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The Hypervariable Region of *Streptococcus pyogenes* M Protein Escapes Antibody Attack by Antigenic Variation and Weak Immunogenicity

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SUMMARY

Sequence variation of antigenic proteins allows pathogens to evade antibody attack. The variable protein commonly includes a hypervariable region (HVR), which represents a key target for antibodies and is therefore predicted to be immunodominant. To understand the mechanism(s) of antibody evasion, we analyzed the clinically important HVR-containing M proteins of the human pathogen *Streptococcus pyogenes*. Antibodies elicited by M proteins were directed almost exclusively against the C-terminal part and not against the N-terminal HVR. Similar results were obtained for mice and humans with invasive *S. pyogenes* infection. Nevertheless, only anti-HVR antibodies protected efficiently against infection, as shown by passive immunizations. The HVR fused to an unrelated protein elicited no antibodies, implying that it is inherently weakly immunogenic. These data indicate that the M protein HVR evades antibody attack not only through antigenic variation but also by weak immunogenicity, a paradoxical observation that may apply to other HVR-containing proteins.

INTRODUCTION

Among the mechanisms pathogens use to evade adaptive immunity, antigenic variation has attracted particular interest, because it occurs in numerous systems and is a major obstacle to vaccine development (Lipsitch and O'Hagan, 2007; Deitsch et al., 2009; Hensley et al., 2009). In its classical form, this immune escape mechanism results from extensive sequence variation in a surface protein, allowing the pathogen to evade antibody attack. In many pathogens, the sequence variability is generated during the course of a single infection, allowing persistence in the individual host, but in other systems, new mutants appear too rarely to allow immune escape within a single individual. In the latter case, the pathogen may persist because new antigenic variants spread in the population, as exemplified by influenza virus (Karlsson Hedestam et al., 2008). In both scenarios, the variable protein commonly includes a hypervariable region (HVR), which represents a key target for antibodies.

Because HVRs are under selective pressure from host immunity, they have been predicted to be immunodominant (Borst, 1991; Barbour and Restrepo, 2000; Forsell et al., 2009), implying that they elicit stronger antibody responses than other parts of the protein (Sercarz et al., 1993). This property could be favorable for the pathogen, if the HVR acts as a decoy, which diverts the antibody response from other targets, as proposed to be the case in some systems (Borst, 1991; Crane et al., 2006; Tobin et al., 2008). However, a strong anti-HVR response should be disadvantageous, if the HVR is a target for opsonizing antibodies and/or has an important function that may be blocked by antibodies (Johnsson et al., 1996; Baruch et al., 1997; Persson et al., 2006; Karlsson Hedestam et al., 2008). This argument and a study of streptococcal surface proteins (Stålhammar-Carlemalm et al., 2007) prompted us to analyze the paradoxical hypothesis that an HVR may escape antibodies not only through antigenic variability, but also by eliciting a weak antibody response. A weak anti-HVR response might be particularly important when a microbial protein does not vary during a single infection, because it could represent the only means for the pathogen to escape anti-HVR attack in the individual host.

We studied the extracellular Gram-positive bacterium *Streptococcus pyogenes* (group A streptococcus), a major pathogen that causes >500,000 deaths and >700 million throat and skin infections each year (Carapetis et al., 2005). Among the virulence factors of *S. pyogenes*, particular interest has been focused on the fibrillar M protein, which is best known for its ability to inhibit phagocytosis (Fischetti, 1989; Smeesters et al., 2010), but promotes virulence also by other mechanisms (Waldemarsson et al., 2009). This classical virulence factor has an N-terminal HVR that exhibits extreme sequence divergence among strains but is largely stable within a strain, allowing the identification of ~200 distinct sequence types (Steer et al., 2009). Of note, the HVR of an M protein does not function as a decoy, because it is a target for opsonizing antibodies (Fischetti, 1989) and actively contributes to virulence (Waldemarsson et al., 2009).

A consensus has emerged that protective anti-HVR antibodies appear promptly in a host exposed to M protein (Fischetti, 1989;

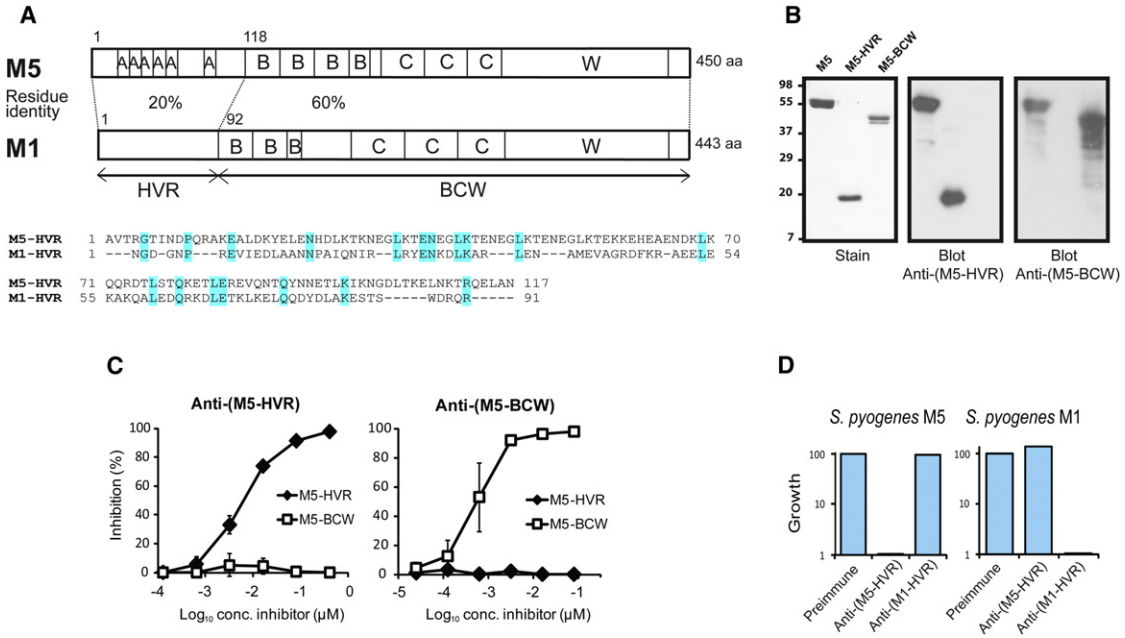

Figure 1. Characterization of the M5 and M1 Proteins

(A) Schematic presentation of the processed forms of M5 and M1. These proteins have predicted molecular masses of 47.5 kDa and 46.7 kDa, respectively. Each protein can be divided into a hypervariable region (HVR) and a region designated BCW, comprised of B- and C-repeat regions and a wall-spanning region (W). The two HVRs have highly divergent sequences, as shown in the alignment, where identical amino acid residues are highlighted.

(B and C) Lack of antigenic cross-reactivity between the HVR and BCW parts of M5. In (B), purified proteins were separated by SDS-PAGE (left), and two identical gels were subjected to western blot analysis with rabbit antisera against the HVR or BCW fragments, as indicated. Numbers indicate molecular mass in kDa. In (C), rabbit antibodies to the HVR or the BCW were used to detect the corresponding protein immobilized in microtiter wells, and antibody binding was inhibited with free HVR or BCW, as indicated. The data in (C) are based on three experiments and show mean values with SD.

(D) Phagocytosis assays in human blood, demonstrating that rabbit antisera to the HVRs promote type-specific opsonization of *S. pyogenes* expressing M5 or M1 (strains M5 Manfredo and MGAS5005, respectively). Preimmune rabbit serum was used in the controls. Data are representative of two experiments. See also Figure S1.

Cunningham, 2000; Dale et al., 2005; Bessen, 2009), and recent reviews describe the HVR as immunodominant (Weiser and Nahm, 2008; Georgousakis et al., 2009). However, the situation is unclear because reports in the literature indicate that the HVR may or may not be immunodominant (Beachey and Seyer, 1986; Fischetti and Windels, 1988; Stålhammar-Carlemalm et al., 2007). Here, we used the clinically important M5 and M1 proteins to study whether M protein escapes protective antibodies by eliciting a weak anti-HVR response. Our analysis showed that the anti-HVR response was very limited, even in humans with invasive *S. pyogenes* infection, while the C-terminal part was immunodominant. These and other data indicate that the HVR of an M protein escapes antibodies by two independent mechanisms, antigenic variation and weak immunogenicity. While the role of antigenic variation in immune escape is well known, our study focuses interest on the ability of an HVR to escape antibody attack by eliciting a weak response, i.e., by being nonimmunodominant.

RESULTS

The Streptococcal M5 and M1 Proteins

The M5 and M1 proteins are epidemiologically associated with rheumatic fever and streptococcal toxic shock syndrome, respectively, the major life-threatening diseases caused by *S. pyogenes* (Carapetis et al., 2005). Like other M proteins, M5 and M1 are fibrillar coiled-coil proteins, and they have similar overall structure (Fischetti, 1989; Smeesters et al., 2010). Each protein has an N-terminal HVR followed by a B-repeat region, a C-repeat region, and a region (W) implicated in cell wall attachment (Figure 1A). The extreme sequence divergence between the HVRs of M5 and M1 is evident from an alignment (Figure 1A), and the variability among HVRs of different M proteins is even more striking in an alignment including four additional HVRs (Figure S1A). For the work presented here, we divided the sequences of M5 and M1 into two parts, designated HVR and BCW, respectively, and used purified recombinant forms of the two intact M proteins and their HVR and BCW fragments (Figure S1B). Good antisera were obtained when the M proteins and their fragments were mixed with Freund's adjuvant (FA) and used to repeatedly immunize rabbits.

Because a major goal of this study was to specifically measure anti-HVR and anti-BCW antibodies, it was essential to exclude cross-reactivity between these two parts within the M5 or M1 protein. Analysis in the M5 system showed that rabbit antibodies to the HVR and the BCW lacked cross-reactivity (Figures 1B and 1C), and similar results were obtained in the M1 system (Figure S1C). Thus, antibodies elicited by intact M5 or M1 can be specifically assigned to either region. Phagocytosis assays with whole human blood showed that rabbit anti-HVR

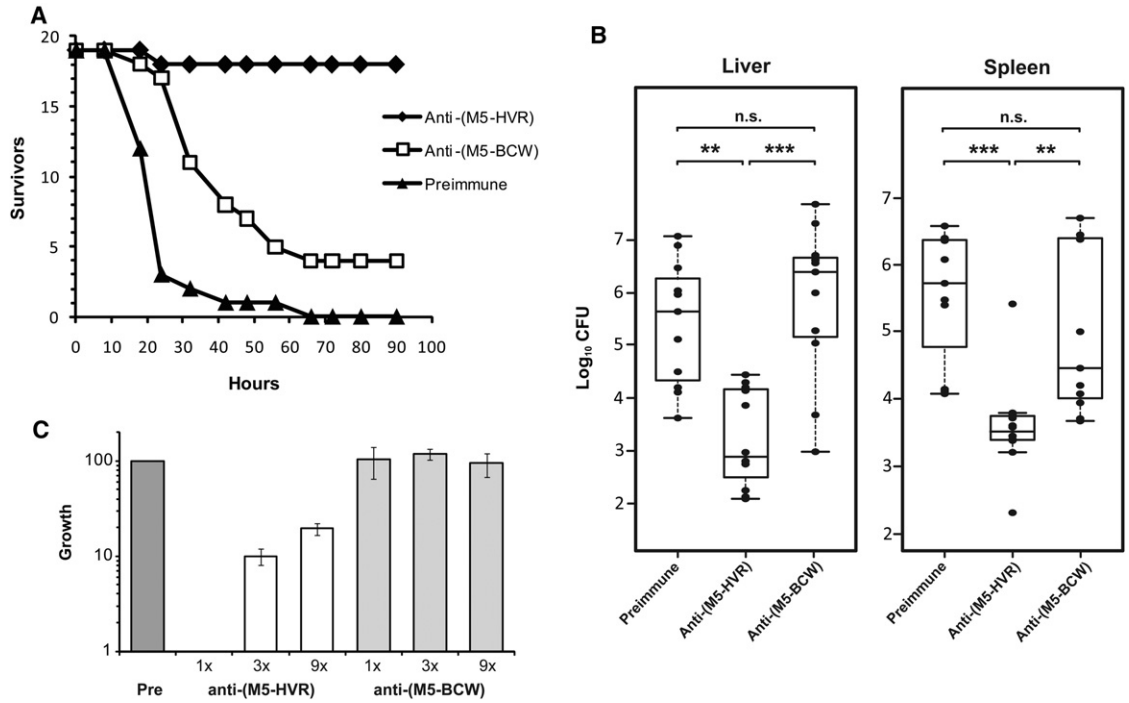

Figure 2. Prevention of Bacterial Growth by Anti-HVR Antibodies

(A) Protection against lethal infection. Three groups of mice (n = 19 per group) were passively vaccinated with the rabbit serum indicated and challenged with an ~LD_{90} dose of M5-expressing *S. pyogenes*. Survival after challenge was recorded during a 90 hr period. Pooled data from two experiments. Anti-HVR versus anti-BCW, $p < 0.0001$; anti-HVR versus preimm, $p < 0.0001$; anti-BCW versus preimm, $p = 0.05$.

(B) Protection against growth in organs. Mice (n = 11–12 per group) were passively vaccinated with the rabbit sera indicated and challenged with a sublethal dose of M5-expressing *S. pyogenes*. The mice were sacrificed 20 hr after challenge, followed by quantification of cfu in livers and spleens. Each dot represents one animal, the rectangle represents 50% of the cfu values in each group, and the horizontal line indicates the median of the group. Significance is indicated as ***$p < 0.001$, ** $p < 0.01$, or n.s. (not significant).

(C) Phagocytosis assay with whole human blood. *S. pyogenes* M5 bacteria were opsonized with rabbit sera, diluted as indicated. The undiluted anti-HVR and anti-BCW sera had the same titer, as determined with intact bacteria, allowing direct comparisons. Bacterial growth was determined after 3 hr and normalized against the preimmune sample (defined as 100). Mean values with SD for three experiments with two different blood donors. See also Figure S2.

antibodies promoted type-specific opsonization, demonstrating that the HVR fragments used here contained biologically relevant epitopes and elicited antibodies with the expected specificity (Figure 1D).

Only Anti-HVR Antibodies Efficiently Prevent Bacterial Growth

Our studies were based on the assumption that antibodies to the HVR of an M protein are particularly important for protection against *S. pyogenes* infection, implying that it is essential for the bacteria to escape attack by such antibodies. It may appear self-evident that anti-HVR antibodies would protect against infection with the homologous *S. pyogenes* strain and would be more efficient than antibodies to the C-terminal part, but the evidence that anti-HVR antibodies protect in vivo is limited (Hall et al., 2004; Penfound et al., 2010). Moreover, even antibodies to the C-terminal part might confer protection (Fischetti, 1989; Pandey et al., 2009). It was therefore important to directly compare the protective ability of polyclonal antibodies elicited by the HVR and the C-terminal region, respectively. We performed such analysis employing a mouse model of passive immunization and the M5-expressing *S. pyogenes* strain (Figures 2A and 2B).

The rabbit anti-HVR and anti-BCW sera used for passive immunizations were adjusted to have the same titer against whole M5-expressing bacteria, allowing direct comparison of their protective capacities (data not shown). The anti-HVR antibodies protected efficiently against lethal infection, while anti-BCW antibodies had little or no protective effect (Figure 2A). Moreover, anti-HVR antibodies, but not anti-BCW antibodies, strongly inhibited bacterial growth in livers and spleens (Figure 2B). In agreement with these in vivo data, only anti-HVR antibodies promoted phagocytosis ex vivo, in whole human blood (Figure 2C). Rabbit antiserum to intact M5 (raised by repeated immunization with the M5 protein mixed with FA) also promoted phagocytosis, but when this antiserum was depleted of anti-HVR antibodies its opsonizing capacity was lost (Figure S2). Thus, it is particularly important for *S. pyogenes* to evade anti-HVR antibodies.

The HVRs of the Intact M5 and M1 Proteins Elicit Weak Antibody Responses

To analyze whether the HVR present in an intact M protein is weakly immunogenic, as compared to the C-terminal part, we analyzed the antibody response in mice immunized with pure

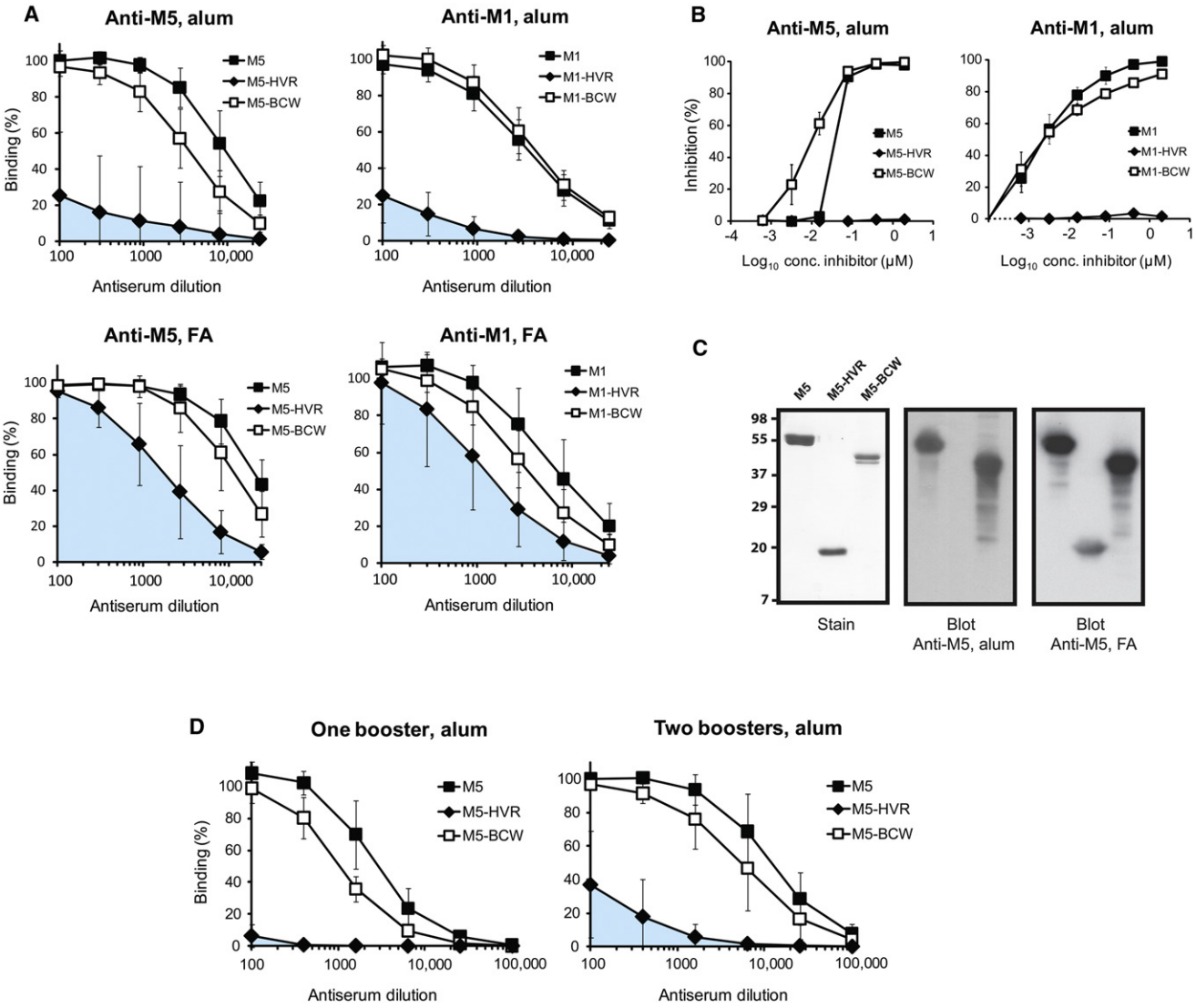

Figure 3. The HVRs of the M5 and M1 Proteins Elicit Weak Antibody Responses

(A) Upper two panels: Pure M5 or M1, mixed with alum, was used to immunize mice (n = 11 for M5, n = 9 for M1). Sera were analyzed for antibodies to the HVR and BCW fragments and to intact M protein. The weak anti-HVR response is emphasized by blue shading. Lower two panels: As in the upper panels, but M protein mixed with FA was used for the immunizations (n = 7 for M5 and n = 7 for M1).

(B) Inhibition tests. One anti-M5 and one anti-M1 serum raised with alum (from A) were used to detect the corresponding M protein immobilized in microtiter wells, and binding was inhibited with the proteins indicated. Similar data were obtained with a second antiserum of each type.

(C) Western blot analysis with antisera from mice immunized with M5 and alum or M5 and FA, as indicated. Similar results were obtained with two sera of each type.

(D) Long-term immunizations. Two groups of mice (n = 6) were immunized with M5 (20 μg) mixed with alum. Each group received one booster (10 μg) after 4 weeks, and the second group received an additional booster (10 μg) 10 weeks later. All mice were bled 16 weeks after the initial immunization. Antibody titers against M5 and its HVR and BCW fragments were determined. The data show mean values with SD for all mice within a group (A and D) or for three experiments with one serum (B). See also Figure S3.

M5 or M1. To avoid the strong inflammatory reaction elicited by FA, the immunizations were performed with alum as the adjuvant. Interestingly, these mouse sera reacted with the corresponding M protein and its BCW fragment, but hardly at all with the HVR fragment (Figure 3A, upper panels). Indeed, <1% of the antibodies elicited by the intact M protein reacted with the HVR. These assays had been optimized to allow accurate detection of anti-HVR and anti-BCW antibodies, indicating that the lack of detection of anti-HVR antibodies was not due to an inability to detect such antibodies (Figures S3A–S3D).

The binding tests indicated that only a small fraction of the antibodies elicited by intact M proteins was directed against the HVR. However, these binding tests employed the isolated HVR fragment for detection of anti-HVR antibodies, and the results did not exclude that the HVR part of the intact M protein elicited antibodies against epitopes not present in the isolated

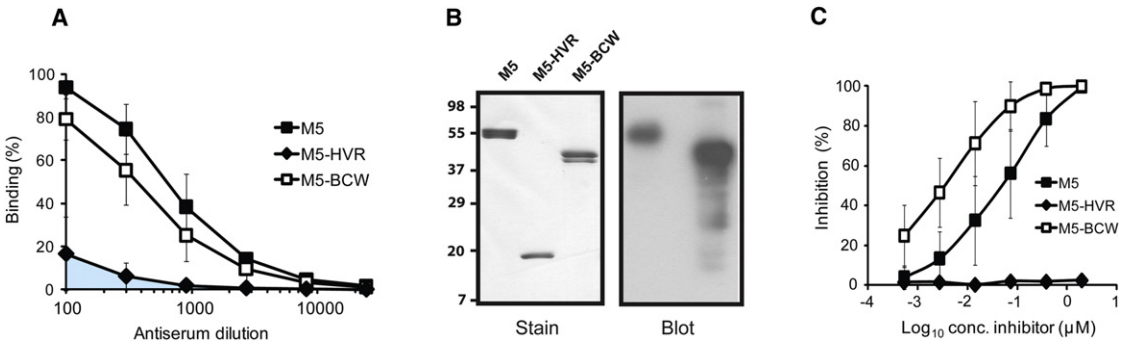

Figure 4. Weak Anti-HVR Response in Mice Infected with *S. pyogenes*

(A) Mice (n = 6) were infected with a sublethal dose of M5-expressing *S. pyogenes*, and sera were recovered after 4 weeks. Antibody titers against M5 and its HVR and BCW parts were determined. Mean values with SD for all mice.

(B) Western blot analysis with the proteins indicated and serum from one infected mouse. Similar results were obtained with serum from a second mouse.

(C) Inhibition tests with antiserum from one infected mouse. This antiserum was used to detect immobilized M5 protein, and binding was inhibited by the addition of M5 or its HVR and BCW fragments, as indicated. The data represent mean values from three experiments, with SD. Similar results were obtained with antisera from two other infected mice.

HVR. In that case, the titer of anti-HVR antibodies might have been strongly underestimated. This explanation was excluded by the demonstration that antibodies elicited by intact M5 or M1 were inhibited virtually completely by the corresponding BCW fragment, but not by the HVR fragment (Figure 3B). Finally, western blot analysis in the M5 system demonstrated that mouse antiserum elicited by intact M5 and alum detected the BCW but not the HVR (Figure 3C, left and middle panels). Thus, analysis by three different methods indicated that mouse antibodies elicited by M5 or M1, mixed with alum, were directed almost exclusively against the BCW region, while the HVR elicited a very weak response. Of note, the M proteins used for immunization had the expected sizes and N-terminal sequences, demonstrating that the lack of anti-HVR response was not due to degradation of the HVR (Figure S1B).

To analyze whether the poor anti-HVR response could be explained by delayed appearance of anti-HVR antibodies, we used the M5 protein. Mice immunized with pure M5 and alum were given one or two boosters and bled 16 weeks after the initial immunization. Also in these mice, <1% of the antibodies were directed against the HVR, demonstrating that the anti-HVR response is not just delayed (Figure 3D). Although the use of two boosters increased overall titers, the fraction of antibodies directed against the HVR remained very low. Preimmune mouse serum virtually lacked reactivity with M proteins (data not shown), indicating that the selective production of anti-BCW antibodies in immunized mice did not reflect a recall response, following previous exposure to an M protein with similar BCW region but different HVR.

A Strong Adjuvant Selectively Enhances the Anti-HVR Response

The weak anti-HVR response after immunization with an M protein is not readily reconciled with the common assumption that *S. pyogenes* infection results in the formation of type-specific and protective anti-HVR antibodies (Lancefield, 1962; Fischetti, 1989). A possible explanation for this apparent contradiction was provided by the observation that the strongly immunostimulatory FA caused selective enhancement of the anti-HVR response in mice immunized with intact M5 or M1, as demonstrated in direct binding tests (Figure 3A, lower panels) and by western blot in the M5 system (Figure 3C, right panel). These mice had been immunized twice, once with complete FA and once with incomplete FA. Also, under these conditions the anti-HVR response was weaker than the anti-BCW response, but the difference was considerably smaller than in immunizations with alum. Indeed, the fraction of antibodies directed against the HVR increased >10-fold when FA was used. However, the anti-HVR response in these mice was not sufficient to protect against infection (Figure S3E), suggesting that a protective anti-HVR response may require prolonged antigen exposure and inflammation.

In contrast to intact M5 and M1 in combination with FA, which enhanced the anti-HVR response, the isolated HVRs of these M proteins in combination with FA did not elicit antibody responses in mice (data not shown). The reason for this lack of antibody response in mice is not known, but a simple explanation could be that the isolated HVRs lacked relevant T cell epitopes.

The HVR Is Weakly Immunogenic in Mice and Humans Infected with *S. pyogenes*

To analyze whether the HVR of an M protein is weakly immunogenic during *S. pyogenes* infection, we analyzed experimentally infected mice and humans with invasive infection. The mice were infected with a sublethal dose of the M5-expressing strain, and sera recovered after 4 weeks were analyzed for antibodies to the HVR and the BCW region of M5. The antibodies showed very low reactivity with the HVR, as demonstrated by direct binding tests and western blot analysis, but reacted well with the BCW and intact M5 protein (Figures 4A and 4B). Inhibition experiments confirmed that the antibodies were directed almost exclusively against the BCW (Figure 4C). In this inhibition analysis, the BCW fragment caused more efficient inhibition than intact M5, for unknown reasons, but both proteins caused virtually complete inhibition. Thus, the HVR of M5 elicits a weak antibody response during experimental infection.

The antibody response to M protein in infected humans was studied with sera from patients with invasive infection caused

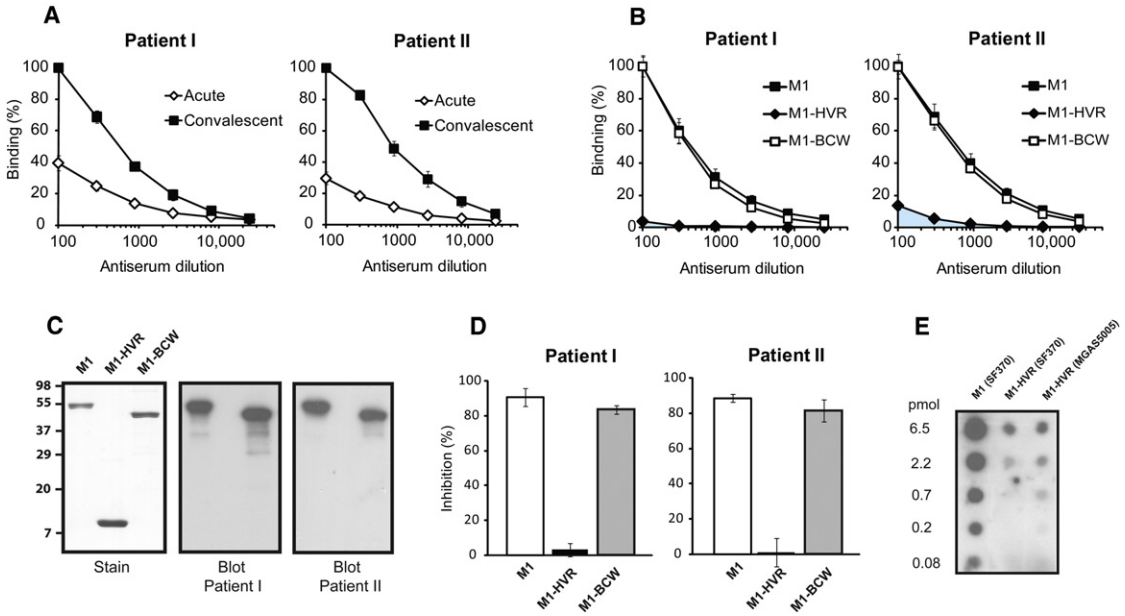

Figure 5. Analysis of Sera from Humans with Invasive *S. pyogenes* Infection

(A) Acute and convalescent sera from two patients with invasive *S. pyogenes* M1 infection were analyzed for reactivity with immobilized full-length M1.

(B) Antibody binding assays with the two convalescent sera and the immobilized antigens indicated.

(C) Western blot analysis with the two convalescent sera.

(D) Inhibition assays with the two convalescent sera. The two sera were used to detect immobilized M1, and binding was inhibited by the addition of M1 or its HVR and BCW fragments, as indicated. Because the human sera were available in limited amounts, the tests could only be performed at one concentration of inhibitor (6 μM), chosen on the basis of a preliminary experiment.

(E) Dot blot analysis with convalescent serum from patient I. This serum reacted with intact M1, derived from strain SF370, but very poorly with the corresponding M1-HVR or with the HVR of M1 in strain MGAS5005. The latter HVR is identical to that in the patient isolates and diverges from the SF370 sequence at one position. The HVRs were used as GST fusions. Similar results were obtained with serum from patient II. The data in (A), (B), and (D) represent mean values with SD from three experiments.

by M1-expressing *S. pyogenes*. Of note, these patients had been treated with antibiotics, including penicillin, upon admission. Accurate analysis made it essential to have access to the *S. pyogenes* strain causing the infection and to paired sera, i.e., acute and convalescent serum. Characterization of the strain was necessary to ensure that it encoded the M1 protein, while comparison of paired sera made it possible to analyze whether the patient had responded to the current infection. Such material was available for six patients, and two of them showed a clear increase in anti-M1 titer between the acute and convalescent samples, unequivocally showing that the antibody response was associated with the M1 infection (Figure 5A). The convalescent sera from these two patients reacted strongly with the BCW but not the HVR of M1, as demonstrated by direct binding tests, western blots, and inhibition tests (Figures 5B–5D). The M1-HVR in the patient strains diverged at one amino acid position from the M1-HVR fragment used in the immunological tests, but this small difference did not affect the reactivity with patient sera, as shown in a dot blot analysis with the relevant constructs (Figure 5E). Thus, the HVR of an M protein is poorly immunogenic even during invasive infection in humans.

The HVR Remains Weakly Immunogenic when Fused to an Unrelated Protein

The weak antibody response elicited by the HVR of an M protein could reflect an inherent property of this region. Alternatively, the weak anti-HVR response could reflect inhibition by the adjacent BCW region. To distinguish between these two alternatives, we analyzed the antibody response to fusion proteins, in which the HVR of M5 had been fused to immunogenic regions derived from other streptococcal surface proteins. In these fusion proteins, the HVR was located at the N-terminal end, as in M proteins (Figure 6A). If the M5-HVR is inherently weakly immunogenic, one would expect such a fusion protein to elicit a weak anti-HVR response, but if the BCW region is inhibitory, the fusion protein would probably elicit a good anti-HVR response. Two different C-terminal fusion partners were studied, to ensure that unequivocal results would be obtained.

One of the fusion partners, designated B6, was derived from the N-terminal B6 region of the *S. agalactiae* β protein (Hedén et al., 1991), while the other fusion partner, referred to here as NN, was derived from the N-terminal regions of the *S. agalactiae* Rib and α proteins (Stålhammar-Carlemalm et al., 2007). These fusion partners were used, because both B6 and NN were known to elicit good antibody responses in mice, when administered with alum (Stålhammar-Carlemalm et al., 2007; our unpublished data). Of note, the size of the two fusion proteins was similar to that of an M protein. As expected, each fusion protein reacted with rabbit antisera raised against the M5-HVR or the fusion partner (Figures S4A and S4B). This analysis also showed that the M5-HVR did not cross-react with any of the fusion partners, implying that antibodies elicited by

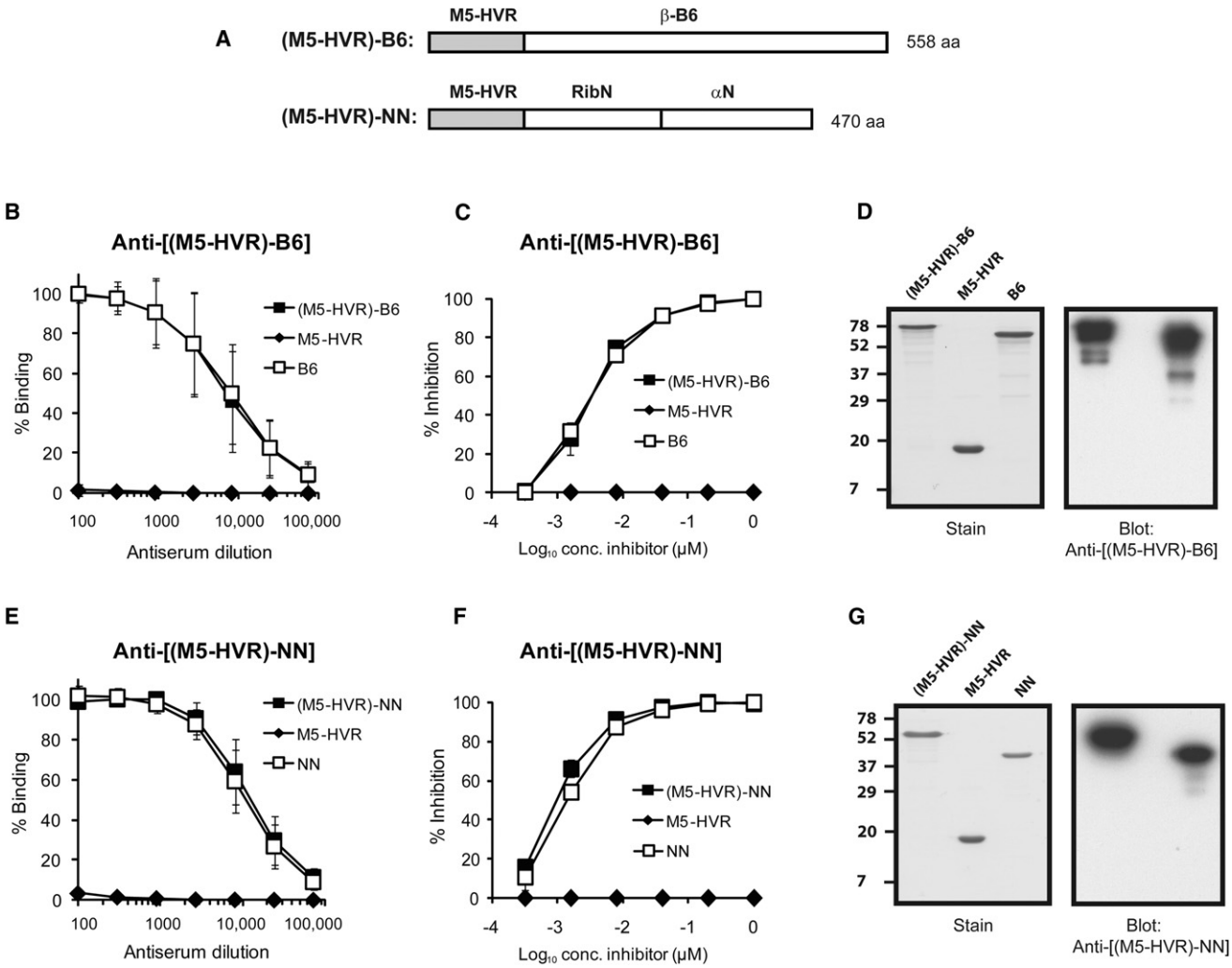

Figure 6. Antibody Response to Fusion Proteins

(A) Schematic presentation of two fusion proteins derived from the M5-HVR and unrelated immunogenic protein regions. These fusion proteins are designated (M5-HVR)-B6 and (M5-HVR)-NN, respectively. The B6 region was derived from the N-terminal part of the *S. agalactiae* β protein, while the NN region was derived from the N-terminal parts of the *S. agalactiae* Rib and α proteins.

(B) C3H/HeN mice (n = 10) immunized with the fusion protein (M5-HVR)-B6 mixed with alum were analyzed for antibody response to the intact fusion protein and its two constituent parts, as indicated.

(C) Inhibition test. Antiserum from a mouse immunized with (M5-HVR)-B6 was used to detect this protein immobilized in microtiter wells, and binding was inhibited with the proteins indicated. Similar data were obtained with antiserum from a second mouse.

(D) Western blot analysis with an antiserum from a mouse immunized with (M5-HVR)-B6. Similar results were obtained with a second antiserum.

(E–G) Same analysis as in (B)–(D), except that the antigen used was the fusion protein (M5-HVR)-NN. For (E), 11 sera were analyzed. For (F) and (G), similar results were obtained with two different sera. The data show mean values with SD for all mice within a group (B and E) or for three experiments with one serum (C and F). See also Figure S4.

a fusion protein could be specifically assigned to either the HVR or the C-terminal part.

The two fusion proteins, (M5-HVR)-B6 and (M5-HVR)-NN, elicited good antibody responses when mixed with alum and used to immunize mice. These antibody responses were directed exclusively against the C-terminal fusion partner, as shown by direct binding tests, inhibition tests, and western blot analysis (Figures 6B–6G). Remarkably, the HVR was even less immunogenic in the fusion proteins than in intact M5 (Figure 3A). This lack of anti-HVR response could not be explained by loss of the HVR from the recombinant fusion proteins, because they reacted with anti-HVR serum and had the expected N-terminal sequences (Figures S4A and S4B). Moreover, the result was not dependent on mouse strain used, because equally weak anti-HVR responses were elicited in C3H/HeN mice (Figures 6B and 6E) and in C57BL/6 mice (Figures S4C and S4D). Finally, the dominating response to the C-terminal fusion partner could not be explained as a recall response, reflecting previous exposure to the fusion partner, because preimmune mouse serum lacked antibodies to the B6 and NN proteins (data not shown). Together, these data indicate that the HVR of an M protein is inherently weakly immunogenic.

DISCUSSION

The work described here was based on the paradoxical hypothesis that the HVR of an M protein may escape antibody attack, not only through antigenic variation but also by eliciting a weak antibody response. Using the well-known M5 and M1 proteins and a variety of experimental conditions, we found strong evidence for the hypothesis. Remarkably, even life-threatening invasive infection in humans was insufficient to elicit a good anti-HVR response, as demonstrated for patients infected with M1-expressing *S. pyogenes*. The biological relevance of these findings was supported by studies with hyperimmune rabbit antisera, raised against the isolated HVR or C-terminal part of M5. While the anti-HVR antibodies prevented bacterial growth in infected mice and promoted opsonization in phagocytosis tests, this was not the case for antibodies to the C-terminal part. Thus, bacterial virulence would be promoted by a mechanism that limits the formation of anti-HVR antibodies and makes the C-terminal part immunodominant.

The phenomenon of immunodominance signifies that an immune response is limited to a proportion of the potential determinants on an antigen (Sercarz et al., 1993). This phenomenon has been extensively studied for T cell responses, where it reflects the fact that only a small fraction of all possible peptides derived from a protein antigen are presented on MHC molecules and trigger T cell responses (Sant et al., 2007). However, the lack of anti-HVR response studied here cannot be explained by absence of T cell epitopes in the HVRs, because the intact M5 and M1 proteins contain T cell epitopes, as indicated by the formation of antibodies to the C-terminal parts. According to standard models, these T cell epitopes may provide help to any B cell epitope in the proteins, including those in the HVRs. Thus, the lack of anti-HVR response does not reflect lack of T cell help but most likely reflects lack of activation of the relevant B cells. This conclusion focuses interest on the poorly understood mechanisms that promote immunodominance, or lack of immunodominance, at the B cell level (Wicker et al., 1984; Sercarz et al., 1993; Nakra et al., 2000).

For the HVR of an M protein, we considered two possible explanations for its lack of immunodominance at the B cell level. In one scenario, the C-terminal region actively interferes with the antibody response to the adjacent HVR, and in another scenario the HVR is inherently weakly immunogenic. Studies of two different fusion proteins, in which the M5-HVR was combined with unrelated immunogenic protein regions, strongly suggested that weak immunogenicity is an inherent property of the HVR, at least when it is located N-terminally, as in an M protein. This conclusion does not exclude that an HVR might elicit a good antibody response under other conditions. For example, the HVR fragments of M5 and M1 elicited good antibody responses in rabbits, when mixed with FA, and multivalent HVR proteins may elicit protective antibody responses (Hall et al., 2004; Penfound et al., 2010).

How can weak immunogenicity be an inherent property of the HVR in an M protein? Properties that may contribute include lack of defined tertiary structure (Dey et al., 2009), selective sensitivity to proteases (Raeder et al., 1998), and affinity for a host component that interferes with antibody formation (Beernink et al., 2011). It is also conceivable that immunological tolerance contributes to the weak immunogenicity, reflecting similarity between the HVR and a host component, but this hypothesis is not readily reconciled with the extreme sequence divergence among HVRs. Moreover, it is unlikely that a modification such as glycosylation can explain the weak immunogenicity of the HVR, because a modification would not have been present in the recombinant proteins used here. Of note, any explanation for the weak immunogenicity must be compatible with the finding that the HVR plays a key role in virulence during the early stages of an infection (Waldemarsson et al., 2009).

The paradoxical finding that the HVR is weakly immunogenic raises the question how the sequence divergence among different HVRs has evolved. A clue to this problem was provided by the observation that anti-HVR responses were selectively enhanced in mice immunized with intact M protein and FA, an adjuvant that causes a robust inflammatory response. Strong inflammation may also accompany an *S. pyogenes* infection (Cunningham, 2000), resulting in conditions that gradually favor the appearance of protective anti-HVR responses and the selection of antigenic escape variants. This argument does not contradict the fact that the existing HVRs are largely stable, because they may have been selected as the most fit ones, making them subject to strong negative selection and favoring sequence conservation (Persson et al., 2006; Lipsitch and O'Hagan, 2007).

The hypothesis that a protective anti-HVR response appears slowly and requires a robust inflammatory response is supported by early observations on rabbits immunized with M protein-containing extracts (Hirst and Lancefield, 1939) and by studies of humans with *S. pyogenes* infection (Denny et al., 1957; Siegel et al., 1961). The studies of humans showed that type-specific antibodies did not appear in patients cured of the infection by treatment with penicillin and appeared only slowly in patients with prolonged infection. When this work was published, it was not known that M proteins have an HVR, and the data did not exclude that all antibodies to M protein appear slowly. However, our findings indicate that only the anti-HVR response requires prolonged antigen exposure and inflammation. Thus, our data are fully compatible with the early studies in humans and provide a molecular insight not previously available. Our data are also compatible with the very few studies in which protective immunity was analyzed in mice immunized with an intact M protein. One of these studies reported that intranasal (i.n.) immunization with M1 elicited protection against i.n. challenge with an M1 strain (Siegert et al., 2006). Although interesting, the significance of this finding is unclear, because i.n immunization may elicit antibody-independent cellular immunity to infection (Basset et al., 2007; Wang et al., 2010). In another study, mice immunized with pure M1 were barely protected against lethal infection, although they had been immunized three times with FA and with 5-fold more M protein than used by us (McNamara et al., 2008). Thus, the available data are compatible with the conclusion that a good anti-HVR response requires prolonged M protein exposure and inflammation. This conclusion implies that many immunization regimens or infections may not result in a protective anti-HVR response.

Collectively, our data contradict the common assumption that the HVR of a microbial surface protein is immunodominant, and they support the paradoxical hypothesis that an HVR may elicit a much weaker antibody response than other parts of the

protein. In particular, our data indicate that the HVR of an M protein is so critical for bacterial virulence that *S. pyogenes* escapes antibodies to this region by employing at least two mechanisms, acting at different stages of an infection. During the establishment of an infection, antigenic variation allows the bacteria to escape attack by any pre-existing anti-HVR antibodies, but antigenic variation cannot be exploited for evasion of antibodies elicited in the newly infected host, because the M protein is remarkably stable during an infection. However, the weak immunogenicity of the HVR in the new host should delay the appearance of protective antibodies and prolong the infection, thereby enhancing chances for bacterial transmission to new hosts. Thus, a single bacterial surface protein, the M protein of *S. pyogenes*, evades the adaptive immune response by two independent mechanisms.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Culture Conditions

S. pyogenes strain M5 Manfredo (Miller et al., 1988), isolated from a patient with rheumatic fever, was from M.A. Kehoe. The reference M1 strain *S. pyogenes* SF370 (Ferretti et al., 2001) was from ATCC (Rockville, MD). The M1 isolate MGAS5005, isolated from the cerebrospinal fluid of a patient, was from J.M. Musser (Sumby et al., 2005). All strains were grown without shaking in Todd-Hewitt broth supplemented with 0.2% yeast extract (THY) in 5% CO_2 at 37°C.

Proteins

The M5 protein was purified from a plasmid-carrying *E. coli* strain, essentially as described (Stenberg et al., 1994). This protein had the same N-terminal sequence as M5 expressed in *S. pyogenes*, because the signal sequence is cleaved off also in *E. coli*. All other constructs were isolated as GST fusions (Amersham, Swansea, UK), and after removal of the GST moiety, they included the N-terminal sequence GPLGS, not present in the original protein. For PCR amplification of DNA encoding M protein regions, we used DNA from strain M5 Manfredo or strain M1 SF370. The region encoding the M1-HVR was also amplified from strain MGAS5005. The DNA fragment encoding the N-terminal B6 region of the *S. agalactiae* β protein was amplified from strain SB35 (Hedén et al., 1991). The construct encoding the NN protein, derived from the N-terminal regions of the *S. agalactiae* Rib and α proteins, has been described (Stålhammar-Carlemalm et al., 2007). The relevant chromosomal regions were amplified with the primers listed in Table S1, and the DNA sequences of all constructs were verified. Determination of N-terminal sequences by Edman degradation was performed at the Protein Analysis Center, Karolinska Institutet, Stockholm.

Animal and Human Antisera

Mice were immunized s.c. with 20 μg protein, boosted after 4 weeks with 10 μg, and bled 2 weeks later, unless otherwise stated. The mouse strain used was C3H/HeN, except for the immunizations reported in Figures S4C–S4D, which employed C57BL/6 mice. Adjuvants were used, as indicated. For use of FA, the first dose and the booster were administered with complete FA (CFA) and incomplete FA (IFA), respectively. Antisera from mice infected with a sublethal dose of *S. pyogenes* M5 were recovered from mice injected i.p. 4 weeks earlier with 10^6 cfu. Rabbit antisera were raised by s.c. immunization with 100 μg protein in CFA, followed by two 50 μg boosters in IFA. The rabbit antisera against the B6 and NN proteins have been described (Hedén et al., 1991; Stålhammar-Carlemalm et al., 2007).

Paired antisera from humans with invasive *S. pyogenes* M1 infection were obtained from six patients included in a placebo group in a study of intravenous IgG therapy (Darenberg et al., 2003). These patients had received i.v. clindamycin in combination with i.v. benzylpenicillin at inclusion in the study. The acute serum was obtained at day 1 and the convalescent serum at day 28 after inclusion. For each patient, the corresponding *S. pyogenes* M1 strain, isolated from a blood culture, was available for analysis. The two patients studied in detail here were 53 and 39 years old, respectively. The study was approved by the regional ethics committee and the drug agency authority in Sweden. Written informed consent was obtained from all subjects or their legal guardians.

Binding Tests with Antibodies, Optimization of Antibody Detection, and Inhibition Tests

Binding tests were performed essentially as described (Stålhammar-Carlemalm et al., 1993; Persson et al., 2006). Microtiter wells were coated overnight at 4°C, using pure proteins at concentrations determined in preliminary tests to give optimal results. Bound rabbit antibodies were detected with radiolabeled protein G, while bound mouse or human antibodies were detected by incubation with secondary antibodies (rabbit anti-mouse Ig and rabbit anti-human Ig, respectively [DakoCytomation, Denmark]) and protein G. Binding was calculated in percent of protein G bound at the lowest antiserum dilution. For mice and rabbits, background values obtained with sera from mock-immunized animals were subtracted.

To ensure that antibodies to the HVR or BCW of an M protein were detected with similar sensitivity, whether the region studied was present in the intact M protein or tested as an isolated fragment, experiments were performed to optimize the test system (Figures S3A–S3D). For analysis of fusion proteins (Figures 6 and S4), wells were coated with 25 ng M5-HVR or an equimolar amount of the other proteins analyzed.

For inhibition assays, antiserum was incubated for 0.5 hr with the inhibiting protein in a volume of 100 μl. The sample was then analyzed for remaining binding activity, as described for binding tests. Coating amounts and antiserum dilutions were optimized for each individual assay.

Phagocytosis Assays

The assays were performed essentially as described (Carlsson et al., 2003), using hirudin as anticoagulant and freshly drawn human blood from nonimmune donors, i.e., blood allowing rapid growth of the M5- and M1-expressing strains. After rotation at 37°C for 3 hr, the increase in titer ("multiplication factor") was calculated for each sample. Growth is plotted in a log scale and expressed relative to the preimmune control, defined as 100. The multiplication factor for the controls varied from 133 to 347.

Protection Experiments in Mice

For analysis of passive protection against lethal infection, C3H/HeN mice were passively vaccinated with rabbit antiserum and challenged i.p. with an $\sim LD_{90}$ dose (5×10^7 cfu) of exponentially growing M5 Manfredo bacteria. The rabbit antiserum (100 μl) was administered twice, 4 hr before the challenge and together with the challenge. Preliminary experiments indicated that administration of rabbit antiserum at these two time points conferred better protection than administration at only one of the time points. Control mice received preimmune serum.

For analysis of active or passive protection against bacterial growth in organs, vaccinated C3H/HeN mice were challenged with a sublethal dose (5×10^6 cfu) of strain M5 Manfredo. Actively immunized mice received M5 protein and FA, while passively immunized mice received rabbit antiserum, as described above. The mice were sacrificed after 20 hr, when spleens and/or livers were homogenized and analyzed for presence of bacteria. All animal experiments were approved by the regional review board on animal studies.

Other Methods

Radiolabeling, SDS-PAGE in 15% gels, western blots, dot blot analysis, and binding tests with whole bacteria were performed essentially as described (Stålhammar-Carlemalm et al., 1993; Persson et al., 2006). In western blots, bound rabbit antibodies were detected by incubation with radiolabeled protein G, followed by autoradiography, while bound mouse and human antibodies were detected by incubation with rabbit anti-mouse and rabbit anti-human Ig, respectively, followed by radiolabeled protein G.

Statistical Analysis

In studies of passive vaccination against lethal infection (Figure 2A), Fisher's exact test was used to calculate p values. For the analysis of protection against bacterial growth in organs (Figures 2B and S3E), the Coin package (Hothorn

et al., 2008) in software R was used to perform exact Kruskal-Wallis ANOVA on ranks to compare the numbers of cfu. Post hoc analyses of all pairwise comparisons were performed using the Nemenyi-Damico-Wolfe-Dunn procedure if a significant result was obtained in the global test. Significance was defined as ***$p < 0.001$ and **$p < 0.01$.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and one table and can be found with this article online at doi:10.1016/j.chom.2011.06.011.

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