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Effect of Modulation of Polymorphonuclear Leukocyte Migration with Anti-CD18 Antibody on Pathogenesis of Experimental Otitis Media in Guinea Pigs

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Guinea pigs were treated with anti-CD18 antibody (M8), and subsequently middle ears (ME) were infected with nontypeable *Haemophilus influenzae*. Forty-eight hours after infection, the ME washes of these animals had significantly lower polymorphonuclear leukocyte numbers but higher bacterial counts compared with washes of animals treated with control antibody (M11) or saline. The edema and epithelial damage of ME tissues correlated directly with polymorphonuclear leukocyte numbers and not bacterial counts.

Otitis media is a common disease of children and adults. Polymorphonuclear leukocytes (PMNs) have been shown to be the most predominant cells involved in the early host responses elicited during the invasion of bacterial pathogens into the middle ear (ME) cavity (1, 11, 21). The beneficial role of PMNs in host defense against otitis media is underscored by the increased incidence of otitis media in patients with congenital or acquired defects in qualitative and quantitative functions of PMNs (17). Conversely, there is evidence that certain products released by PMNs, such as arachidonic acid metabolites and hydrolases, contribute to the adverse sequelae of infection such as tissue edema, ischemia, and necrosis. Some of these products may contribute to persistent inflammation even after bacterial elimination, as manifested by the formation of chronic ME effusions (2, 6, 9, 12).

In order to further study the relative role of PMNs in the pathogenesis of otitis media, we have developed an experimental model of otitis media due to nontypeable *Haemophilus influenzae* (NTHI) in guinea pigs (14). This model was further developed so that we could utilize antibodies to the guinea pig CD18 molecule to control the influx of PMNs into the ME cavity. Anti-CD18 antibodies have been successfully utilized in animal experiments to treat a variety of lesions (3, 7, 19, 22, 23). In this investigation, the impact of absence of PMNs on the growth of instilled bacteria and subsequent histological changes in the ME tissues of guinea pigs were evaluated.

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Two guinea pig-specific hybridoma clones synthesizing anti-CD18 monoclonal antibodies, M8 and M11, were generously supplied by Eileen Remold-O'Donnell at the Center for Blood Research, Boston, Mass. Both monoclonal antibodies are of the immunoglobulin G1 subclass but recognize different epitopes of the CD18 molecule. M11 was previously shown to have no in vitro inhibition of macrophage spreading, whereas M8 produced 50% inhibition (15). The clones were further propagated in pristane-primed BALB/c mice.

The immunoglobulin G antibodies in the ascitic fluid were separated by protein A affinity chromatography (Bio-Rad, Richmond, Calif.), dialyzed in phosphate-buffered saline (PBS), and standardized by protein estimation by spectrophotometry. All animals received saturating amounts of antibodies as determined by flow cytometry.

An isolate of NTHI from a child with otitis media was used for the ME inoculation of guinea pigs. The bacterial inoculum was prepared as previously described (13). A dose of approximately 10^3 CFU of NTHI in a 100-μl volume was used. We have previously determined this to be a subinfectious dose in normal guinea pigs because very few bacteria can be recovered within 48 h of inoculation of ME (14). The rationale for this strategy was based on the assumption that the rapid clearance of bacteria inoculated at a subinfectious dose is primarily due to killing by PMNs. A larger bacterial inoculum was not used because of the possibility that the bacteria would continue to multiply if they were in excess of the PMNs capable of phagocytosis and killing.

One-month-old male Hartley albino guinea pigs (350 g) obtained in virus-antibody-free condition (SASCO, Omaha, Nebr.) were immunized daily by intraperitoneal injection of M8 (2 mg/kg), M11 (2 mg/kg), or saline for 4 days. On the second day, the NTHI inoculum was instilled into the ME via a suprabullar approach under sterile conditions. Forty-eight hours later (day 4), the ME bullae were lavaged with 300 μl of PBS and analyzed for PMN numbers in Neubauer chambers, and the cell density of NTHI was determined by colony count on chocolate agar plates. The minimum sensitivity for the detection of NTHI was 100 CFU/ml. Therefore, the samples that yielded no growth were assigned a value of 90 CFU/ml. Also at this point, the ME bullae were removed, fixed in 10% formalin, decalcified in 0.1 M EDTA, stained with hematoxylin and eosin, and analyzed for histological changes under a light microscope. The ME washes and tissues were evaluated at 48 h after NTHI inoculation because our preliminary studies showed that the PMNs in ME washes and tissues comprise 95% of the inflammatory-cell population at this point, whereas other inflammatory cells begin to appear in larger numbers beyond this point. Therefore, the 48-h examination was expected to reflect the maximal impact of PMNs on bacterial proliferation and
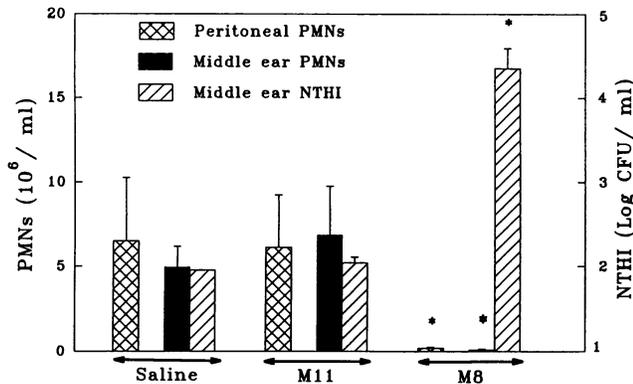
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FIG. 1. Mean PMN and NTHI counts ± standard error in animals receiving saline ($n = 6$), antibody M11 ($n = 6$), and antibody M8 ($n = 6$). *, $P < 0.001$.

tissue inflammation. The results obtained were pooled from two separate experiments.

As an additional measure of the effect of antibody treatment on PMN migration, 1 ml of thioglycolate broth was infused into peritoneum after an ME wash had been performed on day 4. The peritoneum was lavaged with 30 ml of PBS and analyzed for PMN count. On days 3 and 4, samples of peripheral blood obtained by cardiac puncture were analyzed for bacterial growth in Trypticase soy broth.

Statistically significant differences between the means of PMN and NTHI counts were assessed by use of a two-tailed paired Student t test, and a P value of <0.001 was accepted as significant. Linear regression was done by the least-squares method.

The results showed that the mean number of PMNs in the ME washes 48 h after NTHI inoculation in the M8 group was 9.5×10^4/ml, while the numbers of PMNs in the M11 and saline groups were 6.85×10^6/ml and 4.95×10^6/ml, respectively. The number of PMNs in the ME washes of the M8 group was significantly lower than those in washes of the M11 and saline groups ($P < 0.001$) (Fig. 1). The results suggest that PMN migration to the ME cavity is dependent on CD18-based adherence mechanisms.

The mean NTHI count in the ME washes of M8 group was 3.1×10^4 CFU/ml. This count was significantly higher than those for the M11 and saline groups, which had mean NTHI counts of 50 and 18 CFU/ml, respectively ($P < 0.001$) (Fig. 1). Interestingly, the NTHI counts are inversely correlated with PMN counts ($r = 0.63$; $P < 0.05$), suggesting that in normal animals effective PMN migration to the ME is essential in the control of bacteria inoculated at a subinfectious dose. None of the animals had positive blood cultures.

The mean numbers of PMNs in the washes of thioglycolate-inflamed peritoneum of animals treated with M11 and saline were 6.1×10^5/ml and 6.5×10^5/ml, respectively, whereas the number of PMNs in the washes of M8-immunized animals were 2×10^4/ml. The PMN counts in the M11 and saline groups were comparable, while those in the M8 groups were significantly diminished ($P < 0.001$) (Fig. 1). These findings obtained with peritoneal washes are analogous to those obtained with ME washes. As anticipated from the previous in vitro studies with these antibodies (15), the present observations confirm that M8 interferes with a functional epitope on the CD18 molecule and blocks the in vivo migration of PMNs to the peritoneum, whereas M11

binds to a nonfunctional epitope that does not interfere with PMN migration.

Similar to the PMN counts of ME washes, the histologic sections of all saline- and M11-treated animals showed abundant PMNs in the ME cavity and the submucosal tissues (Fig. 2). The ME mucosa of all of these animals were severely edematous with areas of epithelial loss and interstitial hemorrhage. Although other inflammatory cells such as lymphocytes and macrophages were observed, the sparsity of these cells makes it unlikely that they had a major effect on the inflammatory process. Conversely, the histologic sections of the M8 group showed no inflammatory changes in three animals (Fig. 2b), mild edema in two, and moderate edema in one. These animals had little cellular infiltration. Overall, these observations suggest that the degree of tissue edema directly correlates with the number of PMNs present in the section.

Our study clearly demonstrates that PMNs, though important for bacterial control, are responsible for much of the inflammatory reaction in the early phase of otitis media due to bacteria such as NTHI. The mild inflammation present in some of the animals with minimal cellular infiltration may be the result of bacteria or bacterial products and their effect on local tissues. The pathologic findings in the ME tissues are consistent with the observations of Tuomanen et al. (22), who also used anti-CD18 antibodies in a rabbit model of bacterial meningitis to decrease inflammation. In this study, the antibody-induced inhibition of PMN migration was associated with decreased blood-brain barrier injury and cerebral edema and with prevention of mortality in animals also receiving antibiotic therapy.

Similarly, other investigators have utilized anti-CD18 antibodies to define the role of PMNs in various disease processes (3, 7, 19, 22, 23). The CD18 molecule, the common β-subunit of leukocyte integrins (β_2 integrins), exists as a heterodimer noncovalently bound with CD11a, b, and c α-subunits on surface membranes of leukocytes of humans and many other animal species (10, 18). These complexes are adherence molecule receptors which facilitate PMN migration through adhesion-dependent events (20). In the present guinea pig experiments, the anti-CD18 antibodies inhibited PMN migration to sites such as the ME and peritoneum. In this respect, the guinea pig model is preferable to other widely used animal models of experimental otitis media, such as chinchillas, for which anti-CD18 antibodies are currently unavailable.

The role of PMNs in the pathogenesis of acute otitis media is supported by the finding of PMN products capable of producing inflammation, such as leukotrienes, prostaglandins, lysozymes, and proteases (2, 4, 8, 9, 12) in acute ME effusions. In chinchillas, experimental ME inoculation with PMN products such as platelet-activating factor has resulted in the development of acute otitis media with effusion (16). The stimulus for PMN activation and degranulation likely includes both a direct effect of bacterial pathogens in ME and secondary production of mediators by host cells. For example, Garofalo et al. (5) have shown that in vitro incubation of PMNs with NTHI results in the release of leukotriene B_4. However, direct information regarding the most critical cell responsible for early inflammatory changes of the ME is lacking. In this respect, the current study demonstrates that in the acute phase, PMNs are the most important cells contributing to injury of the ME tissues. We also observed relatively few lymphocytes and macrophages, whose numbers were decreased even more in the M8-treated animals. The paucity of these cells makes it unlikely that
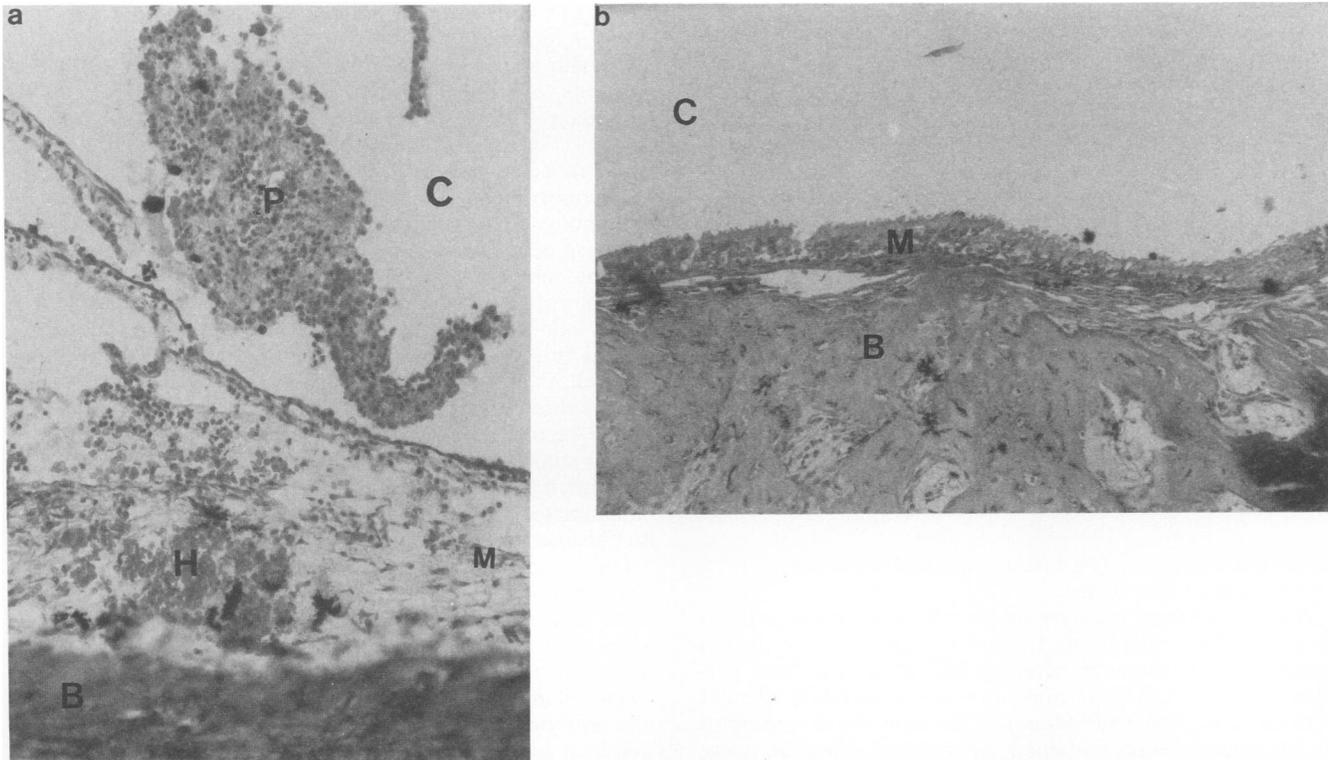
FIG. 2. (a) ME mucosal biopsy 48 h after NTHI inoculation of a guinea pig treated with M11 antibody showing significant inflammatory reaction. B, bone; C, ME cavity; H, hemorrhage; M, mucosa; P, PMN exudate. (b) ME mucosal biopsy 48 h after NTHI inoculation of a guinea pig treated with M8 antibody showing no inflammatory reaction. B, bone; C, ME cavity; M, mucosa.

they are a major cause of the inflammatory lesions in these experiments.

In summary, the present study shows that though PMNs are important in the control of bacteria in the ME, it is the degree of PMN infiltration, and not bacterial quantity, that determines the severity of ME mucosal edema and epithelial damage. Further use of the anti-CD18 antibodies in guinea pigs will allow us to evaluate the effect of interruption of PMN migration at various times on regulation of cytokines and other inflammatory mediators in the ME and the rate of resolution of otitis media.

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