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Antigenic Heterogeneity in High- and Low-Virulence Strains of *Rickettsia rickettsii* Revealed by Monoclonal Antibodies

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Previously it has been reported that strains of *Rickettsia rickettsii* that differ greatly in their ability to cause disease in guinea pigs are similar by serological and sodium dodecyl sulfate-polyacrylamide gel electrophoresis analyses. In this study, we used monoclonal antibodies to the virulent R and the relatively avirulent HLP strains to investigate strain differences which might account for the disparate behavior of the strains in guinea pigs. Coomassie blue-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis profiles of the R and HLP strains were nearly identical for polypeptides with apparent molecular weights greater than 32 kilodaltons (kDa). All of the monoclonal antibodies to a lipopolysaccharide-like antigen reacted equally well with antigen from both strains by immunoblotting. None of the antibodies to the lipopolysaccharide-like antigen protected mice against challenge with viable rickettsiae. Some antibodies reacted with both 120- and 155-kDa polypeptides of both strains in radioimmune precipitation and immunoblotting tests, and other antibodies reacted only with the homologous strain. The monoclonal antibodies cross-reacted with the heterologous strain in the enzyme-linked immunosorbent assay essentially either completely or not at all. The ability of the monoclonal antibodies to the 120- and 155-kDa polypeptides to protect mice against the two strains was correlated with the ability of the antibodies to react with the antigens in the enzyme-linked immunosorbent assay and radioimmune precipitation or immunoblotting tests. These results demonstrate that R and HLP antigens which appear identical in molecular weight differ in their compositions of antigenic determinants.

In the last several decades, numerous members of the spotted fever group of rickettsiae exhibiting considerable differences in virulence and antigenic composition have been isolated from a variety of ticks and from patients residing in various regions of the United States (3, 7, 9, 11–13, 15, 22, 23, 25). As yet, the factors responsible for virulence of spotted fever group rickettsiae have not been identified. In a previous study, no difference was found by one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) among four strains of *Rickettsia rickettsii* isolated from Rocky Mountain spotted fever patients in Montana and North Carolina, although these strains differed in a minor but significant degree in their abilities to produce fever, scrotal reactions, and death in guinea pigs (4). A fifth strain, the HLP strain of *R. rickettsii* isolated from the rabbit tick *Haemaphysalis leporispalustris* (22), by the above criteria was considerably less virulent for guinea pigs than were the four strains from patients, and the SDS-PAGE pattern of the HLP strain differed from those of the virulent strains in the positions of several polypeptides with estimated molecular weights less than 32,000 (4, 22). However, it was not determined whether differences in polypeptide composition were related to the differences in virulence.

A comparison of isogeneic strains which differed only in pathogenicity would perhaps be the easiest way to identify virulence factors of *R. rickettsii*, but, unfortunately, such strains are not available. Therefore, for the present study, we compared two *R. rickettsii* strains which we consider to be the most similar except for their abilities to cause disease in guinea pigs. These two strains are the virulent R strain, which was isolated from a tick collected in the Bitter Root Valley of western Montana (8), and the HLP strain, a low-virulence strain also isolated from the Bitter Root Valley (22). There are several reasons why we believe these strains are closely related. First, as mentioned above, electrophoretic patterns of virulent strains of *R. rickettsii* were very similar to that of the HLP strain (4). Second, sera in high dilution from guinea pigs infected with either the R or the HLP strain protected mice from challenge with the homologous and heterologous strains in cross-neutralization tests (9). Sera from guinea pigs inoculated with other species of the spotted fever group either did not protect against challenge with R and HLP strains or protected only in low dilution. Finally, Philip et al. (24) found that, in the microimmunofluorescence test with mouse antisera, R and HLP strains were somewhat different in serological behavior, but the HLP strain was so similar to the R strain that it was considered a variant of *R. rickettsii*. Thus, the above authors, utilizing the best criteria presently available for classification, support the identification by Parker et al. of the HLP isolate as a strain of *R. rickettsii* (22).

In the study reported below, we found with a panel of monoclonal antibodies (MAbs) that two major surface proteins of the R and HLP strains had both common and unique antigenic determinants. As yet, it has not been determined whether these surface proteins play a significant role in the pathogenesis of *R. rickettsii* infections.

MATERIALS AND METHODS

Rickettsiae. The history of the three strains of *R. rickettsii* used in this study is presented in Table 1. The rickettsiae were grown in the yolk sacs of chicken embryos as previously described (27). For some experiments, the rickettsiae were purified by centrifugation in Renografin density gradients (28), suspended in 10 mM potassium phosphate buffer (pH 7.0) to a concentration of about 5 to 10 mg/ml, and held in 0.1- to 0.2-ml amounts at −75°C until used.

MAbs. Hybridomas prepared as described previously (2,

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TABLE 1. Western Montana strains of *R. rickettsii* studied

Strain	Reference	Host	Yr isolated	No. of passages[a]
R	8	Tick (*Dermacentor andersoni*)	1945	55–56[b] EP
R[c]	8	Tick (*Dermacentor andersoni*)	1945	55[b] EP; 6 TC; 3 EP
Sheila Smith[c]	8	Human	1946	8 EP; 1 TC; 1 GP; 4 TC; 8 EP
HLP[c]	22	Tick (*Haemaphysalis leporispalustris*)	1948	51 EP; 9 TC; 3 EP

[a] EP, Egg passage; TC, tissue culture; GP, guinea pig.
[b] Strain was passed an unknown number of times in eggs and guinea pigs prior to passages recorded here.
[c] Plaque purified.

17) were the source of the MAbs. MAbs designated with the prefix 7- were derived from Hsd mice inoculated with the Sheila Smith strain. Those MAbs designated 8- and 9- came from C57BL/10 SCN and C57BL/10 SNJ mice, respectively, infected with R-strain rickettsiae that had not been plaque purified. MAbs denoted with 10- were obtained from Hsd mice inoculated with plaque-purified R-strain rickettsiae, and MAbs 11- and 12- came from Hsd mice infected with the HLP strain. Isotypes of the MAbs were determined by enzyme-linked immunosorbent assay (ELISA) with a kit from Zymed Laboratories, Inc. (South San Francisco, Calif.). As a negative control, an MAb to *Chlamydia trachomatis*, generously furnished by Harlan Caldwell, was included in tests of anti-*R. rickettsii* MAbs.

Mouse protection test. The ability of MAbs to prevent early death of mice challenged with viable *R. rickettsii* was determined by the methods of Bell and co-workers (8, 10). Although the mechanism of injury has not been defined, this phenomenon is referred to in the literature as toxic death. Briefly, each MAb, concentrated 10-fold by precipitation with ammonium sulfate, was incubated at room temperature for 0.5 h with an equal volume of a suspension of the R or HLP strain diluted to contain four mouse 50% lethal doses per 0.5 ml. Four-week-old male Swiss mice of the Rocky Mountain Laboratories strain in groups of five were inoculated intravenously with 0.5 ml of the mixture. The MAbs were considered protective when at least four of the five mice survived for 24 h and nonprotective when four or more mice were dead at 24 h. Previously, it was shown that antibodies inoculated 30 min before challenge with rickettsiae also protected mice (2), but in this study the antibodies and rickettsiae were combined in vitro for the sake of convenience. Earlier studies demonstrated that doses of spotted fever vaccine that protected mice against toxic death were similar to doses of vaccine that prevented infection of guinea pigs by *R. rickettsii* (1, 10).

Virulence of rickettsiae for guinea pigs. Virulence was assessed as described earlier (3). One thousand PFU of each strain grown in chicken embryos and diluted in Snyder I solution (16) were inoculated intraperitoneally into guinea pigs, which weighed ca. 430 g each, in groups of six. Rectal temperatures were determined daily with a telethermometer (Yellow Springs Instrument Co., Yellow Springs, Ohio). Areas under the fever curves were calculated by Simpson's rule with an IBM PC-XT microcomputer in a manner similar to that described previously (5). Scrotal reactions of infected guinea pigs were assigned a score of 0 to 4+ on the basis of grossly observable responses, from undetectable (0) to severe with necrotic changes (4+). Fever curves, body weights, and scrotal reactions of guinea pigs in the two groups were compared by the Student *t* test.

SDS-PAGE. Samples were analyzed by SDS-PAGE by the procedure of Laemmli (18). The rickettsial suspensions were thawed, mixed with solubilizing buffer containing 4% SDS and 8% 2-mercaptoethanol, and heated in a boiling water bath for 5 min. Samples (25 μl) containing 25 μg of rickettsial protein were added to the wells and electrophoresed on a gel consisting of a 5% stacking gel and a 10 or 12.5% separating gel at a constant current of 40 mA. The gel was fixed in 25% isopropanol–7% acetic acid and stained with Coomassie brilliant blue R-250.

Unlabeled high- and low-molecular-weight protein standards (Bio-Rad Laboratories, Richmond, Calif.) or a ^{14}C-methylated protein mixture (Amersham Corp., Arlington Heights, Ill.) were run along with the rickettsial preparations on the gels.

Specificity of MAbs. Specificity of MAbs was determined either by radioimmune precipitation tests with Nonidet P-40 (NP-40) extracts of extrinsically radioiodinated, purified rickettsiae or by immunoblotting (2, 20). To demonstrate binding of MAbs to the lipopolysaccharide (LPS)-like antigen by immunoblotting, we treated rickettsiae solubilized in SDS–2-mercaptoethanol at 100°C for 5 min (2) with proteinase K (Boehringer Mannheim Biochemicals, Indianapolis, Ind.; 20 μl of a 0.25% solution in solubilizing buffer per 80-μl sample) at 60°C for 1 h (14), electrophoresed them on 10% gels, electroeluted them to nitrocellulose paper, and incubated them with MAbs before detection by radiolabeled protein A (2, 6). Immunoblotting of the 120- and 155-kilodalton (kDa) polypeptide antigens was accomplished by several modifications of the above procedure. First, because of the heat lability of the 120- and 155-kDa proteins (2), rickettsiae used for the demonstration of antibodies to the 120-kDa polypeptide were solubilized at 37°C for 1 min and at room temperature (<25°C) for about 2 h rather than at 100°C for 5 min, and rickettsiae used for the detection of antibodies to the 155-kDa polypeptide were solubilized at 37°C for 15 min and at room temperature for about 1.75 h. Second, the solubilized rickettsiae were not treated with proteinase K.

Two kinds of absorption experiment were performed to compare the 120- and 155-kDa proteins of the R and HLP strains. In the immunoblot experiment, 75-μl portions of an NP-40 extract of the R strain were absorbed twice overnight at 4°C with 50-μl quantities of anti-R MAbs concentrated 10-fold by ammonium sulfate precipitation. One hundred microliters of a 5% suspension of protein A-Sepharose in NP-40 lysing buffer (2) was added to each preparation after absorption; after 1 h at room temperature, the antigen-antibody-protein A complexes were removed by centrifugation for 2 min in a Microfuge (Beckman Instruments, Inc., Fullerton, Calif.). These supernatants were solubilized in an equal volume of sample buffer held at 37°C for 1 min and then electrophoresed on a 10% gel. Subsequent steps in this immunoblotting experiment were the same as described previously (2). For the radioimmune precipitation experiment, 75-μl portions of an NP-40 extract of ^{125}I-labeled rickettsiae were absorbed twice overnight at 4°C with 50-μl samples of 10-fold-concentrated anti-R MAbs. The antigen-antibody complexes were removed with protein A-

Sepharose as describe above. Next, 100-μl quantities of 10-fold-concentrated anti-HLP MAbs were added to each sample, and the mixtures were again incubated overnight at 4°C. The antigen-antibody complexes were again sedimented with protein A-Sepharose, and these last sediments and the sediments obtained after the first absorption were boiled in 75 μl of sample buffer for 5 min. Finally, 25-μl samples were electrophoresed on a 12.5% gel.

ELISA. Titers of hybridoma supernatant fluids to R- and HLP-strain rickettsiae were determined by ELISA by a method published earlier (2).

Binding of MAbs to rickettsiae. Binding of the MAbs to purified rickettsiae was determined with the aid of ^{125}I-protein A (2). Briefly, purified rickettsiae were suspended in 150 mM NaCl in pH 7.4, 67 mM phosphate-buffered saline (PBS) to a concentration of 5×10^9 rickettsiae per ml, and 0.1-ml samples were incubated with 0.25-ml portions of hybridoma culture fluids. These mixtures were washed three times with 1.0 ml of PBS, and then each pellet was suspended in 0.5 ml of PBS and incubated with 20 μl of ^{125}I-labeled protein A (New England Nuclear Corp., Boston, Mass.; 78.3 μCi/ml for experiment 1 and 63.6 μCi/ml for experiment 2 of Table 4). The preparations were then washed with PBS, the final pellets were suspended in 0.5-ml portions of PBS, and 10-μl portions were counted in a Beckman Gamma 4000 counter.

RESULTS

Comparison of strains by SDS-PAGE. Coomassie blue-stained SDS-PAGE profiles of the R and HLP strains were very similar (Fig. 1). This similarity was especially marked for polypeptides with apparent molecular weights greater than or equal to 32 kDa. Slight differences in position of two major bands were noted in the 30- and 18-kDa portions. Positions of minor bands in the 28- and 12-kDa regions also differed somewhat for the two strains. These differences are noted by asterisks in Fig. 1.

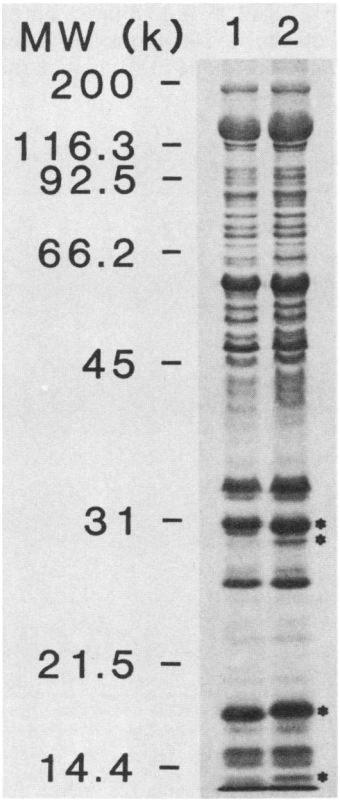

FIG. 1. Coomassie brilliant blue-stained SDS-PAGE profiles of rickettsial strains. Lanes: 1, *R. rickettsii* R; 2, *R. rickettsii* HLP. Asterisks denote major differences in the profiles of the two strains. MW (k), Molecular weight in thousands.

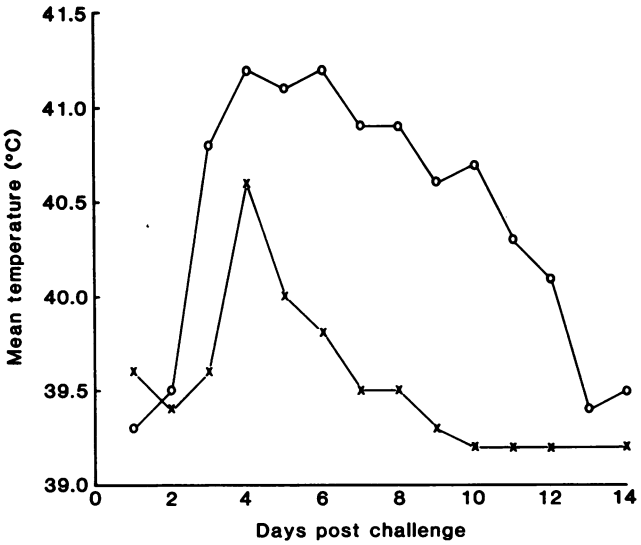

FIG. 2. Fever curves for male Hartley strain guinea pigs inoculated intraperitoneally with 1,000 PFU of the appropriate rickettsia. Each point represents the mean temperature for six animals, except that the points for the R strain represent the mean temperature for the five surviving animals after day 9. O, R strain; X, HLP strain.

Virulence of strains for guinea pigs. Average temperatures of guinea pigs inoculated intraperitoneally with 1,000 PFU of the R and HLP strains are presented in Fig. 2. Analyses of the areas under the fever curves showed that R-strain rickettsiae caused significantly more fever in guinea pigs than did HLP-strain rickettsiae ($P < 0.001$). Scrotal reactions in the guinea pigs correlated well with the fever curves. The mean maximum score for the scrotal reaction in R-strain-infected guinea pigs was 2.7 ± 1.0 (standard deviation), while the score for HLP strain-infected animals was 0.7 ± 0.5 ($P < 0.01$). One animal infected with the R strain died during the 21-day period that the animals were held after challenge; no animals in the HLP group died during the experiment. Mean weights for guinea pigs infected with the R strain were 433.0 ± 37.5 g at the time of infection and 393.6 ± 44.6 g 15 days later, and mean weights for HLP-infected guinea pigs were 435.7 ± 40.6 and 536.3 ± 58.3 g at the corresponding times. Differences in weights of the animals in the two groups were not significant at the beginning of the experiment, but they were significant at the 0.01 level at the second time period.

Specificity of MAbs. MAbs with three specificities were obtained from mice infected with R- or HLP-strain rickettsiae. Several MAbs reacted with an LPS-like antigen prepared by proteinase K digestion of whole-cell lysates of R- and HLP-strain rickettsiae by immunoblotting (Fig. 3). All of the MAbs in this category obtained thus far produced identical patterns with antigen from both the R and HLP strains. Other MAbs were specific for a polypeptide having an apparent molecular weight of 120 kDa. MAb 8-4A3H5 reacted in the immunoblotting test only with the 120-kDa polypeptide from the R strain (Fig. 4), whereas MAb

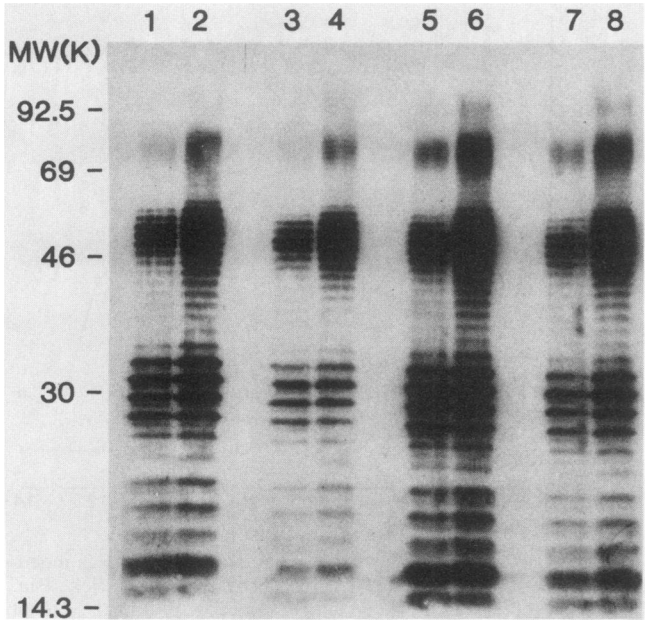

FIG. 3. LPS-like antigen detected in proteinase K-digested whole-cell lysates of R- and HLP-strain rickettsiae by immunoblotting with homologous and heterologous MAbs. Lanes: 1, 3, 5, and 7, R strain; 2, 4, 6, and 8, HLP strain. Anti-R-strain MAbs 10-7C2E11 and 8-2C6G12 were used for lanes 1 and 2 and lanes 3 and 4, respectively. Anti-HLP-strain MAbs 12-3A5A2 and 11-11A6B7 were used for lanes 5 and 6 and lanes 7 and 8, respectively. MW(K), Molecular weight in thousands.

9-7B3G8 reacted with both the homologous and the heterologous antigen. MAb 11-7D3D8 reacted only with the 120-kDa polypeptide from the HLP strain, but anti-HLP MAb 12-2C2E3 bound this antigen from digests of both strains. Anti-R MAb 10-6B6E11 reacted only with the 155-kDa polypeptide from the homologous strain, whereas anti-HLP MAb 12-8A2D5 complexed the 155-kDa antigen from both strains. A control MAb to *C. trachomatis* did not react with any of the antigens (data not shown). The results from the above and other tests to determine specificity of the MAbs are summarized in Table 2.

The above results suggested that the R and HLP strains each (i) had 120- and 155-kDa proteins which shared common epitopes but also had regions which were unique for each strain or (ii) had two 120- and 155-kDa proteins, one member of each pair being common for the two strains and the other being unique for each strain. To distinguish between these possibilities, we performed several absorption experiments. It was reasoned that if there were only one 120- or 155-kDa protein bearing both common and unique epitopes, absorption of an extract containing solubilized antigens of the R strain with anti-R MAb which recognized only the unique portion of the R protein would simultaneously remove from solution the common epitopes recognized by MAbs to HLP. However, if there were two molecules, one common to the two strains and the other unique, absorption of the unique R protein should not affect the ability of an HLP MAb to react with the common R protein.

In the immunoblot experiment (Fig. 5, panel A), an extract of the R strain was treated twice with either anti-R MAb 9-2B3A2 (reactive with the 120-kDa protein of the R strain only) or MAb 9-10C6D5 (reactive with the 155-kDa protein of the R strain only) or with PBS. The absorbed supernatant solutions were subsequently subjected to SDS-PAGE, transferred to nitrocellulose paper, and incubated with anti-HLP MAb 12-2C2E3 (reactive with a common epitope in both the R and HLP strains). The extract absorbed with 9-2B3A2 contained only a trace of a 120-kDa protein identifiable by the HLP antibody (lane 1). Evidence that the putative 120-kDa common protein molecule was not absorbed nonspecifically is presented in lane 3; an extract absorbed with the anti-155-kDa-protein MAb 9-10C6D5 still contained a 120-kDa protein reactive with 12-2C2E3. The reaction of 12-2C2E3 with an extract treated with PBS rather than antibody is shown in lane 2. These results confirmed the first of the above two postulates, which proposed that the R and HLP strains each had a single 120-kDa protein with common and unique regions.

A similar conclusion was reached for the 155-kDa proteins from the results of a radioimmune precipitation test (Fig. 5, panel B). An NP-40 extract of ^{125}I-labeled R-strain rickettsiae was treated twice with either MAb 9-10C6D5 (reactive only with the 155-kDa protein of the R strain) or 9-7B3G8 (reactive with the 120-kDa proteins of both the R and HLP strains) or with PBS. The antigen-antibody complexes were removed with protein A-Sepharose as described above. Finally, MAb 12-8A2D5 (reactive with the 155-kDa proteins of both the R and HLP strains) was added to each sample. Each sample was then treated again with protein A-Sepharose. These last sediments and the sediments obtained after the first absorption were then analyzed in the radioimmune precipitation test. The 155-kDa protein precipitated by MAb 9-10C6D5 during the first absorption is shown in lane 1. Anti-HLP MAb 12-8A2D5 precipitated only a small additional amount of the 155-kDa protein from the extract absorbed with 9-10C6D5 (lane 2). Evidence that the putative

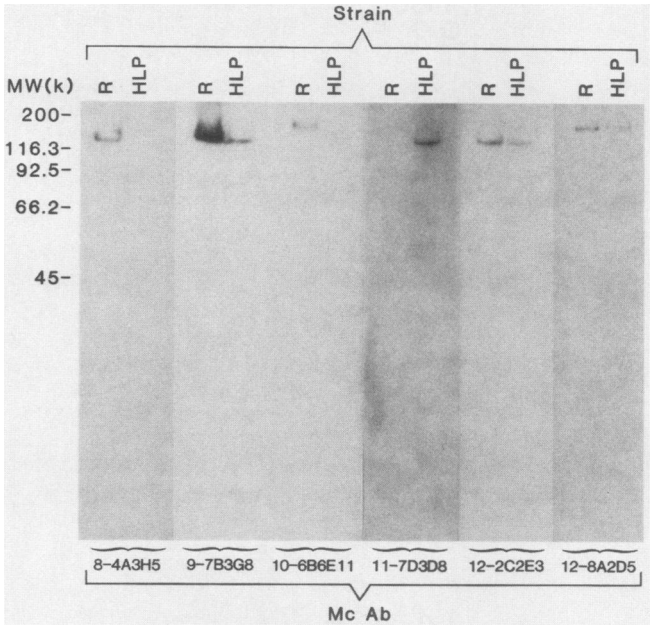

FIG. 4. Specificity of representative MAbs for the 120- and 155-kDa proteins of *R. rickettsii* R and HLP as determined by immunoblotting. MAbs 8-4A3H5, 9-7B3G8, and 10-6B6E11 were derived from mice inoculated with the R strain; MAbs 11-7D3D8, 12-2C2E3, and 12-8A2D5 were from mice inoculated with the HLP strain. MW(k), Molecular weight in thousands.

TABLE 2. Properties of MAbs to strains of *R. rickettsii*

MAb	Isotype	Strain used as immunogen	Specificity of MAb	ELISA titer		Protection against[a]	
				R	HLP	R	HLP
7-2B1B8	IgG2b	Sheila Smith	155 kDa	512	512	−	−
8-1C1A8	IgG2b	R	120 kDa	512	<8	+	−
8-2C6G12	IgG3	R	LPS-like	8	16	−	ND[b]
8-4A3H5	IgG2a	R	120 kDa	4,096	32	+	−
8-13A4A10	IgG2a	R	120 kDa	512	<8	+	−
9-2B3A2	IgG2a	R	120 kDa	1,024	8	+	−
9-3C1F2	IgG2a	R	155 kDa	1,024	<8	+	−
9-7A1H10	IgG2a	R	155 kDa	1,024	<8	+	−
9-7B3G8	IgG2a	R	120 kDa	2,048	2,048	+	+
9-10C6D5	IgG2a	R	155 kDa	4,096	<8	+	−
9-14D5A5	IgG2a	R	120 kDa	4,096	4,096	+	+
10-6B6E11	IgG2a	R	155 kDa	4,096	<8	+	−
10-7C2E11	IgG3	R	LPS-like	32	32	−	−
10-15C2C8	IgG2a	R	155 kDa	512	<8	+	−
10-17A2E8	IgM	R	LPS-like	8	8	−	ND
11-7D3D8	IgG3	HLP	120 kDa	<8	1,024	−	+
11-11A6B7	IgG3	HLP	LPS-like	16	16	−	−
11-15B5H3	IgG2b	HLP	120 kDa	16	512	−	+
12-1A5H10	IgG2b	HLP	155 kDa	1,024	512	+	+
12-2C2E3	IgG2a	HLP	120 kDa	4,096	4,096	+	+
12-3A5A2	IgG3	HLP	LPS-like	64	128	−	−
12-4B1E11	IgG2b	HLP	120 kDa	4,096	4,096	+	+
12-8A2D5	IgG2a	HLP	155 kDa	4,096	2,048	ND	−
12-19D1F5	IgG3	HLP	120 kDa	<8	64	−	+

[a] MAbs were considered protective (+) when at least four of five mice challenged with two 50% lethal doses of rickettsiae survived and nonprotective (−) when at least four of five died.
[b] ND, Not done.

common 155-kDa protein was not nonspecifically removed by entrapment in the complex formed by the putative unique antigen and a heterologous antibody is shown in lanes 3 and 4. MAb 9-7B3G8 precipitated a 120-kDa protein (lane 3), but a 155-kDa protein was still available in the supernatant for precipitation by 12-8A2D5 (lane 4). (The lower band of the doublet in lane 4 presumably represents the soluble remainder of the relatively abundant complex produced by 9-10C6D5.) The 32-kDa band shown in lane 3 is always found in radioimmune precipitation tests with MAbs to the 120-kDa protein (2), and it is believed that the 120- and 32-kDa components are associated in NP-40 extracts. In immunoblotting tests, MAbs to the 120-kDa protein did not recognize the 32-kDa polypeptide (Fig. 4; Fig. 5, panel A), perhaps because the association of the two molecules was disrupted by exposure to SDS.

When PBS rather than antibody was added to the extract, radiolabeled antigen was not sedimented by protein A-Sepharose (lane 5). A 155-kDa protein, however, was precipitated from the PBS-treated supernatant by 12-8A2D5 (lane 6). This band in lane 6 was comparable in density to the 155-kDa band in lane 4 and much denser than that in lane 2. Taken together, the above data support the contention that the R and HLP strains have antigenically similar but not identical 155-kDa proteins.

ELISA. Titers of the various MAbs against the homologous and heterologous rickettsiae are presented in Table 2. Some of the MAbs, such as 7-2B1B8 and 12-2C2E3, reacted at a high dilution with both the R and HLP strains. Other MAbs, for example 8-1C1A8 and 11-15B5H3, reacted at a high dilution with the homologous antigen but not at all or at a very low dilution with the heterologous antigen. Finally, all of the MAbs to the LPS-like antigen reacted at about the same relatively low dilution with both the R and HLP antigens.

Protective activity of antibodies. The various MAbs differed in their abilities to protect mice against the lethal effects of challenge with the two strains (Table 2). MAbs to the LPS-like antigen uniformly failed to protect mice against either homologous or heterologous challenge. MAbs which reacted with 155- or 120-kDa polypeptides of both strains by radioimmune precipitation and by ELISA generally protected against both strains. Two exceptions were MAbs 7-2B1B8 and 12-8A2D5; each reacted with the homologous and heterologous 155-kDa proteins, but neither, for unknown reasons, protected mice against challenge. MAbs specific for epitopes on the homologous strain only usually protected mice against the homologous strain but never against the heterologous strain.

Since the ELISA indicated that antibodies to the LPS-like antigen were present in the stock MAb preparations in lower concentrations than were antibodies to the 120- and 155-kDa proteins, a second experiment was performed to determine whether the observed qualitative differences in protective activity of the various MAbs were the result of quantitative differences in the levels of the antibodies. In this experiment, eight MAb preparations to the 120- or 155-kDa proteins were diluted on the basis of their ELISA titers so that each contained in this protection test the same amount of antibody as the highest-titered anti-LPS MAb (12-3A5A2) did in the first test. In addition, the anti-LPS antibody was tested in this experiment at a concentration twice that used earlier. The results shown in Table 3 suggest that either (i) antibodies to the LPS-like antigen cannot protect mice against challenge with *R. rickettsii* or (ii) antibodies to the LPS-like antigen are much less efficient at protecting mice than are the majority of antibodies to the 120- and 155-kDa proteins.

Binding of MAbs by rickettsiae. The ability of representative MAbs to react with rickettsial surface antigens was indirectly determined by measuring the radioactivity of

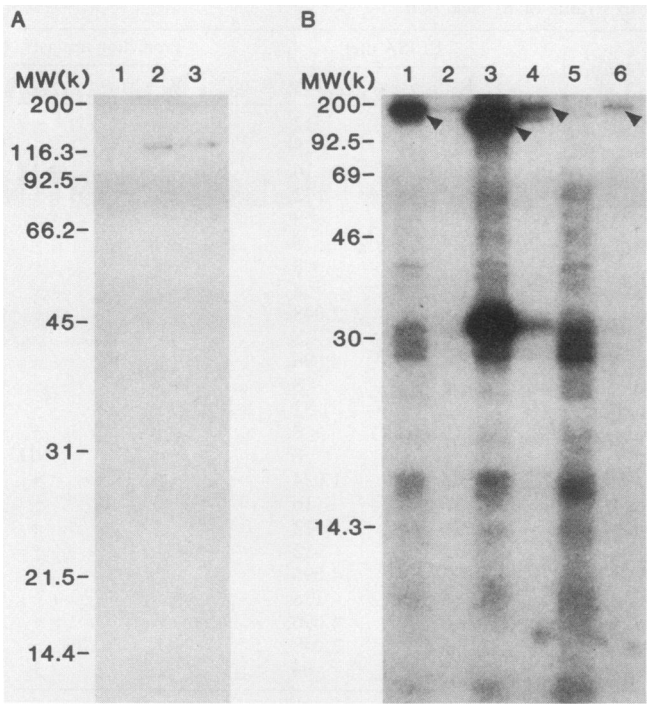

FIG. 5. Demonstration of antigenically related but different 120- and 155-kDa proteins in the R and HLP strains of *R. rickettsii*. Panel A: samples analyzed by immunoblotting. Lanes: 1, R extract absorbed with anti-R MAb 9-2B3A2 (specific for R 120-kDa protein only); 2, R extract absorbed with PBS; 3, R extract absorbed with anti-R MAb 9-10C6D5 (specific for R 155-kDa protein only). All lanes were incubated with anti-HLP MAb 12-2C2E3 (reactive with both R and HLP 120-kDa proteins and then with radiolabeled protein A). Panel B: samples analyzed by radioimmune precipitation. Lanes: 1, sediment from R extract treated with anti-R MAb 9-10C6D5 (specific for R 155-kDa protein only); 2, sediment from supernatant from lane 1 treated with anti-HLP MAb 12-8A2D5 (specific for both R and HLP 155-kDa proteins); 3, sediment from R extract treated with anti-R MAb 9-7B3G8 (specific for both R and HLP 155-kDa proteins); 4, sediment from supernatant from lane 3 treated with anti-HLP MAb 12-8A2D5; 5, sediment from R extract treated with PBS only; 6, sediment from supernatant of lane 5 treated with anti-HLP MAb 12-8A2D5. Arrows indicate specific precipitates in panel B. A variety of constituents was bound nonspecifically by protein A-Sepharose (see lane 5 for the control) during the first absorption. MW(k), Molecular weight in thousands.

whole rickettsiae first incubated with the appropriate MAb and then with ^{125}I-labeled protein A, which complexed to the rickettsia-bound antibodies (Table 4). In experiment 1, several representative MAbs were tested for binding activity with their homologous rickettsiae only; all of the rickettsiae bound at least eight times more protein A than did rickettsiae incubated with the antichlamydial control antibodies. In the second experiment, several additional MAbs were tested for their abilities to bind to both homologous and heterologous rickettsiae. The results presented in the second part of Table 4 indicated that (i) the 120- and 155-kDa proteins and the LPS-like antigens of the R and HLP strains were located on the rickettsial surface and available for interaction with the host, and (ii) MAbs incapable of reacting with heterologous antigen in the ELISA and the radioimmune precipitation and immunoblotting tests were also unable to form antibody-protein A complexes with heterologous rickettsiae.

DISCUSSION

We describe here a panel of MAbs reactive against three surface antigens of the R and HLP strains of *R. rickettsii*. All of the MAbs examined are specific for either LPS-like antigen or the 120- or 155-kDa polypeptides. These antigens were shown earlier to be present on the surface of the Sheila Smith and Morgan strains of *R. rickettsii* (2). (Previously, the apparent molecular weights of these peptides of the Sheila Smith and Morgan strains were reported as 133 and 170 kDa (2), but reexamination of these antigens electrophoresed on 10% separating gels instead of the 12.5% gels used before indicate that the molecular weights of these peptides are 120 and 155 kDa.) These polypeptides from the R and HLP strains have identical M_rs and share some antigenic determinants, but they are not antigenically identical. Some MAbs did indeed react with both the R and HLP strains in radioimmune precipitation tests and the ELISA and also protected mice against challenge with both the homologous and heterologous strains. However, a number of MAbs to these polypeptides reacted in these tests only with the homologous rickettsiae. These results clearly demonstrate that, though the 120- and 155-kDa proteins from the R and HLP strains share common determinants, these proteins are structurally different.

At present we can only speculate about the relationship of these antigens to virulence of *R. rickettsii* for guinea pigs. Although polysaccharides have been implicated in the virulence characteristics of certain bacteria (19, 21), it does not appear that the LPS-like antigen of *R. rickettsii* accounts for the difference in virulence of our two strains, since the immunoblotting experiments suggest that the LPS-like antigens of the virulent R strain and the HLP strain of considerably less virulence are identical. However, since only five MAbs to the LPS-like antigens of these strains were tested, we cannot exclude the possibility that there are minor structural differences in these antigens.

We have no direct evidence that the other surface antigens detected in this study, the 120- and 155-kDa polypeptides, play a role in virulence, but it is conceivable that these antigens are involved. These polypeptides are located on the surface of the organism, where they can readily interact with the host, and where many known virulence factors reside (26). Also, the polypeptides on the virulent R strain were found to differ structurally from the corresponding polypeptides on the HLP strain of lesser virulence. Possibly, the altered structure of these peptides of the HLP strain, though

TABLE 3. Protection of mice against challenge with *R. rickettsii* by MAbs

MAb prepn	Specificity of MAb	Relative concn of MAb[a]	No. of mice surviving[b]/ no. of mice challenged
8-4A3H5	120 kDa	1	5/5
9-2B3A2	120 kDa	1	5/5
9-7B3G8	120 kDa	1	5/5
9-14D5A5	120 kDa	1	5/5
9-3C1F2	155 kDa	1	2/5
9-10C6D5	155 kDa	1	5/5
10-6B6E11	155 kDa	1	4/5
10-15C2C8	155 kDa	1	2/5
12-3A5A2	LPS-like	2	0/5

[a] MAb preparations were diluted on the basis of their titers in an ELISA.
[b] The number of mice surviving intravenous inoculation of a mixture of MAb and two 50% lethal doses of *R. rickettsii* was determined 24 h postchallenge.

TABLE 4. Binding of ^{125}I-protein A to MAb-rickettsia complexes

Expt no. and MAb used	Homologous antigen	Rickettsia used in binding study	ELISA reactivity	Amt of protein A bound (cpm)[a]
1				
8-4A3H5	R-strain 120-kDa protein	R	+	8,466
9-10C6D5	R-strain 155-kDa protein	R	+	3,669
10-7C2E11	R-strain LPS-like	R	+	6,604
L2I-45	*Chlamydia trachomatis*	R	−	429
12-4B1E11	HLP-strain 120-kDa protein	HLP	+	14,398
12-1A5H10	HLP-strain 155-kDa protein	HLP	+	7,816
12-3A5A2	HLP-strain LPS-like	HLP	+	13,551
L2I-45	*Chlamydia trachomatis*	HLP	−	395
2				
8-1C1A8	R-strain 120-kDa protein	R	+	3,855
8-1C1A8	R-strain 120-kDa protein	HLP	−	468
9-7A1H10	R-strain 155-kDa protein	R	+	3,257
9-7A1H10	R-strain 155-kDa protein	HLP	−	624
11-7D3D8	HLP-strain 120-kDa protein	R	−	378
11-7D3D8	HLP-strain 120-kDa protein	HLP	+	18,050
12-8A2D5	HLP-strain 155-kDa protein	R	+	4,863
12-8A2D5	HLP-strain 155-kDa protein	HLP	+	3,467
L2I-45	*Chlamydia trachomatis*	R	−	443
L2I-45	*Chlamydia trachomatis*	HLP	−	513

[a] Purified rickettsiae were incubated with MAbs, washed, and then incubated with ^{125}I-protein A. Radioactivity of the final washed preparations was determined.

no handicap to the rickettsia in its arthropod host, may in some way interfere with the ability of the rickettsia to invade or multiply within susceptible guinea pig cells.

It may be suggested with somewhat greater confidence that the antigenic similarities and differences in the R and HLP strains revealed by MAbs are responsible for the serological behavior of these strains in the microimmunofluorescence test (24) and for the ability of these strains to cross-protect guinea pigs and mice against heterologous challenge (9, 22). Philip and co-workers (24) reported that both R- and HLP-strain rickettsiae reacted in the microimmunofluorescence test with high dilutions of immune mouse sera to both strains, but each strain reacted at a higher dilution of the homologous serum than of the heterologous serum. It seems probable that the common antigenic determinants on the 120- and 155-kDa polypeptides stimulated production of the antibodies responsible for the cross-reactions, and the unique determinants on these same polypeptides induced production of those antibodies which enabled the sera to combine at higher dilutions with the homologous antigen than with the heterologous antigen. Similarly, the common determinants stimulated the production of cross-protective antibodies in guinea pigs and mice (9, 22), but the unique determinants present in each rickettsial strain induced the production of those antibodies responsible for the higher neutralization titers against the homologous challenge in mice.

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