CHEMILUMINESCENCE OF HUMAN LEUKOCYTES STIMULATED BY CLINICAL ISOLATES OF *KLEBSIELLA*

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SUMMARY. Chemiluminescence (CL) of human polymorphonuclear leukocytes was determined after stimulation with 89 clinical isolates of *Klebsiella* which differed in serotype and in their virulence for mice. With K1, K2, K4 and K5 strains, a significantly lower CL response was observed than with K3, K6 and K > 6 strains. These results correlated well with virulence: greater virulence could be explained by greater resistance to phagocytosis.

INTRODUCTION

Previous work with clinical isolates of *Klebsiella* showed that serotypes differed in their virulence for mice (Simoons-Smit *et al.*, 1984). K1 and K5 strains, and most K2 and K4, were more virulent for mice than K6 and serotypes higher than K6, whereas K3 strains were significantly less virulent. A factor which may contribute to their virulence is the degree of resistance to host defence mechanisms, such as phagocytosis. For *Klebsiella* strains, Hall and Humphries (1958) found a close correlation between virulence (intraperitoneal LD50) and susceptibility to phagocytosis by the polymorphonuclear leukocytes (PMNL) of mice, but they tested only four strains. Association between virulence and resistance to the bactericidal activity of human granulocytes has been demonstrated for encapsulated strains of other common pathogens (Howard and Glynn, 1971; Melly *et al.*, 1974; Rottini *et al.*, 1975).

Allen *et al.* (1972) first described chemiluminescence (CL) of PMNL during phagocytosis of bacteria. This CL is the consequence of the generation of electronically excited oxidative radicals by the metabolic burst, and can be enhanced by the addition of luminol (5-amino 2,3-dihydrophthalazinedione) to the phagocytosis system (Allen and Loose, 1976). Many authors have shown a correlation between this luminol-enhanced CL and phagocytosis (Grebner *et al.*, 1977; Stevens and Young, 1977; Welch and Stevens, 1979; Ewetz *et al.*, 1979; Williams *et al.*, 1980). CL response is, therefore, used indirectly to quantitate phagocytosis.

In this study luminol-enhanced CL of human leukocytes was determined after stimulation by various serotypes of *Klebsiella*, to investigate any correlation between resistance to phagocytosis, measured by CL, and virulence for mice.

MATERIALS AND METHODS

Bacterial strains. Of the 93 *Klebsiella* strains described previously (Simoons-Smit *et al.*, 1984), 89 strains were used in this study (see table). The strains were identified to species level by API-20E (API-system S.A., Montalieu-Vercieu, France) in which system *K. pneumoniae* includes all *Klebsiella* biotypes not in the species *K. rhinoscleromatis*, *K. ozaenae* and *K. oxytoca*. They were serotyped by the capsule-swelling technique with rabbit antisera. Strains with a serotype higher than K6, including five non-typable strains, are referred to as K > 6 strains.

Bacterial suspensions. Log-phase cultures of strains in nutrient broth were harvested by centrifugation, and resuspended in phosphate-buffered saline (PBS). Viable counts of these suspensions were determined by plating serial dilutions on nutrient agar. After storage overnight at 4°C the suspensions were diluted to *c.* 10⁸ bacteria/ml, and these concentrations were confirmed retrospectively by viable counts.

Zymosan (Sigma Chemical Co., St Louis, USA) 4 mg was suspended in 2 ml of PBS and heated for 30 min at 100°C in a waterbath. The suspension was washed twice in PBS after which the pellet was suspended in PBS to a concentration of 0.8 mg/ml. This Zymosan suspension was stored in small volumes at -20°C and diluted 1 in 10 before CL experiments.

Serum. Pooled normal human serum from *c.* 50 healthy individuals was stored in small volumes at -70°C and used throughout all experiments at a final concentration of 10% v/v.

Preparation of polymorphonuclear leukocytes. Venous blood was obtained from healthy donors, with heparin (14 units/ml) as anticoagulant. After mixing five parts of the blood with one

TABLE
Klebsiella strains

Serotype	Species*	Number of strains (89)
K1	<i>K. pneumoniae</i>	9
K2	<i>K. pneumoniae</i>	9
K3	<i>K. pneumoniae</i>	5
K3	<i>K. oxytoca</i>	6
K3	<i>K. rhinoscleromatis</i>	1
K4	<i>K. ozaenae</i>	8
K4	<i>K. pneumoniae</i>	1
K5	<i>K. pneumoniae</i>	3
K5	<i>K. pneumoniae</i> (NCTC 9660)	1
K6	<i>K. pneumoniae</i>	5
K7	<i>K. pneumoniae</i>	1
K8	<i>K. pneumoniae</i>	1
K10	<i>K. pneumoniae</i>	1
K18	<i>K. pneumoniae</i>	3
K21	<i>K. pneumoniae</i>	4
K24	<i>K. pneumoniae</i>	1
K26	<i>K. oxytoca</i>	1
K27	<i>K. pneumoniae</i>	3
K27	<i>K. oxytoca</i>	1
K29	<i>K. oxytoca</i>	1
K33	<i>K. pneumoniae</i>	5
K35	<i>K. pneumoniae</i>	2
K43	<i>K. pneumoniae</i>	2
K55	<i>K. oxytoca</i>	2
K61/63/10†	<i>K. pneumoniae</i>	1
K61/63/10†	<i>K. oxytoca</i>	1
K64	<i>K. pneumoniae</i>	2
K69	<i>K. oxytoca</i>	4
NT	<i>K. pneumoniae</i>	5

NT = non-typable

* In contrast with the identifications in our previous study (Simmons-Smit *et al.*, 1984) API-20E now classifies as *K. oxytoca* the indole-positive biotypes of *K. pneumoniae*.

† Reacted with all three antisera.

part of dextran 6% w/v in PBS, the erythrocytes were allowed to sediment for 30 min at 37°C. The leukocyte-rich plasma was centrifuged at 250 *g* for 5 min at 4°C. To the pellet was added 5 ml of a solution of NH_4Cl 1 g, KHCO_3 1 g, and disodiummethylenediamine-tetra-acetic acid 37 mg, per litre of sterile distilled water. After 10 min, to allow lysis of residual erythrocytes, the leukocytes were centrifuged, washed twice in Hanks's medium (Gibco, Paisley, UK) containing gelatin 0.1%, and resuspended in the same medium to give 5×10^6 leukocytes/ml.

Measurement of chemiluminescence. The reaction mixture consisted of: 0.1 ml of the leukocyte suspension (5×10^6 /ml), 0.1 ml of a bacterial suspension (10^8 /ml), 0.1 ml of pooled normal serum (40% v/v) and 0.1 ml of 0.1 mM luminol (Lumac Systems A.G., Basel, Switzerland) in PBS, giving a bacteria:leukocyte ratio of 20:1. The mixtures were incubated in Lumacuvette P-tubes in a shaking waterbath at 37°C. CL was measured every 10 min up to 60 min in a Lumacounter® M2080 (Lumac Systems A.G., Basel, Switzerland) in the integral mode with a preset of 10 s; and the mean of counts during five periods of 2 s each was recorded on a HP model 97 S microcomputer (Hewlett Packard, Corvallis, USA). After curve-area integration, this CL response was expressed as a percentage of the curve-area integration obtained when the bacterial suspension was replaced with the standardised Zymosan suspension in the same reaction mixture.

RESULTS

Typical CL patterns of a virulent strain (K5), avirulent strains (K6 and K69) and the Zymosan suspension are shown in fig. 1. CL was low immediately after mixing, and it increased during the first 10 min; peak CL counts occurred mostly after 20–30 min.

To compare the CL response of PMNL stimulated with different strains, and performed with leukocyte suspensions of different donors on different days, we included the standardised Zymosan suspension in all experiments. The mean

FIG. 1.—Kinetics of chemiluminescence of PMNL activated by *Klebsiella* K5 (●—●), K6 (▲—▲), K69 (Δ—Δ) and a standardized Zymosan suspension (○—○).

FIG. 2.—CL response of PMNL towards different serotypes of *Klebsiella* strains (curve-area integration expressed as a percentage of the curve-area integration of a standardised Zymosan suspension.)

curve-area integration of the CL response caused by Zymosan was 2.08×10^6 , with a standard deviation of 0.90×10^6 (43%). Despite this rather high variation with Zymosan between different experiments, the CL response (curve-area integration) caused by bacterial suspensions, expressed as a percentage of the curve-area integration of Zymosan tested on the same occasion, was reproducible.

Fig. 2 shows the CL responses of PMNL towards different *Klebsiella* isolates, calculated as described above. Each point is the mean CL value of at least two experiments with one strain, obtained with different leukocyte suspensions on different days. All K1 and K5 strains and nearly all K2 and K4 strains gave a low CL response, whereas nearly all K3, K6 and K > 6 strains gave a higher CL response.

Statistical analysis by the Wilcoxon test, at a significance level of $p=0.01$, showed that the CL response of the K1, K2, K4 and K5 strains differed from that of the K > 6 strains, whereas that of the K6 strains did not ($p>0.05$); K3 strains differed from K > 6 strains at a significance level of $p=0.05$.

The CL response of PMNL, stimulated with different strains, was enhanced by the presence of serum in the reaction mixture. Without serum, the CL response caused by 36 strains was 5–34% (mean 14%) of the CL response with the same strains tested in 10% serum. In a few experiments with serum inactivated for 30 min at 56°C, the CL responses were at the same low level as, or somewhat higher than, those obtained without serum.

DISCUSSION

Clinical isolates of *Klebsiella* were able to stimulate human PMNL in the presence of serum. The measurement of the resulting CL was facilitated by the reducing agent, luminol. The lowest CL response was observed with all K1 and K5 strains and nearly all K2 and K4 strains, whereas the CL response of nearly all K3, K6 and K > 6 strains was higher and showed a wider scatter. The low CL response with K1, K2, K4 and K5 correlates with their virulence for mice (Simoons-Smit *et al.*, 1984). In skin pathogenicity, K1, K2, K4 and K5 were significantly more virulent than K3, K6 and K > 6. The good correlation between the CL response of human PMNL and the virulence of the same strains in mice is demonstrated by the calculated correlation coefficient $r = -0.9175$ for the mean of the values for CL and for virulence with each group of strains (K1, K2, K3, K4, K5, K6 and K > 6).

CL reflects the metabolic activity of leukocytes during phagocytosis (Cheson *et al.*, 1976; Schadelin *et al.*, 1981), and is often used as an indirect measure of phagocytosis. The necessity of an intact complement system and other factors like immunoglobulins to obtain an optimal CL response, was demonstrated by the low CL responses with 36 strains tested without serum and a few strains tested with complement-inactivated serum. These observation reject the role of random adherence of bacteria to human PMNL, independent of the phagocytic process, as the source of the CL response.

Our results indicate that K1, K2, K4 and K5 strains were more resistant to phagocytosis than K3, K6 and K > 6 strains. The in-vitro resistance to phagocytosis might explain the greater virulence of these strains for mice, if we assume a low rate of phagocytosis *in vivo* too. The observation that strains virulent in the mouse are poorly phagocytosed by human PMNL, and *vice versa*, suggests that the mouse model may be useful in evaluating the virulence of *Klebsiella* strains in man.

The overall correspondence of certain K types with high virulence and high resistance to phagocytosis clearly illustrates the importance of certain K antigens in virulence of *Klebsiella*. However, not all strains fit into this scheme. Single strains of some serotypes of K > 6 with low virulence for mice (fig. 2) gave a CL response as low as with most virulent strains (K1, K2, K4 or K5). This overlap indicates that resistance to phagocytosis is not always the decisive determinant in pathogenicity. The ultimate evidence for the role of K antigens in virulence and resistance to phagocytosis will be investigated with variants of the virulent strains, devoid of K antigen.

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