Role of Neutrophils in Controlling Early Stages of a *Chlamydia trachomatis* Infection

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Inoculation of mice with the granulocyte-depleting monoclonal antibody RB6-8C5 showed that neutrophils are critical protective effector cells during a *Chlamydia trachomatis* infection. In addition, administration of monoclonal antibody 2E6 demonstrated that extravasation of neutrophils into the peritoneal cavity in response to inoculation with the *C. trachomatis* MoPn biovar is dependent on the surface β -2 integrin molecule CD18.

Chlamydia trachomatis is a gram-negative obligate intracellular bacterium that can infect a wide range of hosts and cause a variety of diseases in humans including sexually transmitted diseases and trachoma (3, 15, 26). Neutrophils are the most predominant effector cells recruited to the infectious foci during the early stages of a chlamydial infection (1, 4, 19, 20, 27). In spite of this, the role of neutrophils in a chlamydial infection is not clearly understood (13, 28, 29). With the advent of monoclonal antibodies (MAb), the possibility of assessing the functional role of neutrophils in vivo has now become feasible. Here, we utilized two MAb, RB6-8C5 and 2E6, to determine the role that neutrophils may have during a chlamydial infection. RB6-8C5 is a rat immunoglobulin G2b (IgG2b) antibody that binds and depletes mature mouse neutrophils and eosinophils (23). 2E6 is an IgG hamster MAb that inhibits extravasation by binding to the surface β -2 integrin molecules of mouse neutrophils, thereby blocking their interaction with the intercellular adhesion molecule 1 of the vascular endothelial cells (16).

Seven- to 8-week-old female BALB/c (*H-2^d*) mice were purchased from Simonsen Laboratories (Gilroy, Calif.). HeLa-229 cells were obtained from the American Type Culture Collection (ATCC) (Rockville, Md.), and McCoy cells were from Viomed (Minneapolis, Minn.). These cells were grown as previously described (19). The hybridoma RB6-8C5 was a gift from R. Coffman (DNAX Research Institute, Palo Alto, Calif.), and the hamster hybridoma 2E6 was obtained from ATCC. The MAb RB6-8C5 was purified by affinity chromatography with protein A- or G-coupled Sepharose (Pierce, Rockford, Ill.), and the MAb 2E6 was purified by hydroxyapatite column chromatography. The hamster IgG and the rat IgG, which served as controls, were purchased from Cappel (Durham, N.C.). A 4% (wt/vol) solution of sodium caseinate (casein digest; Difco Laboratories, Detroit, Mich.) was prepared in saline and buffered to a pH of 7.0. The *C. trachomatis* mouse pneumonitis (MoPn) serovar (Nigg II strain) was obtained from ATCC, propagated in HeLa-229 cells, and purified as previously described (19, 20). *C. trachomatis* MoPn was heat killed at 63°C for 60 min in a water bath. Mice inoculated intraperitoneally (i.p.) with different doses of *C. trachomatis* (see below) were killed at 3, 5, and 14 days after inoculation,

and the liver, spleen, and lungs were harvested and processed for culture in McCoy cells as previously described (30). For genital inoculation, mice received 10^6 *C. trachomatis* MoPn inclusion-forming units (IFU) intravaginally, and subsequently vaginal swabs were collected on a weekly basis for culture (20). The intravaginally inoculated animals received 0.2 mg of MAb RB6-8C5 i.p. 1 day before the challenge, on the day of the challenge, and on days 3, 5, and 7 postchallenge. The control mice received rat IgG on the same dates, by the same route, and at the same dosage. In each experiment, three to five mice were included in each group, and experiments were repeated two to three times.

In order to assess the ability of *C. trachomatis* MoPn to induce a neutrophil response, three groups of mice were inoculated i.p. with 10^5 , 10^6 , and 10^7 IFU of *C. trachomatis* MoPn, and a control group of mice was inoculated with a solution containing 0.2 M sucrose, 0.02 M phosphate, and 5 mM glutamate (SPG). At 9 h after inoculation, the peritoneal cavity was washed with phosphate-buffered saline and the number of neutrophils per mouse was determined. The animals inoculated with SPG yielded $(5.0 \pm 3) \times 10^4$ neutrophils per mouse, while the mice inoculated with 10^5 *C. trachomatis* IFU yielded $(2.4 \pm 0.6) \times 10^5$, and those inoculated with 10^6 and 10^7 yielded $(1.21 \pm 0.21) \times 10^6$ and $(1.59 \pm 0.43) \times 10^6$, respectively. On the basis of these results, we decided to inoculate the mice i.p. with 5×10^6 *C. trachomatis* IFU to determine the effect of depleting neutrophils with MAb RB6-8C5. Mice were given 0.2 mg of MAb RB6-8C5 intravenously at 1 day and 6 h before bacterial challenge. At 3, 5, and 14 days postchallenge, the liver, spleen, and lungs from the mice were harvested and the yield of IFU was determined. As shown in Table 1, the number of chlamydial IFU recovered at 3 and 5 days postchallenge was approximately 10- to 500-fold greater in the mice treated with MAb RB6-8C5 than in the control mice inoculated with a nonspecific rat IgG. By 14 days postinfection, no *C. trachomatis* IFU were recovered from any of the three organs (limit of detection, 5×10^3 IFU per g of tissue).

Similarly, mice that were intravaginally inoculated with 10^6 *C. trachomatis* MoPn IFU and received MAb RB6-8C5 had a more intense infection than the group receiving the nonspecific rat IgG (Fig. 1). For example, starting on the second week after the challenge, the percentage of mice treated with RB6-8C5 that had a positive vaginal culture was on the average two- to fourfold higher than the mice treated with the nonspecific rat IgG ($P < 0.05$). Also, the number of IFU shed per mouse was

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TABLE 1. Effect of depleting neutrophils with MAb RB6-8C5 on the recovery of *C. trachomatis* MoPn from the spleen, liver, and lungs at 3 and 5 days postinfection^a

Day	<i>C. trachomatis</i> log ₁₀ IFU ($\pm$ SE)/g of tissue					
	Spleen		Liver		Lungs	
	Rat IgG	RB6-8C5	Rat IgG	RB6-8C5	Rat IgG	RB6-8C5
3	7.3 $\pm$ 0.2	8.7 $\pm$ 0.3 ^b	5.3 $\pm$ 0.4	7.1 $\pm$ 0.5 ^b	5.2 $\pm$ 0.4	6.4 $\pm$ 0.3 ^b
5 ^c	5.9 $\pm$ 0.3	7.0 $\pm$ 0.3 ^b	4.6 $\pm$ 0.3	6.0 $\pm$ 0.4 ^b	5.3 $\pm$ 0.2	5.6 $\pm$ 0.3

^a The experiment was performed three times with three to five mice each time per group.

^b Significantly different from values obtained with rat IgG (control) ($P < 0.05$ by the Wilcoxon rank sum test).

^c All cultures were negative from the animals euthanized at 14 days postinoculation.

approximately 2- to 100-fold higher in the experimental group treated with RB6-8C5 than in the control group ($P < 0.05$).

To determine the mechanisms involved in the migration of neutrophils to the peritoneal cavity following a *C. trachomatis* infection, we utilized MAb 2E6. It has been previously shown that neutrophil recruitment into the peritoneal cavity after i.p. inoculation of caseinate involves β -2 integrins, namely, CD18, since MAb to this antigen completely abrogates neutrophil influx. To test the biological activity of the MAb 2E6 prepared in our laboratory, we inoculated mice intravenously with 0.5 mg of the MAb 2E6 or with a nonspecific hamster IgG antibody as a control and subsequently inoculated the mice i.p. with caseinate. In the mice injected with sodium caseinate and the control antibody, we recovered $(6.10 \pm 1.01) \times 10^6$ and $(8.43 \pm 1.58) \times 10^6$ neutrophils from the peritoneal cavity at 6 and 12 h postinoculation, respectively. On the other hand, in the mice inoculated with sodium caseinate and MAb 2E6, we retrieved $(5.4 \pm 1.1) \times 10^5$ and $(9.6 \pm 1.6) \times 10^5$ neutrophils at 6 and 12 h postinoculation, respectively ($P < 0.05$). On the basis of these results, we decided to test the ability of MAb 2E6 to block the influx of neutrophils at 9 h following a *C. trachomatis* MoPn i.p. challenge. From mice inoculated with 5×10^6 *C. trachomatis* IFU and MAb 2E6, we recovered $(5.0 \pm 3) \times 10^4$ neutrophils, while in the mice injected with *C. trachomatis* and the control hamster IgG, we retrieved $(1.84 \pm 0.37) \times 10^6$ neutrophils ($P < 0.05$). Similarly, in the group of mice inoculated with heat-killed *C. trachomatis* and treated with MAb 2E6, we recovered $(4.0 \pm 3.0) \times 10^4$ neutrophils, while in the group inoculated with heat-killed *C. trachomatis* and treated with the control hamster IgG we retrieved $(1.11 \pm 0.19) \times 10^6$ neutrophils ($P < 0.05$).

The critical role of neutrophils during the early stages of infection with a facultative intracellular bacterium was recently reported (5–7, 8, 21, 22). However, there is no direct evidence that neutrophils are important in controlling infections due to obligate intracellular bacteria. Here, our results showed that mice inoculated intravaginally and i.p. with *C. trachomatis* MoPn and treated with MAb RB6-8C5 have a more severe infection, demonstrating the critical role that these cells play in defense against this pathogen. The fact that the mice inoculated with this MAb, like those treated with nonspecific antibody, eventually controlled the infection suggests that neutrophils restrained the infection, once MAb RB6-8C5 was catabolized, or that other immunological mechanisms were involved in abrogating the spread of the organism.

An increase in the number of eosinophils has also been observed particularly during perinatal chlamydial infections (2). Thus, the effect of MAb RB6-8C5 may be due to the

combined depletion of eosinophils and neutrophils, although we think that most likely the effect is predominantly due to neutrophil depletion. Furthermore, some reports have indicated that RB6-8C5 induces a decrease in the number of CD8⁺ cells. Initially, Fleming et al. (11) described the apparent cross-reactivity of RB6-8C5 with Ly-6C, an antigenic component present in some of the CD8⁺ cells. This decrease, however, does not appear to be due to a direct effect of the MAb itself but to the effects that neutrophil depletion may have on the T-cell immune response. This mechanism was postulated based on the fact that RB6-8C5 does not stain CD8⁺ cells in an immunofluorescence assay and on the observation that the decrease of CD8⁺ occurred several days after the administration of the MAb, while the depletion of neutrophils was detected a few hours after inoculation with MAb RB6-8C5 (8, 25). Furthermore, in the case of a *C. trachomatis* infection, although the role of CD8⁺ cells is still controversial,

FIG. 1. Progression of the infection following intravaginal inoculation with *C. trachomatis* MoPn. (Top) Percentage of mice treated with RB6-8C5 or a nonspecific rat IgG (control) with positive vaginal *C. trachomatis* cultures. (Bottom) Average number of *C. trachomatis* MoPn IFU shed vaginally by the mice treated with RB6-8C5 and by the control mice.

a report by Morrison et al. (17) appears to indicate that these cells may have a limited role in protection.

Using MAb 2E6, we have shown that in response to an i.p. inoculation with *C. trachomatis* there is a rapid influx of neutrophils to the peritoneal cavity that is at least in part mediated by CD18-dependent mechanisms. Conlan and North (7) demonstrated a similar response with *Salmonella typhimurium* but that was not the case in a *Listeria monocytogenes* infection, since MAb 2E6 failed to prevent neutrophil accumulation in the peritoneal cavity following inoculation with this organism. This finding contradicted the notion that neutrophil recruitment into the peritoneal cavity was mediated by only CD18. Conlan and North (7) postulated that the type of response could be influenced by the localization of the two organisms in different compartments of the host macrophages. *S. typhimurium* remains viable inside the phagocytic vacuole of the macrophage and as a result induces a CD18-dependent recruitment of neutrophils. In the case of a chlamydial infection, the interaction with macrophages seems to be fairly complex depending on the species, biovars, and strains of *Chlamydia* and on the stage of maturation of the host cells (12, 14, 18). Thus, at this point we cannot make conclusions that could apply to the recruitment of neutrophils via a CD18 mechanism based on the interaction between *Chlamydia* and macrophages.

With heat-killed *C. trachomatis* we also observed an increase in the number of neutrophils, a finding similar to that previously reported with endotoxins and other killed organisms including *Escherichia coli* (10). This observation and the fact that we made our measurements at 9 h postinoculation, a period of time too short for *C. trachomatis* to complete even a single cycle of replication, implies that neutrophil migration was mediated by a preformed chemotactic factor such as the chlamydial lipopolysaccharide. No attempt was made at determining the long-term effect of MAb 2E6, since this antibody temporarily blocks but does not deplete neutrophils and *C. trachomatis* requires a period of 2 to 4 days to go through one cycle of replication. Also, there are several reports indicating that MAb 2E6 does not prevent extravasation of neutrophils to all organs (24).

In conclusion, we have shown that neutrophils play a critical role in the control of the early stages of a chlamydial infection and that the recruitment of neutrophils to the site of infection is mediated by CD18. The pathogenic mechanisms that lead to the long-term sequelae of a chlamydial infection, namely, abdominal pain, ectopic pregnancy, and infertility are poorly understood. Recently, Morrison et al. (17) have shown that knockout mice for the β 2-microglobulin, I-A, or CD4 gene all developed hydrosalpinx following a genital infection, thus casting doubts on the role of T cells in the pathogenesis of the disease. Similarly, in humans, a majority of patients with chronic sequelae become infertile after a single acute episode of pelvic inflammatory disease (3, 26). Thus, it will be important to determine if, by blocking the migration of neutrophils to the genital tract, we can prevent infertility following a chlamydial infection. In this respect, recent evidence suggests that CD18, as part of the CD11b/CD18 glycoprotein complex, can be involved in a neutrophil-mediated immune injury that may result in male infertility (9).

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