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Research Article

IL-10 in Antilipopolysaccharide Immunity Against Systemic *Klebsiella* Infections

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Aim. This study was undertaken in order to determine whether anti-inflammatory cytokine interleukin-10 is responsible for a previously described protection against *Klebsiella* infection mediated by antilipopolysaccharide antibodies. *Methods*. BALB/c mice were infected intraperitoneally with a lethal challenge of *Klebsiella pneumoniae* Caroli. One group was protected with monoclonal antibodies prior to infection and the second was not. We measured plasma levels of interleukin-10 at different time points by enzyme immunoassay and analyzed the relation between interleukin-10 and proinflammatory cytokines interleukin-6 and tumor necrosis factor-α in order to determine the association of these ratios with the outcome of infection. *Major findings and conclusions*. We found different pattern of interleukin-10 production in protected mice compared with unprotected ones. The difference is greatest 24 hours postinfection. The ratios between IL-10 and proinflammatory cytokines confirmed the suppressed proinflammatory response in protected animals, especially 24 hours postinfection. Hence the mortality in unprotected mice begins immediately after we conclude that such cytokine relation and IL-10 production are, at least partially, responsible for the destiny of infected animals and the outcome of infection.

INTRODUCTION

Klebsiella pneumoniae (*K pneumoniae*) is the important cause of community-acquired and nosocomial infections. In spite of modern antibiotic therapy, some of these infections are still characterized by high mortality rates [1, 2]. Therefore various researches have been conducted for the determination and characterization of possible virulence factors that are involved in pathogenicity and might serve as target molecules for the construction of active or passive immunologic tools as an alternative to antibiotic therapy. The prominent morphologic and immunologic feature of *Klebsiella spp* is a polysaccharide capsule. Capsular (K) antigen plays a significant role in the pathogenicity of *K pneumoniae* [3] and induces protective immune response [4]. The obstacle for the successful construction of such K antigen-based immunologic tool is the existence of more than 70 K antigens on clinical isolates. Therefore, a more effective *Klebsiella* vaccine should probably contain some other surface determinants with less complex seroepidemiology than that of the K antigen. Lipopolysaccharide (LPS; O antigen) is another promising candidate surface molecule for the development of such immunologic tool since there are evidences that antibodies directed against LPS are capable to penetrate through bacterial capsule [5, 6] and are protective in a mouse model of lethal systemic *Klebsiella* infection [7]. Further, the number of O antigens is relatively low compared to the number of K antigens. Several seroepidemiological studies have shown that a great proportion of all *Klebsiella* clinical isolates belongs to only few O antigenic groups [8, 9].

We have previously described an O1 antigen-specific murine monoclonal antibody (clone Ru-O1, immunoglobulin G2b; IgG2b) that exerted protection in a murine model of lethal systemic *Klebsiella* infection [7]. The exact mechanisms involved in this protection remained unknown. A part of its protective effect could be contributed to the ability of coating the encapsulated bacteria which was demonstrated by in vitro experiments [10]. Besides that, it may also exert protection by several other mechanisms. One of the possible mechanisms is neutralization of circulating free LPS and its biological effects. Cytokines themselves play the important role in the pathogenesis of *Klebsiella* and other gram-negative infections. Their production is partly induced by LPS. The data regarding the role of some cytokines in the pathogenesis of infections are often controversial [11]. In a previous study we analyzed the involvement of proinflammatory cytokines

in the protection of mice against lethal *Klebsiella* challenge. We found that animals protected with anti-LPS MAb had lower concentrations of all cytokines analyzed, especially 24 hours after the infection [12]. We concluded that such proinflammatory cytokine pattern is important for the outcome of infection. Cytokine network consists of both, pro- and anti-inflammatory cytokines. Interleukin-10 (IL-10) functions as an anti-inflammatory cytokine that suppresses production of certain proinflammatory mediators [13–15] and exerted anti-inflammatory properties in experimental models of LPS-induced lethality [16, 17]. Moreover, the relation between IL-10 and some proinflammatory cytokines seems to be important for the outcome of infection [18, 19].

Therefore, in the present study, we tried to determine whether the protective effect of anti-LPS Ru-O1 MAb could be a consequence of modulated IL-10 production. We analyzed plasma concentrations at different time points after a lethal intraperitoneal (IP) bacterial challenge with *K pneumoniae* Caroli (O1 : K2). We also evaluated the balance between IL-10 and certain proinflammatory cytokines which were reported to be important and even predictable for the outcome of infection at the same time points.

MATERIALS AND METHODS

Animals

Eight- to ten-week-old pathogen-free male BALB/c mice weighing 20 to 25 grams each were used through study. Animals were obtained from the breeding colony at the Medical Faculty, University of Rijeka. They were kept in plastic cages and given standard laboratory food (standard pellets, Faculty of Biotechnology, Domžale, Slovenia) and water ad libitum. The experiments were conducted according to the laws and principles found in the *International Guiding Principles of Biomedical Research Involving Animals* by the Council of International Organisations of Medical Science. The principles are also in accordance with the *Statute for Laboratory Animals of the Croatian Society for Laboratory Animals*.

Bacteria

Experimental infections were performed using the highly virulent variant of the strain *K pneumoniae* Caroli (O1 : K2) which has been used before by us [7, 12] and by other authors as well [4].

Experimental Klebsiella infection

The bacterial suspensions were prepared as described previously [7]. The experimental groups of mice were pretreated four hours before the infection with an IP injection of purified MAb Ru-O1 (protected group) at the dose of 40 μg/g that was determined to be protective, or with PBS (unprotected group). Animals were injected IP with an estimated dose of 50 organisms of *K pneumoniae* Caroli, corresponding to five times the LD_{50}. According to our previous findings, all animals from unprotected group died within 4 days, with the mortality of approximately 50% after 2 days. Pretreatment with MAb Ru-O1 resulted in 70 percent survival.

Plasma cytokine analysis

The animals were euthanized by inhalation of CO_2 and the blood was obtained immediately after by cardiac puncture at 2, 6, 12, and 24 hours postinfection. Plasma samples were separated and stored at -20°C until assayed. Plasma levels of IL-10 were determined by commercially available mouse cytokine ELISA kit (Bender MedSystems, Austria) according to the manufacturer's instructions. According to data supplied by the manufacturer, detection limit for specified kit was 14.52 pg/mL. The overall intra-assay and interassay reproducibilities, expressed by coefficient of variation, were declared to be $< 5\%$ and $< 10\%$, respectively. The results are presented as mean values ± SE of the mean (SEM) of cytokine concentration.

Determination of IL-10 versus proinflammatory cytokine ratios

Ratios between IL-10 and proinflammatory cytokines interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) were calculated from absolute values of IL-10 concentrations obtained in this study and from results for the mentioned proinflammatory cytokines reported in a previous article obtained from plasma samples of the same experimental animals [12]. We calculated IL-6/IL-10 and IL-10/TNF-α ratios. The plasma concentrations of IL-6 in experimental animals were in range from 0.3 to 14.7 ng/mL for unprotected group and from 0.1 to 2.3 ng/mL for protected animals. The values of TNF-α ranged from 0.5 to 3.2 ng/mL for unprotected group and from 0.1 to 1.1 ng/mL for the protected group.

Statistical analysis

Statistical significance of the difference between cytokine concentrations of unprotected, protected, and uninfected control groups was determined by two-tailed Student *t* test.

RESULTS

Kinetics of IL-10 concentration

Two hours after the infection, IL-10 concentration in both experimental groups increased above the level in uninfected control animals (Figure 1). After six hours, the concentration in unprotected group further increased while the concentration in protected animals remained almost unchanged (1389 ± 137 versus 971 ± 101 pg/mL; NS). Twelve hours postinfection concentrations in both infected groups increased almost to the same level, reaching the maximum in unprotected animals (1683 ± 109 pg/mL). During the next twelve hours, IL-10 concentration continues to increase in the protected group (2230 ± 253 pg/mL) while in unprotected animals the concentration decreases to the level of 1314 ± 111 pg/mL ($P < .02$).

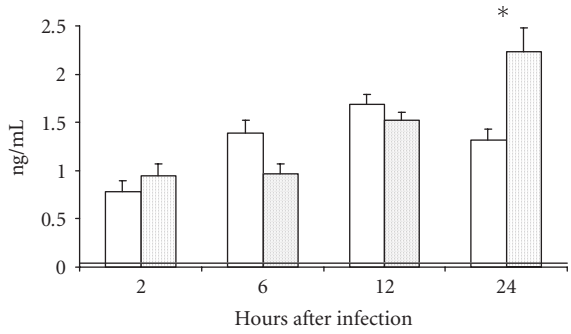

Figure 1: Plasma concentration of IL-10 in the blood of BALB/c mice protected with anti-LPS MAb Ru-O1 (gray bars) and unprotected mice (white bars) after the IP infection with 50 CFU of *K pneumoniae* Caroli (O1 : K2) at different time points. Results are expressed as mean values ± SEM ng/mL. Six animals in each group were infected. *Above bars represents the statistical significance between the groups at the level of $P < .02$. Horizontal line represents the mean value of plasma IL-10 concentration in the blood of uninfected control mice.

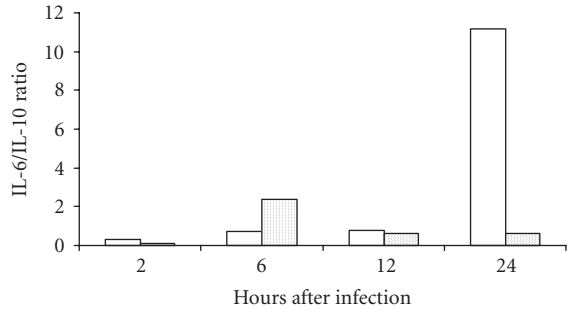

Figure 2: IL-6/IL-10 ratio in BALB/c mice protected with anti-LPS MAb Ru-O1 (gray bars) and unprotected mice (white bars) after the IP infection with 50 CFU of *K pneumoniae* Caroli (O1 : K2) at different time points.

IL-6/IL-10 ratio

Two, six, and twelve hours after the infection, IL-6/IL-10 ratio remained lower than 1 in unprotected group (Figure 2). Twenty four hours postinfection, the value dramatically increased to the level of 11.2. In the protected group, the ratio was lower compared to unprotected animals at all time points except 6 hours after the infection when it reached the highest value of 2.4. The most prominent difference between two groups was 24 hours postinfection, when in protected animals the ratio value was approximately 19-fold lower than that in unprotected group.

IL-10/TNF-α ratio

IL-10/TNF-α ratio (Figure 3) in unprotected group remained at all time points within the range from 0.4 (12 hours postinfection) to 1.6 (2 hours postinfection). In the protected group, the ratio was the highest two hours after the infection

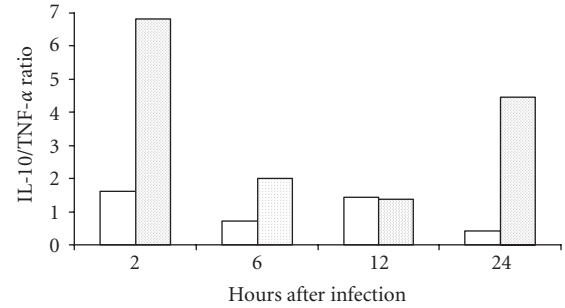

Figure 3: IL-10/TNF-α ratio in BALB/c mice protected with anti-LPS MAb Ru-O1 (gray bars) and unprotected mice (white bars) after the IP infection with 50 CFU of *K pneumoniae* Caroli (O1 : K2) at different time points.

(6.8) and then markedly decreased to the value of 2 (6 hours postinfection). The final ratio value in this group reached the level of 4.4. This value is 11-fold higher than that in the unprotected group.

DISCUSSION

We have previously reported that anti-LPS MAb Ru-O1 exerted protection in a murine model of lethal systemic *Klebsiella* infection [7]. In a previous article, we reported that this effect can partially be explained by modulation of proinflammatory cytokine response [12]. Since the production of proinflammatory cytokines during the course of infection can be harmful and may lead to shock, multiple-organ failure and death [20, 21], anti-inflammatory cytokines such as IL-10 are necessary for downregulating inflammatory process and maintaining homeostasis for proper function of vital organs [16, 22].

We have also reported that after the IP challenge, according to our experimental design, bacteria appear in blood two and six hours after the infection in unprotected and protected groups, respectively [12]. The results of the present study suggest that the kinetics of IL-10 production depends also on the beginning of bacteremia. After the initial rise in plasma IL-10 concentration (2 hours postinfection) that was detected in both groups compared to uninfected animals, in unprotected group the concentration continues to increase reaching its maximum 12 hours postinfection. On the contrary, in the protected group IL-10 concentration begins to increase later (between 6 and 12 hours) and continues to increase until 24 hours of infection. Elevated concentration in this group remained at almost unchanged level during the next 24 hours and then slightly begun to decrease (data not shown). We presumed that the degree of bacteremia correlates with a quantity of liberated bacterial compounds including LPS. Our presumption is that the quantity of circulating LPS and its effects are diminished in the protected group because of its binding to MAb. We also presumed that a part of MAb has coated the circulating bacteria resulting in activation of other available defense mechanisms. These events may result in different IL-10 production

patterns between groups. We speculate that the dynamic of greater IL-10 production in unprotected animals during the early phase is responsible for inadequate inflammatory reaction and control of infectious agent. On the other hand, anti-inflammatory cytokine response in protected animals was lower during the first six hours. Such condition may be responsible for the development of more effective inflammation and better control of infectious agent during that phase.

LPS is known to be a stimulator of IL-10 production and IL-10 was described to exert beneficial effects in several experimental models [16, 23]. Our results partially support such findings. IL-10 level in our experiment was different between two groups especially 24 hours postinfection with higher concentration in the protected group. Hence the mortality in unprotected group begins immediately after we concluded that the cytokine profile at this stage is important for the outcome of infection. We speculate that higher IL-10 concentration in the protected group 24 hours postinfection efficiently downregulates the production of proinflammatory cytokines which we analyzed previously [12]. The highest level of IL-10 in unprotected group was detected after 12 hours. At this time point, proinflammatory cytokines production started to exceed the production of IL-10. We presumed that the excessive proinflammatory production, especially 24 hours postinfection, could not be counteracted by anti-inflammatory effect of IL-10 and other anti-inflammatory cytokines. Therefore, we speculate that such cytokine pattern expressed deleterious effects that contributed mortality.

Several clinical studies reported that high anti-inflammatory cytokine levels at certain stages of infection are associated with the poor outcome of infection [18, 19]. On the contrary, the association of high IL-10 level with increased mortality was not confirmed in laboratory models of sepsis since exogenous administration of IL-10 conferred protection against excessive proinflammatory cytokine production and mortality [16, 22, 24]. Our results support these experimental data. Possible explanation for such difference between mentioned clinical and experimental results may be the statement that IL-10 effect depends on timing, dose, and location of expression [25]. These parameters could be controlled and followed in experimental conditions only, so the comparison with infected patients' data is sometimes difficult or impossible.

Further, different studies suggested the importance of the balance between anti- and proinflammatory cytokines for the outcome of systemic infection. To date, the interaction between these cytokine groups in response to sepsis remains a controversial subject. Proinflammatory cytokines IL-6 and TNF-α are capable to induce a strong inflammatory reaction that may lead to severe hypotension, multiple-organ dysfunction, and death [26, 27]. Such response ultimately triggers a compensatory anti-inflammatory reaction involving antagonist mediators including IL-10. IL-10 has the ability to suppress the synthesis of proinflammatory cytokines and effectively downregulates the proinflammatory reaction [14, 28]. However, the highest values of the IL-10/TNF-α ratio were reported to be associated with the poor outcome in sepsis [18] and in febrile hospitalized patients [19]. Our results are in contrast with such findings since the IL-10/TNF-α ratio is higher in surviving protected animals, especially 24 hours postinfection. On the contrary, our experimental results are in accordance with results of several studies that reported the correlation of high IL-6/IL-10 ratio and the poor outcome in patients with systemic inflammation [29, 30]. Such findings can probably be explained by the exaggerated proinflammatory response that is associated with inadequate anti-inflammatory compensation [30].

The exact mechanisms involved in different cytokine production patterns between two groups in our experiments require more detailed studies. They should certainly consider the fact that the main difference between these groups is the presence of anti-O1 MAb of the IgG2b subclass in the protected group. LPS molecule itself is known to activate surface receptors on the cells of innate immune system resulting in activation of series of signaling events that potentiate the production of molecular mediators. Ru-O1 MAb by its binding to circulating LPS most probably reduces the level of LPS interaction with the endotoxin sensing apparatus, leading to modified cytokine production in protected animals. On the other hand, Fc fragment of IgG in immune complexes was described to be involved in upregulation of IL-10 production through its binding to Fc receptors [31]. We presume that such upregulation is present in protected animals. However, modified IL-10 production with beneficial effects in protected animals may also be the consequence of some other regulatory mechanisms involved. Several recently published articles suggested the close cooperation between innate and adaptive immune responses [32, 33]. We believe that in our experimental model, this cooperation of MAbs as effector molecules of the adaptive immune response with the innate immune response to LPS is possible, but this hypothesis requires confirmation by future research.

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