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Anti-Lipid A Monoclonal Antibody Centoxin (HA-1A) Binds to a Wide Variety of Hydrophobic Ligands

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This note describes the binding specificities of four lipid A monoclonal antibodies (MAbs) including Centoxin (HA-1A); these MAb s display similar binding properties. MAb s reacted with lipid A and heat-killed smooth bacteria, whereas no reactivity was observed with smooth lipopolysaccharide (LPS). Immunoblotting of bacterial extracts separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed that the MAb s bound to many polyepitope bands including the molecular weight markers. Denaturation of bovine serum albumin (BSA) by boiling or dithiothreitol treatment unmasked antibody epitopes. In addition, binding both to a hydrophobic aliphatic C12 chain covalently coupled to BSA and to single-stranded DNA was observed. The polyreactivity of these clones is most likely mediated by a preferential reactivity with hydrophobic molecular patches.

Severe infections with gram-negative organisms are still an important cause of death. One of the outer membrane components of such bacteria, endotoxin (LPS) plays a pivotal role in the pathogenesis of GNB. There is substantial evidence that LPS initiates a cascade of events leading to the onset of the sepsis syndrome (13). Since lipid A is the toxic moiety of LPS, many attempts to prepare protective agents against LPS have focused on the preparation of ligands to the lipid A moiety. It has been postulated that antibodies directed against the conserved core or lipid A region of LPS may cross-react with LPS from many different bacteria, whereas no reactivity was observed with smooth LPS. Immunoblotting of bacterial extracts separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed that the MAb s bound to many polyepitope bands including the molecular weight markers. Denaturation of bovine serum albumin (BSA) by boiling or dithiothreitol treatment unmasked antibody epitopes. In addition, binding both to a hydrophobic aliphatic C12 chain covalently coupled to BSA and to single-stranded DNA was observed. The polyreactivity of these clones is most likely mediated by a preferential reactivity with hydrophobic molecular patches.
Tween 80 (Sigma), and incubated with MAb 40 (1.8 μg/ml), Centoxin (20 μg/ml), or α-Rc MAb 3 (3 μg/ml). Figure 1a shows that MAb 40 binds to a wide variety of OMPs (lane 2). No binding to proteinase K-treated OMPs was observed (lane 1). A wide range of protein bands was also recognized in heat-killed R5 bacteria (lane 3); the banding pattern was comparable to that produced by total protein staining with Congo red (data not shown). For proteinase K-treated R5 bacteria, only one predominant band of 36 kDa recognized by the antibody was left (lane 4). This band could represent either a proteinase K-resistant protein or a nonprotein component. Remarkably, all protein molecular weight markers (phosphorylase B, BSA, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme; Pharmacia) were also recognized by MAb 40 (lane 5). Just like MAb 40, Centoxin recognized many proteins in heat-killed S. minnesota R5 (Fig. 1b, left panel, lane 3) and one band in the proteolytic digest (lane 2). In accordance with the binding studies performed by ELISA, no binding of Centoxin to R5 LPS (lane 1), which was near the front of the gel and which was visualized with α-Rc LPS antibody, was seen (Fig. 1b, right panel, lanes 1 and 3). In contrast to what was seen with MAb 40 and Centoxin, proteinase K treatment of whole bacteria did not abolish the epitope of MAb 3 (lane 2). The binding of Centoxin to coextracted porin proteins in LPS samples has previously been described by Mascelli et al. (15). Our binding studies further emphasize the great affinity of Centoxin for proteins separated by SDS-PAGE. These proteins have undergone a series of denaturing treatments, such as breaking of cysteine bridges in DTT, dissolution in SDS, and heating. To study whether the unfolding of proteins could unmask antibody epitopes, a well-defined system was chosen for this subject of investigation. BSA (Sigma) was exposed to a number of conditions that affect its conformational characteristics: incubation in 2, 5, and 8 M urea overnight at room temperature, incubation for 1 h in 200 mM DTT at room temperature, boiling for 10 min, and a combination of these.
conditions. In a dot spot experiment (Fig. 2), no binding of clone 40 to native BSA (1 mg/ml; spot 10) or to BSA incubated with urea alone (spots 2, 3, and 4) was observed. Clear immunoreactivity was observed with BSA treated with 200 mM DTT (spot 8) and with boiled BSA (spot 11). By these treatments the protein is expected to unfold to expose the inner parts of the molecule to the outside. It was concluded that partial denaturation of the protein, achieved by either boiling or DTT treatment, uncovers protein patches that are hidden in native BSA. These inner parts, that are known to have a hydrophobic character, are recognized by MAb 40.

The binding of MAbs 40 and HA-1A to all molecular weight markers and to bacterial proteins in SDS-PAGE and to denatured BSA but not to native BSA in a dot spot assay suggests that hydrophobic protein patches are recognized. In additional ELISA experiments it was observed that MAbs 28, 37, 40, and HA-1A also have great affinity for non-protein-derived hydrophobic ligands, such as a polymeric aliphatic chain (C12, dodecyl) covalently coupled to BSA (9) (Fig. 3). No binding to native BSA was observed (data not shown). In addition to observing the binding to dodecyl-BSA, we confirmed the finding of Bhat et al. (6) that Centoxin binds to ssDNA (origin: calf thymus type I; Sigma) and found that MAbs 28, 37, and 40 also bind to ssDNA (data not shown); DNase treatment of the DNA sample completely abolished the binding (data not shown). The binding of MAb 40 and Centoxin to solid-phase bound ssDNA could be inhibited by fluid-phase dodecyl-BSA and heat-killed bacteria (data not shown); this suggests that the same antigen-binding site is involved in the interaction with the various ligands. Bhat et al. (6) provided convincing evidence for a highly specific binding of Centoxin to i antigen, a carbohydrate structure present on cord erythrocytes. In agreement with this was the observation that the MAb was coded for by VH4.21, a gene often utilized by anti-i antibodies (7). They explained the cross-reactivity with lipid A by assuming that an acyl-substituted disaccharide structure, present both in i antigen and lipid A, was the common epitope (4). Alternatively, the data provided by us suggest that the hydrophobicity of the antigen-binding site is responsible for its polyreactivity. The specificity of MAbs 28 differs from that of Centoxin. i.e., MAb 28 reacts specifically and strongly with LPS of S. minnesota R595 (2) and does not bind to LPS of other chemotypes. Yet MAb 28 displays a polyreactivity similar to that of Centoxin; in Centoxin and MAb 28 hydrophobicity is a common property.

Our study emphasizes the preferential reactivity of Centoxin and MAbs 28, 37, and 40 with hydrophobic structures. These are present in lipid A (acyl chains), in unfolded proteins (hydrophobic amino acid side chains), and in dodecyl-BSA (polymeric aliphatic chain). The binding of Centoxin to ssDNA can be explained by the hydrophobic nature of the DNA bases, which are only exposed in the single-stranded conformation. Indeed Centoxin does not bind to double-stranded DNA (7, 8). Moreover it was found that MAbs 37 and Centoxin had a greater affinity for monophosphoryl lipid A than for diposphoryl lipid A (data not shown), which is less hydrophobic than monophosphoryl lipid A.

In summary, this binding study shows that a number of MAbs raised against rough mutants of bacteria involved in GNB and selected on binding to lipid A are polyreactive. The multispecific binding pattern can be explained by a strong interaction with hydrophobic molecular patches, and in addition, these Mabs might react more specifically with carbohydrate epitopes such as those present in the i antigen or in R595 LPS (2). As it has now been demonstrated that Centoxin has a great affinity for denatured proteins and likely also for hydrophobic patches present in native proteins such as bacterial porins, contamination of LPS samples with coextracted proteins may have led to misinterpretations in studies of Centoxin binding to LPS performed in the past.

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