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Resistance to Meningococcemia Apparently Conferred by Anti-H.8 Monoclonal Antibody Is Due to Contaminating Endotoxin and Not to Specific Immunoprotection

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We evaluated the ability of a monoclonal antibody directed against the common H.8 antigen of pathogenic *Neisseria* sp. to confer passive protection against meningococcal disease in mice. The apparent protection conferred by antibody purified from tissue culture supernatant was actually the result of endotoxin contamination of buffers and tissue culture media. Endotoxin-free anti-H.8 antibody was not protective. The possibility of endotoxin contamination should be considered when evaluating immunity conferred by passively administered antibody in animal models.

Neisseria meningitidis causes significant human disease for which there is no completely effective vaccine, in part due to the marked heterogeneity and variability of meningococcal surface structures (6–8). The H.8 antigen is an outer membrane protein possessing several characteristics that make it attractive as a meningococcal vaccine component. Anti-H.8 monoclonal antibodies (MAbs) bind to all pathogenic neisseriae, including meningococci of different serogroups and serotypes (3). The antigen is expressed in vivo and is immunogenic in people during meningococcal infection (2). There is evidence for surface exposure of the antigen in vitro in the closely related gonococcus (3, 9).

We tested whether an anti-H.8 MAb would confer passive protection in the well-described adult mouse model of meningococcal infection (5, 6). Two recent human blood isolates were used for challenge: FAM18 (serogroup C, serotype 2a) and JB515 (B, nontypable). Meningococci were grown overnight from frozen stocks on GC medium base agar (Difco Laboratories) with Kellogg supplements (10). Bacteria were suspended in GCB broth (10) and grown to log phase with constant agitation at 37°C in a 5% CO_2 atmosphere. A suspension at a concentration of 4×10^8 to 1×10^9 CFU/ml (always confirmed by viable counts) was diluted in phosphate-buffered saline, and 0.5 ml was injected intraperitoneally into female BALB/c mice (Charles River Laboratories) 6 to 12 weeks of age. Antibody or control preparations (100 μg of affinity-purified antibody, 1.0 mg of ascites protein, or an equal volume of buffer) were administered intraperitoneally in a volume of 0.1 to 0.5 ml 4 h before meningococcal challenge. Mice were tail bled 4 h after meningococcal challenge as follows: after the tail was cleansed with povidone iodine (Clinidine; Clinipad Corp.) and ethanol, a vein was nicked with a sterilized razor, and 5 to 20 μl of blood was plated in duplicate on GC agar or was serially diluted before plating. The level of bacteremia was expressed as CFU per milliliter of blood.

In initial experiments, MAb was purified from tissue culture supernatant by affinity chromatography (11) using staphylococcal protein A-agarose (Sigma Chemical Corp.). Protein concentrations were determined by using the Bio-Rad assay according to the manufacturer's directions. Anti-H.8 MAb H.101 (immunoglobulin G1) significantly reduced the incidence and level of bacteremia caused by both meningococcal strains, whereas a control antibody did not provide such protection (Table 1). These results were reported previously (J. R. Black and J. G. Cannon, Program Abstr. 24th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 96, 1984).

When we attempted to repeat the passive-protection experiments using new preparations of antibody and buffer, we found that we could not duplicate the initial results. There was a low incidence of bacteremia in mice receiving any treatment (buffer, control antibody, or H.101) before injection of meningococci, whereas most mice did become bacteremic when injected with meningococci alone (data not shown). We suspected that endotoxin, which is a common contaminant of even sterile laboratory materials and tissue culture media (1, 15) could be influencing the results, since endotoxin is known to increase the nonspecific resistance of animals to a number of infectious agents (4, 12). Using the E-toxate *Limulus* amebocyte lysate assay (Sigma) according to the manufacturer's instructions, we determined that both H.101 and control antibody solutions, as well as diluent HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (United States Biochemical Corp.) and sterile water from a tissue culture facility, were contaminated with endotoxin. Assay of dilutions of the antibody preparations in pyrogen-free water (Travenol) indicated contamination of at least 50 to 200 ng/ml, with *Shigella flexneri* endotoxin used as a standard (data not shown). Different lots of HEPES prepared in pyrogen-free water (Travenol) all were contaminated with endotoxin but varied in the concentration of endotoxin.

Purified *Shigella* endotoxin (Sigma) elicited resistance to meningococcal infection similar to that seen in the initial passive-protection experiments. In two experiments, 12 mice in groups of three were injected intraperitoneally with strain FAM18, or with 200 ng of endotoxin followed 4 h later by strain FAM18. All of these mice became bacteremic, but the level of bacteremia in endotoxin-injected mice averaged only 2 and 17% of that in control mice. Reduction in bacteremia caused by strain JB515 was seen after injection

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TABLE 1. Summary of protection experiments using affinity-purified, tissue culture-derived MAbs

Challenge strain	MAb	No. of mice challenged	No. of mice bacteremic (%)	Mean CFU/ml in blood of bacteremic mice
JB515[a]	None (buffer)	20	20 (100)	7.8×10^4
	Control MAb	23	19 (83)	1.8×10^4
	H.101	18	2 (11)	6.5×10^2
FAM18[b]	None (buffer)	28	26 (93)	1.9×10^6
	Control MAb	16	16 (100)	7.3×10^5
	H.101	29	16 (55)	8.3×10^3

[a] Inoculum, approximately 9×10^3 CFU.
[b] Inoculum, approximately 3×10^5 CFU.

of as little as 50 pg of endotoxin (data not shown). The protection provided by injection of purified endotoxin and of endotoxin-contaminated HEPES buffer was transient, lasting at least 4 h but less than 36 h (data not shown). Pyrogen-free saline (Travenol), which was endotoxin negative in the *Limulus* amebocyte lysate assay, did not display this protective effect when injected before meningococci.

To reassess immunoprotection by the H.101 antibody, we needed an endotoxin-free preparation of antibody. Attempts to remove endotoxin completely from the tissue culture-derived preparations, using repeated passages over a Detoxi-Gel affinity column (Pierce Chemical Co.), were unsuccessful. Hybridoma-induced ascites fluid (13) was negative in the *Limulus* amebocyte lysate assay and was used in further passive-protection experiments. Mice injected with ascites received at least as much antibody as the mice in the initial experiments, and the antibody did enter the blood (data not shown). All mice receiving H.101 or control ascites or saline became bacteremic after challenge with strain FAM18 (ca. 3×10^4 CFU), and the levels of bacteremia in the three groups (six mice each) were similar (2.0×10^4, 4.6×10^4, and 3.4×10^4 CFU/ml, respectively).

The results of the passive-protection experiments using endotoxin-free antibody from ascites indicate that the anti-H.8 MAb did not protect mice from bacteremia caused by intraperitoneally injected meningococci. These results do not disprove a role for the H.8 antigen in pathogenesis, nor do they eliminate the possibility that the antigen can be a target of an effective immune response. Active immunization studies using the H.8 antigen may provide better information as to the utility of this antigen in a vaccine. Recent progress in the purification of the H.8 antigen (14; unpublished data) should make such experiments possible soon.

In these studies, we found that small quantities of endotoxin could have a dramatic effect on the murine model of meningococcal infection. Chong and Huston also detected trace amounts of endotoxin in MAb preparations from tissue culture and showed that such amounts could protect mice from lethal *Escherichia coli* sepsis (K. Chong and M. Huston, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, B188, p. 151). Since endotoxin contamination of materials that may be used in passive-protection experiments using animal models is not unlikely, and since we have shown that endotoxin can remarkably mimic protective antibody in reducing meningococcal infection, we suggest that the possibility of endotoxin contamination be considered in the design of any such experiments and the interpretation of results.

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