 6.8×10^8 cells could survive. These results suggested that the challenge with 2.7×10^8 or 6.8×10^8 cells of *K. pneumoniae* might be suitable for estimating the defense after immunization with several antigens. Table 1. shows the results of protection against *K. pneumoniae* in mice which were previously immunized with *K. pneumoniae* or its antigens. Intraperitoneal injection of less than 1.1×10^7 viable cells was not effective for immunization. From these results, it is obvious that the number of viable bacteria to get enough immunity is important. The killed cells of 6.4×10^8 which were mixed with adjuvant and injected twice subcutaneously, were enough to get protection against the challenge of *K. pneumoniae*. To find out the responsible antigen, DOC extract of bacteria was prepared and injected to mice, followed by the challenge of *K. pneumoniae*. All the mice examined showed defense, suggesting that a potent antigen to protect against infection was included in the DOC extract or LPS. One of the antigenic fractions was purified from the DOC extract by using monoclonal antibody to *K. pneumoniae* 3296 (Kp-62) and tested for the immunization effect. As shown in Table 1, it is clear that Kp-62 antigen is effective to protect against *K. pneumoniae* infection.

b) *Serum antibody levels in immunized mice*

The antibody levels in each immunization were shown in Fig. 1. Compared to the saline group, the radioactivities associated with sera of immunized mice were significantly high. The dilution of each serum which showed 50% radioactivity of undiluted serum was taken as the antibody titre, and the results were summarized in Table 2. Even in the case of Kp-62 antigen, the antibody titre was significantly high.

c) *Cross-reactivity of several antigens with Kp-62 monoclonal antibody*

Cross-reactions of bacterial antigens with Kp-62 antibody were examined by RIA after absorbing the antibody with several antigens. Results were shown in Fig. 2. All the tested antigens including viable cells, dead cells, DOC extract, LPS and Kp-62 antigens cross-reacted with Kp-62 antibody.

d) *The antigens detected by the monoclonal antibody or immunized serum*

Table 1 Survival ratio after immunizing with various antigens*

	Antigen					
	Viable** cells	Dead cells	LPS	DOC extract	Kp-62 antigen	Saline
Survival ratio***	3/3	3/3	3/3	3/3	3/3	0/3

* The challenge dose was 2.7×10^8 cells/mouse.
** Immunization with 1.4×10^8, 6.8×10^7 or 5.5×10^7 viable cells per mouse gave the same survival ratio
*** number of survived mice/number of challenged mice.

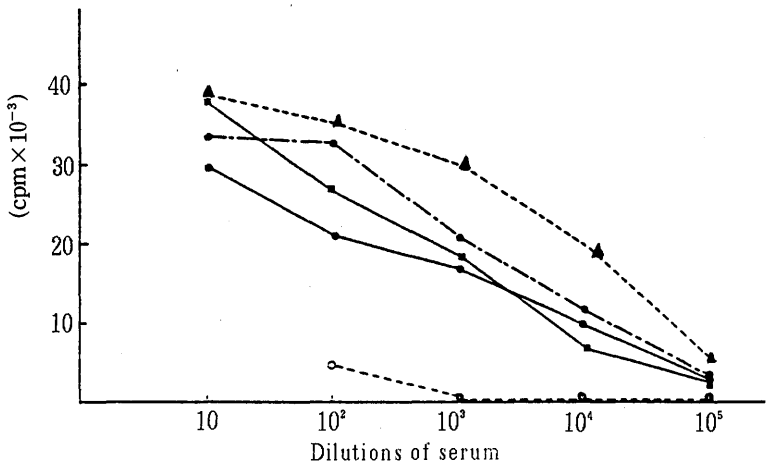

Fig. 1 Serum antibody levels in mice immunized with various *K. pneumoniae* antigens. Mouse sera immunized with dead cells (●———●), LPS (■———■), DOC extract (▲·····▲), Kp-62 antigen (●—·—●), or saline control (○·····○) were analysed by RIA.

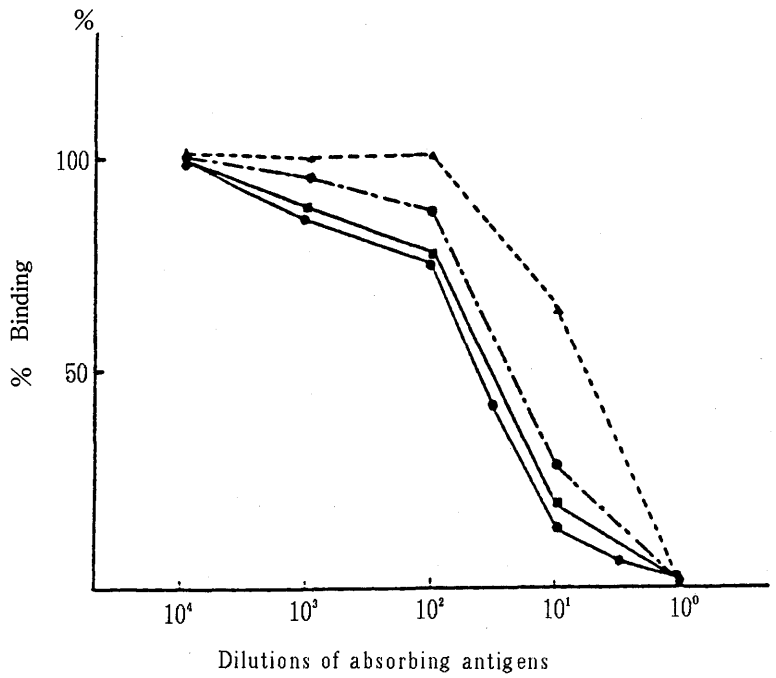

Fig. 2 Cross-reaction of various antigens with Kp-62 determined by competitive binding assay. Kp-62 antibody was absorbed with serially diluted dead cells (●———●), LPS (■———■), DOC extract (▲·····▲) or Kp-62 antigen (●—·—●) and incubated with *K. pneumoniae*-coated beads. The beads-bound monoclonal antibody (Kp-62) was determined with ^{125}I-labelled anti-mouse IgG. The radioactivities obtained with 10^4 and 10^0 dilutions of each antigen were taken as 100% and 0% binding respectively.

Table 2 Antibody titres of mice injected with various antigens

Antigen	Number of animals	Antibody titre: $\log_{10}$n in 50% binding	Antibody titre: Mean ± standard errors
Viable cells	3	3.1, 3.1, 5.4	3.8 ± 0.77
Dead cells	3	2.1, 2.3, 3.6	2.7 ± 0.47
LPS	2	3.2, 2.4	2.8 ± 0.40
DOC extract	3	3.1, 3.6, 4.2, 3.4	3.7 ± 0.24
Kp-62 antigen	4	3.3, 3.1, 3.1, 3.3	3.2 ± 0.05
0.9 % saline	3	0.7, 0.0, 0.0, 0.2	0.2 ± 0.16

The results of immunoblotting of DOC extract with which the DOC-extract-immunized mice serum or monoclonal antibody Kp-62 reacted are shown in Fig. 3. The serum of mice immunized with DOC extract reacted with most of the bands of the antigen (Fig. 3, lanes B and C), which include a band of the molecular weight similar to Kp-62 antigen (Fig. 3, lane D). Similar results were obtained in the sera immunized with viable cells, dead cells, LPS and Kp-62 antigens (data not shown).

e) *Effects of Kp-62 antigen on the local lymph nodes of mice*

When a mouse was injected with Kp-62 antigen at the foot pad, the foot pad became necrotic on day 5 or 6, whereas such a change was not observed in a control mouse injected with saline. The popliteal lymph node significantly enlarged as compared to that of the saline control. When 100 μg of Kp-62 antigen was injected, the weight on day 7 was 7 mg, whereas it was 1.7 mg in the control mouse. In another experiment, 100 μg of the antigen was injected on day 1, 50μg of the antigen on day 7, and the weight of popliteal lymph node was determined on day 14, which was 4.7 mg as compared to 1.4 mg in the control mouse. In the third experiment, 100μg of the antigen was injected on day 1 and the weight of popliteal lymph node was determined on day 14 , which was 14.7 mg as compared to 2.3 mg in the control mouse. Histological changes of the popliteal lymph nodes from the mice injected with Kp-62 antigen in foot pad, were examined (Fig. 4). The numbers and sizes of secondary nodules in the injected groups increased as compared to the saline control. Large pyroninophilic cells in secondary nodules as well as in parafollicular regions were observed predominantly in the injected mice (Fig. 4a), whereas such cells

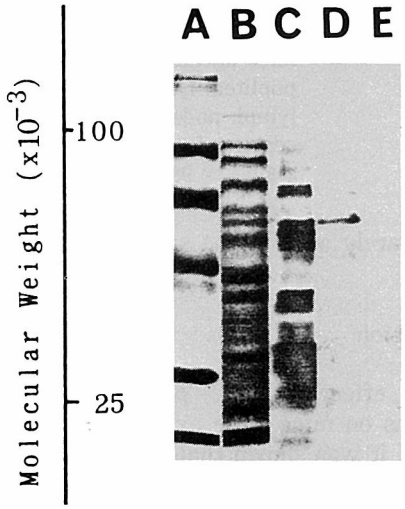

Fig. 3 Immunoblotting of DOC extract of *K. pneumoniae* reacted with DOC-extract-immunized mouse serum or Kp-62. DOC extract was electrophoresed on SDS-PAGE and trans-blotted on a cellulose acetate membrane. Samples on a cellulose acetate membrane were reacted with mouse serum immunized with DOC-extract (lane C), Kp-62 monoclonal antibody (lane D) or unimmunized mouse serum (lane E). Lane A and B show the standard markers and DOC extract, respectively, which were trans-blotted on a cellulose acetate membrane and stained with Coomassie blue.

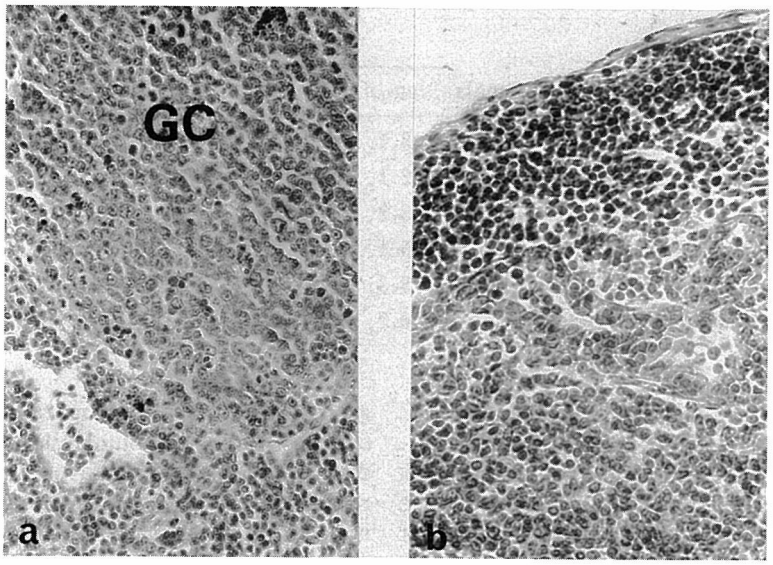

Fig. 4 Histological findings of the popliteal lymph nodes of the mice injected with Kp-62 antigen or saline. *a*, a representative popliteal lymph node of the antigen-injected mice, b, a popliteal lymph node of the control mouse, GC represents the germinal center of the secondary nodules (Methyl green and pyronin stain ; x 96).

were rarely seen in the saline control (Fig. 4b).

Discussion

The effects of *K. pneumonae* or its antigens on mice were examined. From the results, it was shown that the defense could be obtained after immunizing with optimal numbers of viable cells. Furthermore, this could be replaced by the dead cells or even by the DOC extract of *K. pneumoniae*, suggesting that, some antigen components, probably membrane fractions, were seriously involved in immunizing mice against *K. pneumoniae* infection.

To determine the effective components for immunizing mice to *K. pneumoniae* in such DOC extract, LPS and affinity-purified antigen (Kp-62 antigen) were examined. Table 1 showed clearly that these fractions are also effective for obtaining protection against *K. pneumoniae*. In fact, the antibody titres of the mice immunized with each antigen were significantly high as in the serum level of the mice immunized with whole bacteria. From Fig. 2, it is quite clear that DOC extract and LPS cross-reacted with Kp-62 monoclonal antibody suggesting that Kp-62 antigen is included in these antigens, and Kp-62 antigen itself can induce enough immunization. Thus, we believe that Kp-62 antigen might be the responsible antigen for the protection against *K. pneumoniae*. However, the Kp-62 antigen was not pure when analysed by SDS-PAGE (data not shown). One of the reasons why we could not obtain the pure antigen with monoclonal antibody-immunoaffinity column might be that we have used ascitic fluid of Kp-62-producing hybridoma, and the acites might contain other antibodies which could cross-react to the other antigens of *K. pneumoniae*. To avoid this problem, we should have used culture supernatant of Kp-62 to prepare immunoglobulin for immunoaffinity column.

The Kp-62 antigen fraction appeared to be a quite strong antigen, as we could get strong

reaction in local lymph nodes after administraion of this antigen into foot pads of mice. Strong adjuvant activity of capsular polysaccharides of *K. pneumoniae* on antibody response was reported previously[7]. However, only a few reports exist on the protection against *K. pneumoniae* using bacterial antigens or on monoclonal antibody analysis on *K. pneumoniae* antigens[8),9)]. Thus, we are on the way to analyse further the biological activities of Kp-62 antigen as well as to purify the antigen. At the moment, Kp-62 antigen appeared to be included in the LPS preparation, but the nature of the antigen with respect to the contents of protein, carbohydrate or lipid was left to be resolved.

The author acknowledges to Prof. Akira Koshiro for his suggestion and encouragement and to Prof. Tetsuo Fukumoto, Dr. Hiromichi Kuniki, Dr. Yoshihisa Fujikura and Dr. Tomoo Sawada, First Department of Anatomy for their supports of this work. The author also thanks Prof. Teruko Nakazawa and Dr. Hisanori Konishi of Department of Microbiology for their help of conducting defense experiments.

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