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ORIGINAL ARTICLE

Increasing opsonizing and killing effect of serum from patients with recurrent K1 *Klebsiella pneumoniae* liver abscess

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Background: Klebsiella pneumoniae liver abscess (KLA) is an emerging infectious disease caused by the virulent *K pneumoniae* strains of capsular serotype K1 and commonly associated with diabetes mellitus. Recurrent KLA is rarely reported and the mechanism of recurrence is uncertain. In this study we evaluated both phagocytosis by neutrophils and serum killing ability of serum from recurrent K1 KLA patients compared to normal healthy subjects (NHS).
Methods: This prospective study included six cases of recurrent K1 KLA consisting of three male and three female patients with a mean age of 67.2 years (range, 56-88 years). The different serotypes of *K pneumoniae* were reacted with serum from patients with recurrent KLA and NHS. Subsequent phagocytosis by neutrophils was determined using flow cytometry and serum killing assays were performed.
Results: The most common underlying disease in patients with recurrent KLA was diabetes mellitus, occurring in about 83.3% (5/6) of patients. The antibiogram of the strains associated with recurrent KLA remained uniquely resistant to ampicillin. The average percentage derived from the serum killing assays showed serotype K1 and K2 resistance to serum from NHS (1281% and 621%, respectively); however, serum susceptibly was observed in the serum of patients with recurrent K1 KLA (0.3% and 1.1%, respectively). A significant increase in neutrophil phagocytosis of serotype K1 was observed following opsonisation with serum from patients with

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recurrent KLA compared with serum from NHS ($p = 0.008$). No significant difference in the phagocytic rate of non-K1/K2 or K2 serotypes was observed between NHS and patients with recurrent KLA ($p = 0.76$ and $p = 0.132$, respectively).
Conclusion: These preliminary results showed possible immunologic protection in patients with recurrent KLA due to increasing opsonization and serum killing.

Introduction

Pyogenic liver abscesses caused by *K pneumoniae* are an emerging and common intra-abdominal infection. *K pneumoniae* has been identified as the predominant pathogen of liver abscess in Taiwan.[1–3] In the United States an increasing incidence of *K pneumoniae* liver abscess (KLA) as high as 41% has been observed. Among the 77 identified capsular serotypes, K1 and K2 have been reported to play a virulent role in *K pneumoniae* infection. Epidemiologic studies have demonstrated that capsular serotypes K1 and K2 are the most prevalent serotypes and are associated with about 78% of liver abscess. Furthermore, diabetes mellitus is a risk factor for KLA and is present in approximately 50—75% of patients with KLA.[1,2,4–6] In previous reports, patients with diabetes were shown to be more susceptible to *K pneumoniae* infection due to the impairment of opsonophagocytic and neutrophil functions.[7,8] Poor glycemic control also plays an important role in phagocytic resistance of virulent K1/K2 *K pneumoniae*.

Recurrent KLA was very rare in English literature, and only six patients with different serotypes isolated from both primary and recurrent KLA have been reported.[9] The detailed mechanism involving recurrent KLA has not been clarified. Nevertheless, production of humeral or cellular immune responses targeted against infectious microorganisms might be considered. The thesis of this study was to demonstrate the possible immunologic protective reaction in recurrent KLA. In this prospective study, six recurrent KLA patients with confirmed K1 serotypes were enrolled to compare clinical information with normal healthy subjects (NHS) at Tri-Service General Hospital from 1996 to 2011. Clinical information that was collected included bacterial virulent factor genes *rmpA*/*magA*, serum killing, and phagocytosis reactions.

Materials and methods

Patient enrollment and bacterial isolates

We prospectively enrolled patients with liver abscesses and traced their past history. *K pneumoniae* strains were isolated from patients at Tri-Service General Hospital in Taiwan from 1996 to 2011. Liver abscess was diagnosed by clinical evaluation of the patient's condition (e.g., fever, chills), imaging studies (e.g., abdominal sonography, computer tomography), and the presence of pathogen(s) from an abscess or blood sample. Patients with a second occurrence of KLA after completing treatment were defined as recurrent KLA. Patients with relapse of liver abscess due to insufficient treatment duration were excluded and demographic data of patients were also analyzed. All *K pneumoniae* strains were stored at -80°C before use. The study protocol was approved by the ethics committees of our institutes and informed consent was provided by all of the participants in the study.

K pneumoniae serotype determination

A total of 15 *K pneumoniae* strains with different capsular serotypes were obtained from patients with primary liver abscesses, patients with recurrent KLA, or from the American Type Culture Collection (Rockville, MD, USA). Serotyping was assessed by polymerase chain reaction (PCR) and the capsular swelling technique.[1] Nine strains of capsular serotype K1 isolated from patients with recurrent KLA were identified. Two strains of K1, two strains of K2, one strain of non-K1/K2 (K38) obtained from clinical blood cultures, and one strain of ATCC K47 were also used for the experiments to exclude bacterial bias in the serum killing assay and phagocytosis reaction.

Determination of the magA and rmpA genes

PCR was used to determine the prevalence of *magA* and *rmpA*. Bacterial colonies were cultured overnight, added to 300 μL water and boiled for 15 min to release the DNA template. Previously published primers were used for PCR: *magA* forward 5′-GGTGCTCTTTACATCATTGC-3′, *magA* reverse 5′-GCAATGGCCATTTGCGTTAG-3′, *rmpA* forward 5′-ACTGGGCTACCTCTGCTTCA-3′, and *rmpA* reverse 5′-CTTGCATGAGCCATCTTTCA-3′. The reaction mixture was kept at 95°C for 5 min; followed by 40 cycles of 95°C for 1 min, 50°C for 1 min, and 72°C for 2 min; and a final incubation at 72°C for 7 min. The expected PCR products of *magA* and *rmpA* were 1282 bp and 535 bp in length, respectively.[10]

Phagocytosis reaction

Neutrophils from NHS without acute infection were separated as previously described.[11] Viability was shown to be greater than 95% determined by trypan blue exclusion assays. Human sera from 10 healthy volunteers without acute infection or previous history of liver abscess as well as sera from 6 patients with recurrent KLA were prepared. Pooled NHS serum was obtained and stored in aliquots at −70°C until required. To evaluate the serum effect of

patients with K1 recurrent KLA, the primary K1 KLA had been not used due to insufficient humoral production in the acute infection stage. Fluorescence (FITC) labeling of 2×10^8 cells of *K pneumoniae* per mL in phosphate buffer solution (PBS) was performed as previously described and stored at −70°C prior to use. Phagocytosis was measured using a standard assay.[11] A FACScan (BD Biosciences, Franklin Lakes, NJ, USA) was used to measure phagocytic rates. Phagocytosis percentage was counted at 0, 30, and 60 minutes. A non-FITC-labeled tube served as the 0-minute control. The experimental procedures and FACS settings were performed as previously described.[11]

Serum killing assay

Serum resistance or susceptibility was assayed using a modified method of Hughes and Podschun.[12,13] Different strains of *K pneumoniae* were reacted with sera from patients with recurrent KLA as well as NHS. All experiments were performed in triplicate. Bacteria grown in nutrient broth were collected during the early logarithmic phase. The viable bacterial concentration was adjusted to 1×10^6 colony forming units/mL and reacted with 75% pooled human sera. A strain was considered serum resistant or serum sensitive if the grading was the same in all experiments. Each isolate was classified as highly sensitive (grade 1 or 2), intermediately sensitive (grade 3 or 4), or resistant (grade 5 or 6). For grade 1, viable count (VC) after the 1st and 2nd hour was <10% of the inoculum; after the third hour, VC was <0.1%. For grade 2, the VC after the 1st hour was 10−100%; after third hour, VC was <10%. For grade 3, the VC after the 1st hour was >100% of inoculum; after the 2nd and 3rd hour VC was <100%. For grade 4, the VC after the 1st and 2nd hour was >100%; after the 3rd hour the VC was <100%. For grade 5, the VC after 1st, 2nd, and 3rd hour was >100%, but the VC fell sometime during the 3-hour period. For grade 6, the VC was >100% of the inoculum after 1st, 2nd, and 3rd hour. Each strain was tested at least three times.

Statistical analysis

Data was expressed as the mean ± standard deviation (SD). One-way analysis of variance (ANOVA) with repeated measures was performed to compare the differences in phagocytosis from 0 to 60 minutes between groups. Student's t-test was used to assess the differences in phagocytosis rates at 60 minutes between groups. *P* values <0.05 were considered statically significant.

Results

Patients

Six patients from 1996 to 2010 were identified with recurrent K1 KLA, including three male and three female patients with a mean age of 67.2 years (range, 56−88 years). The average time between first occurrence and recurrent liver abscess was 5.7 years (range, 0.33−20 years). The most common underlying diseases in the patients with recurrent KLA were diabetes (83.3%, 5/6) and hypertension (67%, 4/6). Gall stones, liver cirrhosis, and peptic ulcer were also present in 18% (1/6) of these patients. Poor glycemic control was observed in 80% (4/5) of patients with recurrent KLA and hemoglobin A1c above 8.0% (average, 10.4%; range, 6.9−14.7%). The recurrent KLA had been almost found in right lobe of liver. The complication of metastasis, including brain abscess, pulmonary embolism, and endophthalmitis was found in 16.7% (1/6) of patients with primary K1 KLA. There were no metastatic complications observed in the patients with recurrent KLA and bacteremia was observed in 50% of these patients. A 100% survival rate without mortality was observed in patients with recurrent KLA. The demographic information and data gathered from patients are shown in Table 1.

Serotype and virulent genes of recurrent KLA

Paired bacteria from primary and recurrent infections of three patients with recurrent K1 KLA were isolated. The other three patients with recurrent KLA had their primary liver abscesses diagnosed at other hospitals. The antibiogram indicated that the primary and recurrent strains were uniquely resistant to ampicillin. All primary and recurrent strains were serotype K1 and harbored *magA* and *rmpA* genes (Table 2).

Serum killing assays

The average percentage of serum killing showed serum resistance of serotypes K1 and K2 when incubated with serum from NHS (1281% and 621%, respectively). In contrast, serum susceptibility was demonstrated when these strains were incubated with serum from patients with recurrent KLA (0.3% and 1.1%, respectively; Table 3). However, there was no difference in percentage of serum killing for non-K1/K2 strains between patients with recurrent KLA and NHS (0% and 0%, respectively).

Phagocytosis reaction

To evaluate the serum effect of patients with recurrent KLA, we compared opsonization of different *K pneumoniae* serotypes following incubation with serum from patients with recurrent KLA and NHS. For opsonized non-K1/K2 isolates, phagocytosis rates between NHS and patients with recurrent KLA were both similarly high, with 60-min phagocytic uptake rates of 51.7 ± 3.2% and 53.0 ± 2.8%, respectively. No significant difference in phagocytic rate of non-K1/K2 isolates was observed between NHS and patients with recurrent KLA ($p = 0.76$; Fig. 1). For K2 isolates, the phagocytosis rates between NHS and patients with recurrent KLA were also demonstrated to be similar, with 60-min phagocytic uptake rates of 51.3 ± 1.8% and 56.5 ± 2.7%, respectively ($p = 0.132$; Fig. 2). In contrast, there was a significantly increased phagocytosis rate for serotype K1 strains in patients with recurrent KLA compared to NHS, with 60-min uptake rates of 54.0 ± 2.3% versus 43.6 ± 2.4%, respectively ($p = 0.008$; Fig. 3).

Table 1 Demographic data and clinical presentations of patients with recurrent serotype K1 *K pneumoniae* liver abscess (KLA)

	Case1	Case 2	Case 3	Case 4	Case 5	Case 6
Sex/Age (y)	M/62	F/58	M/88	M/56	F/58	F/81
Underlying Diseases	Diabetes Liver cirrhosis Hypertension	Gastric ulcer	Diabetes Gallstones HCVD	Diabetes Hypertension	Diabetes Hypertension	Diabetes Hypertension
HgbA1c (%)	14.7	ND	9.2	6.9	13.7	7.4
Interval of onset (y)	7	2	3	20	2	0.33
1st abscess						
size (cm)	5.8	8	3	Unknown	9.2	5
Site (segment)	4	4	6	Unknown	6&7	3&4&5
Bacteremia	Yes	Unknown	No	Unknown	No	No
Drainage	No	Yes	No	No	Yes	Yes
Complications	Brain abscess Endophthalmitis Pulmonary emboli	None	None	None	None	None
Recurrent abscess						
size (cm)	2	6.2	8	7.5	7.1	3.9
Site(segment)	6	6	6&7	8	6	8
Bacteremia	Yes	No	No	Yes	No	Yes
Drainage	No	Yes	Yes	Yes	Yes	Yes
Complication	None	None	None	None	None	None
Prognosis	Survival	Survival	Survival	Survival	Survival	Survival

Discussion

K pneumoniae has been identified as the predominant bacteria responsible for community-acquired liver abscesses in Taiwan and the United States.[2,14] Epidemiologic studies have demonstrated that the capsular serotypes K1 (64%) and K2 (14%) are the most prevalent in community-acquired primary KLA and complicated metastasis infections including endophthalmitis.[3] Based on the current literature, recurrent KLA has not been reported. Previously, six patients with recurrent KLA (K1 and non-K1) had been identified in our prospective study from 1996 to 2008. The predominant serotype in these patients was K1 at about 84%. The most common underlying disease was diabetes mellitus, occurring in about 84% of these patients. The focus in this current study was serotype K1 due to its higher prevalence in patients with recurrent KLA. The antibiogram of the strains isolated from patients with recurrent K1 KLA remained uniquely resistant to ampicillin. Bacteremia was found in 50% of the patients with recurrent K1 KLA and there was no metastatic complications or mortality in these patients. We suggest that diabetes mellitus, poor glycemic control, and the K1 serotype were still common risk factors for recurrent KLA.

Among the 77 recognized capsular serotypes, K1 and K2 have been reported to have a clear relationship with virulence and infection. Capsular serotypes K1 and K2 were also the most prevalent when isolated from blood, sputum, urine, pus, and liver abscess. Capsular antigens related to antiphagocytosis and serum resistance to bactericidal activity in *K pneumoniae* have been reported for clinical infections. In both *in vitro* and *in vivo* studies, serotypes K1 and K2 have been demonstrated to be more lethal. Resistance of capsular serotypes K1 or K2 to phagocytosis and intracellular killing presumably contributes to their high prevalence in infections. Serum from patients with

Table 2 Bacterial information of recurrent serotype K1 *K pneumoniae* liver abscess (KLA)

	Case1	Case 2	Case 3	Case 4	Case 5	Case 6
1st isolate						
Antibiogram result	Ampicillin resistance only	Ampicillin resistance only	Ampicillin resistance only	Ampicillin resistance only	Ampicillin resistance only	Ampicillin resistance only
magA	Positive	ND	ND	ND	Positive	Positive
rmpA	Positive	ND	ND	ND	Positive	Positive
2nd isolate						
Antibiogram result	Ampicillin resistance only	Ampicillin resistance only	Ampicillin resistance only	Ampicillin resistance only	Ampicillin resistance only	Ampicillin resistance only
magA	Positive	Positive	Positive	Positive	Positive	Positive
rmpA	Positive	Positive	Positive	Positive	Positive	Positive

ND = no data

Table 3 Percentage of surviving *K pneumoniae* serotypes in serum killing assays from recurrent *K pneumoniae* liver abscess (KLA) and normal healthy subjects (NHS)

	0 hour	1 hour (%)		2 hour (%)		3 hour (%)	
(NHS)							
K1	32×10^5	10×10^6	(323)	20×10^6	(625)	41×10^6	(1281)
K2	37×10^5	11×10^6	(297)	34×10^6	(930)	23×10^6	(621)
Non-K1/K2	22×10^5	14×10^5	(64)	2×10^5	(9)	0	(0)
(patient)							
K1	32×10^5	13×10^4	(4)	5×10^4	(1.6)	1×10^4	(0.3)
K2	38×10^5	17×10^4	(4.5)	7×10^4	(1.8)	4×10^4	(1.1)
Non-K1/K2	29×10^5	7×10^4	(2.4)	0	(0)	0	(0)

recurrent K1 KLA showed increased serum killing and oposonization of neutrophils to phagocytose with higher anti-phagocytosis and serum resistance of capsular serotype K1.

Considering the antibiogram analysis of strains from patients with recurrent KLA, the unique characteristic of ampicillin resistance was found for both primary and recurrent KLA. In a previous study of KLA in Taiwan, community-acquired KLA with a unique antibiogram indicative of resistance only to ampicillin was discovered. There was no difference in the antibiogram among primary, recurrent, and community-acquired KLA. The drug sensitivity tests of the *K pneumoniae* strains isolated from patients with recurrent K1 KLA were all susceptible to nearly all antibiotics except ampicillin.[1,4,5] There were no instances of distant metastasis in the patients with recurrent KLA from this study possibly because they had received early treatment by broad spectrum antibiotics due to suspicion of liver abscess as a result of previous KLA. All patients in this study received antibiotic treatment and drainage without mortality or morbidity and all recovered completely. Adequate antibiotic treatment and early drainage were the main treatment for patients with KLA. Six independent factors, including; normal platelet count, alkaline phosphatase less than 300 U/liter, no gas formation in the abscess, an APACHE III score less than 40, use of extended-spectrum cephalosporin, and early drainage were good predictors against severe complications in patients with KLA.[15]

KLA is common in Taiwan but recurrence is very rare in clinical practice.[1–3] To date, the pathogenesis of liver abscesses is not completely understood. However, previous studies have noted that diabetes mellitus is correlated to a high KLA prevalence rate (78%), especially with the additional complication of septic endophthalmitis.[16] Among patients with distant metastasis to septic endophthalmitis, 92% also had diabetes. Poor neutrophil function and impairment of opsonophagocytic function were noted in patients with diabetes and poor glycemic control.[7,8] In our patients, five were diabetic with poor glycemic control and high hemoglobin A1c. We assumed that diabetes mellitus was also a risk factor for recurrence which may explain why patients with diabetes were more susceptible to KLA and recurrent KLA.

Previous reports have shown that recurrent liver abscesses are rare and are mostly related to granulomatous disease,[17–19] immunocompromised diseases,[18,20] and amebic infections.[21,22] The rate of amebic liver abscess recurrence is lower than 0.04% per year and the recurrence of KLA has rarely been reported.[9] The specific adaptive

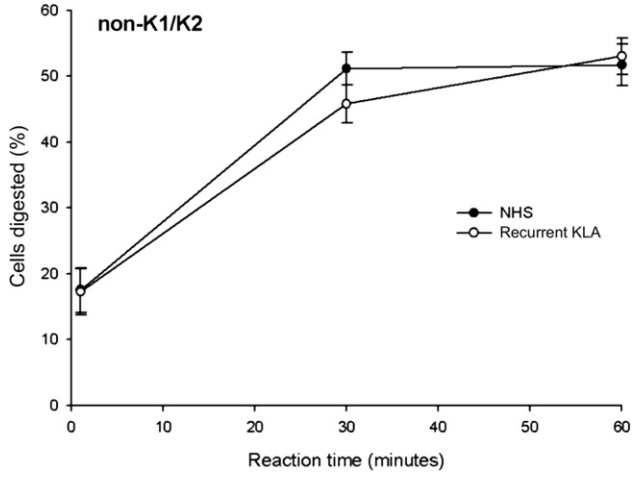

Figure 1. Comparison of neutrophil phagocytosis with serotype non-K1/K2 *K pneumoniae* by oposonization of recurrent *K pneumoniae* liver abscess (KLA) and normal healthy subjects (NHS) ($p = 0.765$).

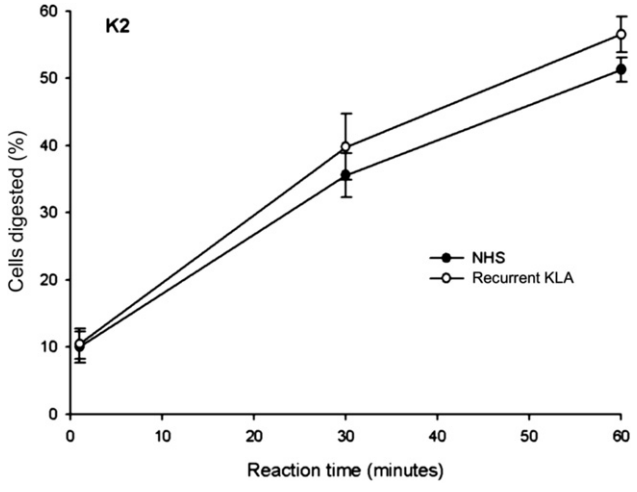

Figure 2. Comparison of neutrophil phagocytosis with serotype K2 *K pneumoniae* by oposonization of recurrent *K pneumoniae* liver abscess (KLA) and normal healthy subjects (NHS) ($p = 0.132$).

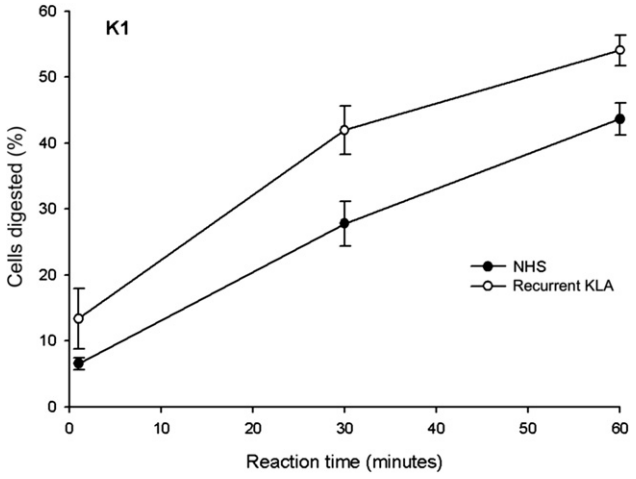

Figure 3. Comparison of neutrophil phagocytosis with serotype K1 *K pneumoniae* by oposonization of recurrent *K pneumoniae* liver abscess (KLA) and normal healthy subjects (NHS) ($p = 0.008$).

immune response is the hallmark of recognition of foreign antigens. Immunoglobulin can be induced after attacking the KLA. The protection of the host with a specific immune response has been considered. A significant increase in neutrophil phagocytosis of serotypes K1 *K pneumoniae* opsonized with serum from patients with recurrent KLA was observed when compared with serum from NHS. These results might explain why a low incidence of recurrent K1 KLA is observed. The possibility of recurrence has been thought to be related to inadequate treatment, immunological incompetence, and repeated exposure to infectious organisms.[21] The exact relationship between the environment and host still requires more clarification. The limitations of our study included the limited number of patients, lack of serum component analysis, and a lack of protein qualification. The detailed mechanism involved in recurrent KLA still requires further clarification.

In conclusion, our results showed increasing opsonization and serum killing in patients with recurrent K1 KLA. Although patients with recurrent K1 KLA were commonly diabetic with poor glycemic control, they might have higher immunologic protection against K1 strains after primary KLA. Further studies to link the recurrence of liver abscess and enhanced immunological response are needed.

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