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Neutralizing Activity of Monoclonal Antibodies to Heat-Sensitive and Heat-Resistant Epitopes of *Rickettsia rickettsii* Surface Proteins

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Antiprotein monoclonal antibodies derived from mice inoculated with *Rickettsia rickettsii* heated at 56°C for 15 min are of two types: one is type specific for epitopes denatured by moderate temperatures, and the other is specific for epitopes resistant to 100°C for 5 min. The heat-resistant epitopes are found by immunoblotting on multiple polypeptides after solubilization of the rickettsiae at temperatures of 56°C or higher. Most, but not all, antibodies to the heat-sensitive epitopes passively protected mice against two 50% lethal doses of *R. rickettsii*, whereas none of the antibodies to heat-resistant epitopes did.

Reports from several laboratories indicate that antiprotein monoclonal antibodies derived from mice inoculated with rickettsiae are specific either for heat-sensitive or for heat-resistant epitopes on those proteins. Monoclonal antibodies from mice inoculated with viable *Rickettsia rickettsii* reacted with 120- and 155-kilodalton (kDa) protein antigens of that organism by immunoblotting when the rickettsiae were solubilized in sample buffer for 1 min at 37°C but not when the rickettsiae were boiled for 5 min (2). Dasch and coworkers found that some of their monoclonal antibodies to typhus group rickettsiae combined with heat-denatured proteins in enzyme-linked immunosorbent assay and immunoblotting tests, whereas other monoclonal antibodies did not (G. A. Dasch, C. D. Benjamin, J. P. Burans, and R. I. Jaffe, Abstr. Annu. Meet. Am. Soc. Rickettsiol. 1985). Only those antibodies which recognized native antigen were able to neutralize the lethal effects of typhus rickettsiae for mice. In addition, these workers also discovered by immunoblotting tests that the heat-resistant epitopes were present on molecules of a variety of sizes (G. A. Dasch, personal communication). In this report, we describe monoclonal antibodies specific for heat-sensitive and heat-resistant epitopes of the 155-kDa surface protein of *R. rickettsii*. The heat-resistant epitopes of the 155-kDa protein of *R. rickettsii*, like those of typhus group rickettsiae, were found by using immunoblotting tests on molecules of a variety of sizes.

The Sheila Smith, Morgan, and R strains of *R. rickettsii* used in this study were described earlier (2, 3). All but two of the monoclonal antibodies were prepared as described earlier (1), except that the mice were inoculated subcutaneously with 80 μg (dry wt) of either Sheila Smith or Morgan strain rickettsiae grown in embryonated hen eggs (10), purified by Renografin density gradient centrifugation (12), held at 56°C for 15 min, and emulsified in incomplete Freund adjuvant before injection. Two and three weeks later, the mice were given intravenous booster injections of rickettsiae (80 μg) heated similarly. Two monoclonal antibodies used in this study, 10-14A5A10 and 7-7A1B11B10, were originally obtained in earlier experiments (1, 2). Neutralization of rickettsiae by monoclonal antibodies was determined in mice as described previously (2, 5), except that the rickettsiae were incubated in undiluted hybridoma culture supernatant before injection. Samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis by the procedure of Laemmli (9). Immunoblotting was done by the method of Batteiger et al. (4) with 3% bovine serum albumin as the blocking agent (4). Rickettsiae were treated with proteinase K by the method of Hitchcock and Brown (7). Reactivity of monoclonal antibodies with rickettsiae treated in various ways was determined with the Bio-Dot microfiltration apparatus according to the instructions of the manufacturer (Bio-Rad Laboratories, Richmond, Calif.), except that the washing solution consisted of 0.05 M phosphate buffer (pH 7.4) plus 0.15 M NaCl without Tween 20.

In the present experiments, three basic kinds of monoclonal antibodies were obtained from mice inoculated with heat-treated rickettsiae, the same kinds that were obtained previously when mice were inoculated with viable rickettsiae (1, 2) and a new variety. These monoclonal antibodies were specific for two proteins, a 120-kDa protein (Fig. 1, lane 1) and a 155-kDa protein (Fig. 1, lanes 3, 5, 6, 7, and 8), and a lipopolysaccharide (LPS)-like antigen (data not shown) exactly like the one illustrated previously (2, 3). However, in contrast to the monoclonal antibody shown in lane 3 of Fig. 1 and to previously obtained monoclonal antibodies which recognized only proteins solubilized at temperatures less than 100°C by immunoblotting (2), most of the antibodies obtained in the present study reacted much better by immunoblotting with the 155-kDa protein solubilized in Laemmli buffer at 100°C for 5 min (lanes 6 and 8) than with the protein solubilized at 37°C (lanes 5 and 7). In addition, these monoclonal antibodies reactive with the epitopes resistant to boiling temperatures also reacted in immunoblotting experiments with polypeptides of a variety of molecular sizes (lane 6). Two patterns of reactivity were observed with antibodies derived from mice inoculated with either heated Sheila Smith or heated Morgan strains of *R. rickettsii*, although only the patterns developed with anti-Morgan strain antibodies are shown. In one pattern, many dense bands in the molecular mass range of 80 to 120 kDa were seen (Fig. 1, lane 6). In the other pattern, two faint bands were seen on the X-ray film in addition to the major 155-kDa band, but only the major band was successfully reproduced on the photograph (lane 8). Thus far, we have not observed the monoclonal antibodies which detect heat-resistant epitopes of the 120-kDa protein.

The band in lane 3 has a lower apparent molecular mass

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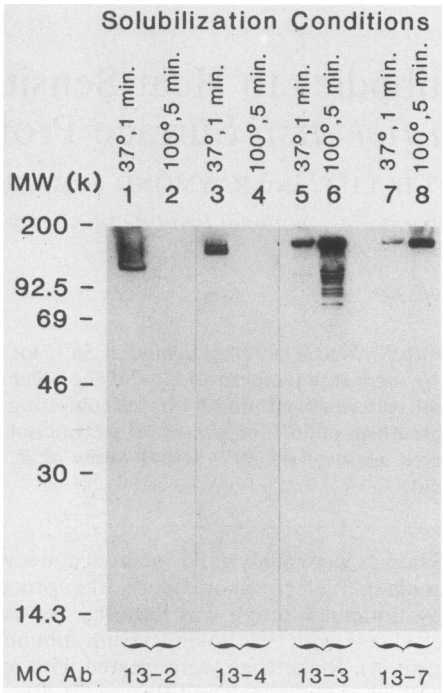

FIG. 1. Representative immunoblots obtained with monoclonal antibodies. The antibodies were derived from mice inoculated with *R. rickettsii* Morgan heated at 56°C for 15 min and then reacted with *R. rickettsii* Morgan solubilized either at 37 or at 100°C. Abbreviations: k, kilodalton, MC Ab, monoclonal antibody.

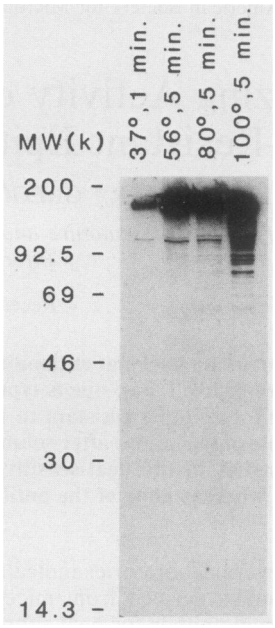

FIG. 2. Effect of temperature of solubilization of rickettsiae on immunoblot patterns. Rickettsiae of the Morgan strain solubilized at various temperatures and electrophoresed on a 12.5% polyacrylamide gel were developed with monoclonal antibody 13-3.

than do the principal bands in lanes 6 and 8 of Fig. 1, although all of the principal bands are considered to have a nominal molecular mass of 155 kDa. The reason for this apparent discrepancy is that the 155-kDa protein is heat modifiable (see Fig. 2 of reference 1). Antibodies used in lanes 5 through 8 were specific for heat-resistant epitopes on the modified 155-kDa protein prepared by boiling rickettsiae in Laemmli buffer for 5 min, whereas the antibody used in lane 3 reacted with a heat-sensitive epitope present only on the unmodified protein exposed to 37°C for 1 min (Fig. 1). The unmodified form of the protein had an apparent molecular mass of about 130 kDa.

The effect of heat on the immunoblot patterns developed with one monoclonal antibody to a heat-resistant epitope was determined by solubilizing rickettsiae of the Morgan strain either at 37°C for 1 min or at 56, 80, or 100°C for 5 min. All samples were held at room temperature for about 2 h before electrophoresis. With increased temperature, there was an increase in the number and density of bands demonstrable (Fig. 2). This epitope presumably was protein and not carbohydrate, since bands were not observed after treatment of the rickettsiae with proteinase K (data not shown).

A similar effect was noted in a dot blot analysis when intact rickettsiae suspended in phosphate-buffered saline were heated at various temperatures (Fig. 3). Antibodies to the heat-sensitive epitopes of the 120- and 155-kDa proteins reacted with rickettsiae heated at 37 or 56°C but reacted poorly or not at all with rickettsiae heated at 80°C or higher. The reverse order of reactivity was observed for antibodies to heat-resistant epitopes of the 155-kDa protein. The immunoreactivity of the 155-kDa antigen solubilized at 100°C was much better than that of antigen solubilized at 37°C. Antibody to the LPS-like antigen reacted well in the dot blot analysis regardless of the temperature of solubilization.

Although some monoclonal antibodies to heat-sensitive epitopes of the 120- and 155-kDa proteins failed to protect mice against challenge with two 50% lethal doses of *R. rickettsii* (Fig. 3), most antibodies to these heat-resistant epitopes were protective. Of the antibodies obtained previously to the 120- and 155-kDa proteins from mice inoculated with viable rickettsiae (1, 2) and reexamined in connection with this study, less than 10% of the antibodies to heat-sensitive epitopes were nonprotective. Of the 32 clones obtained in the current experiments from mice inoculated

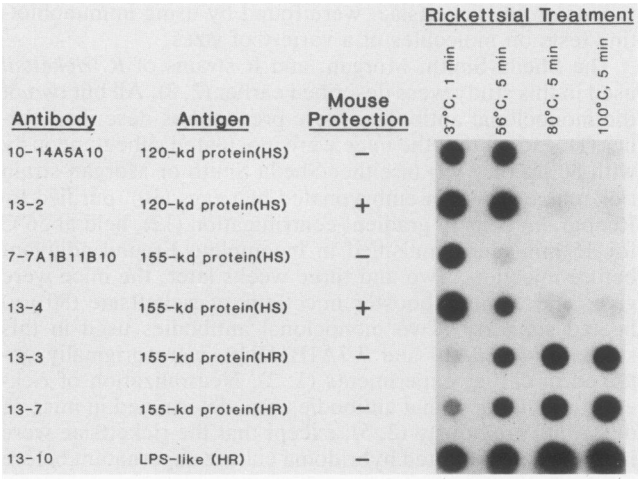

FIG. 3. Dot blot analysis of antigenicity of rickettsiae (Morgan strain) heated at various temperatures in phosphate-buffered saline. HS, Heat sensitive; HR, heat resistant, kd, kilodalton.

with heat-treated rickettsiae, all 6 of the clones to heat-sensitive epitopes (3 for each protein) were protective. In contrast, none of the 9 clones to heat-resistant epitopes of the 155-kDa protein or 17 clones to the LPS-like antigen protected mice.

These data and data obtained previously (3) indicate that at least two changes occur when the 155-kDa protein is heated at moderate temperatures (≥56°C) in the presence of the solubilizing buffer. First, the apparent molecular mass of the protein increases from approximately 130 kDa in its unmodified form to about 155 kDa in its heat-modified configuration. Second, immunoblots of the protein heated at temperatures ranging from 37 to 100°C reveal that the protein fragments in a specific and reproducible way, each fragment bearing at least one specific heat-resistant epitope. It is presently unknown whether the fragmentation is due to exposure of the heat-modified protein molecule to an endogenous protease, to autocatalytic degradation such as that seen with subtilisin (11), or to some other cause. Incorporation of the protease inhibitor phenylmethylsulfonyl fluoride did not inhibit the appearance of the multiple reactive polypeptides. Thyroglobulin hydrolyzed by trypsin and then reacted with a monoclonal antibody in an immunoblotting test gave a pattern of multiple polypeptides containing the specific epitope not unlike the pattern reported here (8).

Thus far, no antibody to a heat-resistant epitope of the 155-kDa protein has been found to passively protect mice from challenge with *R. rickettsii* as do most antibodies to the heat-sensitive epitopes on the 120- and 155-kDa proteins. Conceivably, the heat-resistant epitopes are exposed by denaturing influences, such as heat, and are not immunoaccessible to any appreciable extent on the surface of normal infectious rickettsiae. A somewhat similar finding has been reported by Ferguson et al. (6) for poliovirus. Monoclonal antibodies capable of reacting with the infectious poliovirus in an antigen-blocking test generally were able to prevent poliovirus infection of tissue culture cells and were unable to react with denatured protein in an immunoblotting test. In contrast, monoclonal antibodies that reacted with purified poliovirus capsid in the blocking test and with denatured protein in an immunoblotting test did not neutralize the tissue culture infectivity of the poliovirus.

Although the above data indicate strongly that monoclonal antibodies to heat-resistant epitopes cannot protect mice, the role of antibodies to heat-sensitive epitopes is not understood. Since some antibodies to surface proteins and all antibodies to the LPS-like antigen combined with their epitopes on rickettsiae but did not prevent death of mice challenged with *R. rickettsii*, it is clear that combination antibody and a surface constituent is not necessarily sufficient for neutralization. These data support the hypothesis that there is a critical site(s) on the surface proteins essential for infectivity. The function of the postulated site(s) could be related to adsorption to and penetration of host cellular membranes. Antibodies specific for the site(s) would neutralize infectivity, but antibodies to noncritical portions of these proteins or to other surface constituents, such as the LPS-like antigen, would not materially affect the infectious process. Obviously, much more work will be required to resolve this important problem.

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