Insight of Host Immune Evasion Mediated by Two Variants of Group A Streptococcus Mac Protein*  

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Group A Streptococcus has evolved numerous mechanisms to evade the host immune system to survive, disseminate, and cause disease. Recently a secreted protein named Mac-1 was identified and shown to enhance survival of the pathogen. A new variant of Mac-1 (designated Mac-2) also was recently described and shown to differ from Mac-1 by ~50% amino acid sequence divergence in the middle one-third of the molecule. To gain new information about the role of Mac-1 and Mac-2 in host-pathogen interactions, solution binding experiments were performed using surface plasmon resonance and purified Mac proteins. Mac-1 bound the same lower hinge region of human IgG as Fc receptors with 2.5 μM affinity, which lead to proteolytic cleavage of the antibody. Similar $K_m$ (6.8–18.9 μM) and $k_{cat}$ (0.02–0.13 s$^{-1}$) values of the Mac-1 endopeptidase activity were obtained for IgG1, IgG2, IgG3, and IgG4. Mac-2 variant, in contrast, bound human IgG poorly ($K_m = 16$ μM) and had weak endopeptidase activity against IgG. Instead, Mac-2 bound FcyRII and FcyRIII with 5 and 75 μM affinity, respectively. This binding competitively blocked IgG from recognition by Fc receptors. Taken together, Mac proteins block immunoglobulin recognition by Fc receptors and degrade immunoglobulins, thereby enhancing survival of the pathogen through the inhibition of phagocytosis, endocytosis of IgG-opsonized particles, and antibody-dependent cell-mediated cytotoxicity. Consequently, these proteins may be potential therapeutic targets.

Group A Streptococcus (GAS) is a very common human pathogenic bacterium that causes infections such as tonsillopharyngitis, cervical lymphadentitis, cellulitis, scarlet fever, osteomyelitis, necrotizing fasciitis (“flesh-eating” syndrome), and streptococcal toxic shock syndrome (1). It remains a public health problem in both developed and developing countries. In general, the human immune system has evolved with effective mechanisms to clear bacterial infections (1, 2). Immunoglobulins are a central part of the adaptive immune system that specifically recognize and mediate the elimination of invading microorganisms. Upon recognition of antigen, immune complexes, through Fc receptor signaling pathway, activate a variety of effector cell functions, including antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cellular cytotoxicity, phagocytosis and endocytosis of IgG-opsonized particles, oxidative burst, and release of inflammatory mediators (1, 2).

GAS has evolved several molecular mechanisms to evade host immune defenses (3–6). Many GAS virulence factors are extracellular proteins that can be grouped into three distinct categories (1, 7, 8). The first category involves proteins that have signal peptides and are actively secreted into the extracellular environment. Examples of these proteins include the exotoxins A and C superantigens. The second category includes extracellular proteins bound to the bacterium cell wall by an (Leu-Pro-X-Thr-Gly(LPXTG)) anchor sequence. Proteins of a third category lack both an apparent secretion signal sequence and the LPXTG membrane anchor motif (9–11).

Recently, a new member of GAS-secreted virulent factors, Mac (also known as Ides) (12, 13), with limited homology to the α subunit of human leukocyte adhesion glycoprotein Mac-1, was described. Characterization of the mac gene showed the existence of two divergent Mac variants designated Mac-1 and Mac-2 (12, 14). Mac-1 and Mac-2 differ mainly in the amino acid residues located within the middle one-third of the molecule, with ~50% sequence divergence in this region (14). Mac-1 binds to the surface of human polymorphonuclear leukocytes (PMNs) and inhibits opsonophagocytosis and production of reactive oxygen species (12). Recently, Mac-1 was shown to contain immunoglobulin G (IgG)-endopeptidase activity that may contribute to its ability to block PMN opsonophagocytosis (13). In this work, we sought to gain new insight into the mechanism of Mac-mediated host immune evasion by characterizing the interaction of the Mac variants with host immunoglobulins and their receptors.

**EXPERIMENTAL PROCEDURES**

Expression, Purification of Recombinant Mac-1 and Mac-2, and Preparation of Fc by Papain Digestion—Recombinant Mac-1 and -2 were expressed and purified to apparent homogeneity as described previously (14). The Fc fragment of human monoclonal IgG1 was isolated by papain digestion. In brief, IgG, at a concentration of 10 mg/ml was incubated for 2 h at 37 °C with 6.6% (w/w) papain at pH 7.1 in the presence of 1 mM cysteamine. The Fc fragment was separated from the Fab fragments on a protein A affinity column (Amersham Biosciences) with MAPS II (Bio-Rad) binding and elution buffers at a flow rate of 0.5 ml/min. The Fc fragment was further purified from undigested IgG on a Superdex 200 HR 10/30 gel filtration column (Amersham Biosciences)
with 50 mM NaCl, 50 mM Tris at pH 8.0 as a running buffer at a 0.5 ml/min flow rate.

**SDS-PAGE Endopeptidase Cleavage Assay and N-terminal Sequence Determination**—The endopeptidase activity of Mac-1 and Mac-2 was analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) under reducing condition using Coomassie Blue staining. For N-terminal amino acid sequence analysis, proteins were separated by 10% SDS-PAGE and blotted onto a polyvinylidene difluoride membrane. Protein bands were visualized in Ponceau Red dye, destained with water, and cut out for N-terminal sequencing using a model 477A protein sequencer coupled to a model 120A PTH analyzer (PerkinElmer Life Sciences/Applied Biosystems).

**Surface Plasmon Resonance Measurements**—Surface plasmon resonance (SPR) measurements were performed using BIAcore 3000 instrument (BIAcore AB). Mac-1, Mac-2, and recombinant human Fc receptor (FcγRIII) were immobilized on a CM5 sensor chip at concentrations of 3 μM in 5 mM sodium acetate, pH 5.5–6.0, using N-hydroxysuccinimide/1-ethyl-3(-3-dimethlaminopropyl)carbodiimide hydrochloride at a flow rate of 10 μl/min. Flow cell 1 of every sensor chip was mocked with N-hydroxysuccinimide/1-ethyl-3(-3-dimethlaminopropyl)carbodiimide hydrochloride as a blank control. As a further control for nonspecific binding, soluble Ig-like transcript 4 (ILT4), an immunoglobulin-like receptor expressed on myeloid cells, was immobilized in one of the flow cell in every experiment. All binding and kinetic experiments were performed at room temperature with HBS-EP (BIAcore AB) as running buffer. The immobilization level varied from 500 to 2500 response units. Dissociation constants \(K_D\) were obtained by linear regression of steady state response versus 1/C plots or from kinetic rate constants fitted with the BIAevaluation software package (BIAcore AB).

The binding of IgG-Fc fragment, IgG, and IgM to immobilized Mac-1 was measured with serial dilutions of Fc, IgG, and IgM varying from 3

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**TABLE I**

| Analyte     | Concentration of analyte | \(K_D\) Mac-1 | \(K_D\) Mac-1 (C94A) | \(K_D\) Mac-2 |
|-------------|--------------------------|---------------|----------------------|---------------|
| Fc          | 3.0–0.094 μM             | 5.4           | 5.6                  | ND            |
| IgG         | 1.0–0.015 μM             | 2.5           | 2.6                  | ND            |
| IgM         | 6.6–0.206 μM             | 34            | ND                   | ND            |
| IgA         | ND                       | ND            | ND                   | ND            |
| FcγRII (CD32)| 25.0–0.39 μM             | ND            | 4.4                  | ND            |
| FcγRIII (CD16)| 47.6–1.49 μM             | ND            | 70.9                 | ND            |

ND, not detectable.

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**FIG. 1. Binding of Mac-1 and Mac-2 to immunoglobulins and Fcγ receptors.**

a, Fc binding to immobilized Mac-1. Measurements were performed using serial dilutions of Fc from 3 to 0.094 μM. b, IgG binding to Mac-1 with serial dilutions of IgG from 3.5 to 0.05 μM. c, IgM binding to Mac-1. The IgM concentration was varied from 6.6 to 0.21 μM. d, FcγRII binding to Mac-2 with FcγRIII concentration from 25 to 0.39 μM. e, FcγRII binding to Mac-2. FcγRIII concentration was varied from 47 to 1.49 μM. f, binding of Fc to Cys94→Ala mutant. Serial dilutions of Fc between 3 and 0.009 μM concentration were used as the analyte.

**FIG. 2. Mac-1 competition with FcγRIII.**

a, effect of FcγRIII competition on Fc binding to immobilized Mac-1. The mixture of 4 μM Fc with concentrations of Mac-1 from 0 to 3.8 μM was used as the analyte. Ru indicates binding of Fc to Mac-1. b, effect of Mac-1 competition on Fc binding to immobilized FcγRIII. The mixture of 3 μM Fc with concentrations of Mac-1 from 0 to 2.32 nM was used as the analyte. Ru indicates Fc binding to FcγRIII.

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The binding of IgG-Fc fragment, IgG, and IgM to immobilized Mac-1 was measured with serial dilutions of Fc, IgG, and IgM varying from 3
Residues potentially involved in Mac-1 binding are 6, purified Mac-1 incubated with sheep IgG. Incubated with mouse IgG. The arrow indicates proteolytic product. Mac-2 treated with DTT and incubated with human IgG. The purified Mac-1; incubated with purified human IgG. The arrow indicates proteolytic product. d, IgG endopeptidase activity of purified Mac-1 on pig and mouse IgG. Lane 1, low molecular weight marker; lane 2, purified Mac-1; lane 3, pig IgG; lane 4, purified Mac-1 incubated with pig IgG; lane 5, mouse IgG; and lane 6, purified Mac-1 incubated with mouse IgG. The arrow indicates proteolytic product. e, IgG endopeptidase activity of Mac-1 on rabbit and sheep IgG. Lane 1, low molecular weight marker; lane 2, purified Mac-1; lane 3, rabbit IgG; lane 4, purified Mac-1 incubated with rabbit IgG; lane 5, sheep IgG; and lane 6, purified Mac-1 incubated with sheep IgG. f, sequence alignment of the N-terminal Fc portion of human, monkey, rabbit, pig, and mouse IgG. Residues potentially involved in Mac-1 binding are boxed.

To 0.94 μM, 3.5 to 0.05 μM, and 6.6 to 0.21 μM, respectively. Binding of FcγRII and FcγRIII to immobilized Mac-2 was analyzed with receptor concentrations varying from 25 to 0.39 μM and 47 to 1.49 μM, respectively. Binding of Mac-1 mutant protein (Cys44 → Ala) to Fc was measured with Fc concentration varied in serial dilution from 3 to 0.99 μM. For experiments conducted with reduced Mac-2, the protein was treated overnight with 1 mM DTT and immobilized.

Kinetic Experiments on Immobilized FcγRII, FcγRII, and Mac-1—In the case of kinetic experiments on immobilized FcγRIII, 6 μM Fc incubated with 2.6 nM Mac-1 was flown as the analyte every 0.5 h from 1.5 to 4.5 h. 6 μM Fc alone was flown at 0, 1, and 5 h as a control to probe the surface binding capacity of immobilized FcγRII-Mac-1. At a concentration of 2.6 nM was flown at 0.5 h to show that it did not bind FcγRII. For a similar experiment on human IgG, the analyte consisted of 3 μM IgG incubated with 1 nM Mac-1. In the time course experiments with immobilized Mac-1, 3 μM IgG incubated with 1.3 nM Mac-1 was used as the analyte every 0.5 h from 1.5 to 4.5 h. Mac-1 at a concentration of 1.3 nM was injected at 0.5 h to show that Mac-1 did not bind to itself nonspecifically. IgG at 3 μM concentration was used as the analyte at 0, 1, and 5 h after the injection of Mac-1 to assess the surface binding capacity of the immobilized Mac-1.

Enzymatic Rate Constants—To determine the enzyme kinetic parameters of Mac-1 for substrates IgG, IgG, IgG, and IgG, kinetic binding experiments were performed on CM5 BIAcore sensor chips immobilized with 1) an enzymatic inactive C94A Mac-1 mutant that still retained IgG isotype binding, 2) protein G, and 3) protein A in separate flow cells. The analyte for IgG, IgG, and IgG consisted of 2 μM immunoglobulin mixed with 10 nM Mac-1 while the IgG analyte contained 2 μM immunoglobulin and 50 nM Mac-1. The residual binding of immunoglobulins to the immobilized reporter proteins (C94A, protein G, and protein A) upon Mac-1 digestion was assayed kinetically every 5–10 min at room temperature to detect and quantify the residual substrate concentration and thus the concentration of product formed. A 20 mM glycine buffer at pH 9.5 was used for regenerating the C94A Mac-1 flow cell, while 25 mM NaOH was used for regenerating the protein G and protein A flow cells. The kinetic constants (V, V, and kcat) were determined by Lineweaver-Burk plot.

Competition Experiments with Immobilized FcγRII, Mac-1, and Mac-2—The competition experiment with Mac-1 and Fc receptors was performed on a FcγRIII immobilized chip using analytes consisting of 3 μM Fc mixed with an increasing concentration of Mac-1 from 0 to 2.32 μM. Alternatively, the competition was also measured on an immobilized Mac-1 sensor chip with the analytes containing 2 μM Fc mixed with an increasing concentration of FcγRIII from 0 to 3.8 μM. To evaluate the competition between Mac-2 and IgG for Fc receptor binding, 13 μM FcγRIII mixed with an increasing concentration of IgG from 2.8 to 20 μM were flown over immobilized Mac-2.

RESULTS

Mac-1 and Mac-2 Bind to Different Components of the Human Immune System—Mac-1 was reported to mimic parts of the CD11b/CD18 complement receptor and to inhibit phagocyte function by binding to FcγRIII, thereby blocking receptor-antibody interaction (12). Surprisingly, no detectable binding was observed between FcγRIII and immobilized Mac-1 under direct solution binding conditions using a BIAcore 3000 instrument. Instead, Mac-1 bound to a human IgG with 2.5 μM affinity (Fig. 1b, Table 1). In addition to IgG, Mac-1 also recognized IgM with an affinity of 34 μM, albeit with a lower affinity (Fig. 1). No detectable binding was observed between Mac-1 and human IgA.

In striking contrast, Mac-2 did not bind human IgG, IgM, and IgA. Rather, Mac-2 bound to the immunoglobulin receptors, FcγRII, and FcγRIII with 5 and 70 μM affinities, respectively (Fig. 1). Overnight treatment of Mac-2 with 1 mM DTT resulted in weak binding of Mac-2 to IgG with an affinity of 16...
mM as compared with the micromolar affinity of Mac-1. Reduced Mac-2 retains its micromolar affinity to FcγRII and FcγRIII. Taken together, these results demonstrate the ability of Mac-1 and Mac-2 to recognize human immunoglobulins and their receptors (Table I), thereby potentially blocking the receptor-antibody interactions and preventing Fcγ receptor-mediated phagocytosis and NK cell-mediated ADCC.

Mac-1 Recognizes the Lower Hinge Region of IgG and Blocks the Binding of Fcγ Receptors—To test the hypothesis that Mac-1 bound the Fc fragment prepared by papain digestion of a human IgG, solution binding experiments were performed using a serial dilution of Fc varying in concentration from 3 to 0.094 μM. Mac-1 bound Fc with an affinity of ~5.4 μM, similar to that of IgG (Fig. 1a). To further investigate whether Mac-1 and Fcγ receptors have overlapping binding epitopes on Fc, direct binding competition experiments were performed on a Mac-1 immobilized CM5 sensor chip. The analyte contained 4 μM Fc mixed with increasing concentration of FcγRIII from 0 to 3.8 μM. If Mac-1 bound to the same lower hinge region of Fc as FcγRIII, Fc binding to the immobilized Mac-1 would decrease as the FcγRIII concentration increases. Alternatively, if Mac-1 bound to a site distinct from the FcγRIII recognition site, the SPR response will be independent of or will increase with the FcγRIII concentration due to a higher molecular weight of the FcγRIII-Fc complex. The results of the competition experiment showed that Fc binding to Mac-1 decreased with increasing concentrations of FcγRIII (Fig. 2a). This dose-dependent decrease in binding of Fc to Mac-1 indicated that Mac-1 bound to the same lower hinge region of Fc as FcγRIII.

Mac Proteins Display IgG Endopeptidase Activity—The endopeptidase activity of both Mac-1 and Mac-2 on human IgG was probed by SDS-PAGE. Consistent with published results
Mac-1 and Mac-2-mediated Recognition of IgG and Fc Receptors

(13), Mac-1 cleaved the heavy chain of human IgG but not IgA and IgM. The N terminus of the cleaved fragment (GPSVFLFP) corresponded to amino acid residues 237–244 of the IgG heavy chain. No endopeptidase cleavage was observed when Mac-2 was incubated with various immunoglobulins under the same condition. However, lower level endopeptidase activity of Mac-2 against IgG was observed under reducing conditions in the presence of 1 mM DTT (Fig. 3b). This result is consistent with a previous study showing that Mac-2 is more susceptible to oxidation than Mac-1 as a result of oxidation of its cysteine residues (14). When the oxidized cysteine side chain of Mac-2 was reduced by overnight incubation with DTT, Mac-2 gained IgG endopeptidase activity, which was significantly less efficient than Mac-1 (Fig. 3b). Reduced Mac-2 did not cleave immunoglobulins IgM and IgA nor FcγRIII and FcγRIIb.

To further define the specificity of the endopeptidase activity of Mac-1, IgG from mouse, sheep, pig, monkey, and rabbit were studied in the cleavage assay. In addition to human IgG, Mac-1 cleaved monkey and rabbit but not mouse, sheep, or pig IgG1 (Fig. 3, a, c, d, and e). Sequence comparison revealed that the human, monkey, and rabbit IgG1 had a conserved sequence (232PELLGG237) located at the lower hinge region near the site of cleavage (Fig. 3f), suggesting that this PELLG sequence functions as a binding site for Mac-1. Interestingly, the same LLGG at the lower hinge region of IgG has been implicated as the residues involved in Fcγ receptor binding to antibody in the crystal structure of the FcFcyRIII complex (16).

To test whether Mac-1 cleaved IgG-Fc fragment retain FcγRIII binding ability, a time-dependent binding experiment was performed with an FcγRIII-immobilized CM5 sensor chip. In this experiment, the analyte consisted of IgG-Fc fragment incubated with Mac-1 for various length of time. If Fc cleaved by Mac-1 lost the ability to bind FcγRIII, the BLAcore response will decrease with time as Fc is digested by Mac-1. Alternatively, if the endopeptidase activity of Mac-1 did not affect the ability of Fc fragment to bind FcγRIII, the SPR response will be independent of Mac-1 incubation time. The results showed decreased receptor binding with time, suggesting that the cleaved Fc undergoes a conformational change such that it no longer binds FcγRIII (Fig. 4a). A control binding with IgG alone as the analyte performed at the end of the kinetic experiment showed a SPR response similar to the initial response indicating that the immobilized FcγRIII retained its ligand binding capacity. A consistent result was obtained when an intact human IgG incubated with Mac-1 was used as the analyte (Fig. 4b). Similarly, to verify whether Mac-1 treated Fc can still bind to Mac-1, the latter was immobilized on a CM5 sensor chip, and Fc treated with Mac-1 was used as the analyte. The result showed that Mac-1 digested Fc lost its binding to Mac-1 and that the digested product is released by Mac-1 (Fig. 4c).

**Mac-1 Binding to IgG Is Independent of Its Endopeptidase Activity**—To determine whether Mac-1 binding to Fc can be separated from its endopeptidase activity, an enzymatic inactive Mac-1 with Cys94 mutated to Ala was immobilized on the sensor chip and serial dilutions of Fc between 3 and 0.09 μM concentration were used as the analyte. The observed affinity of the mutant Mac-1 to Fc was essentially the same as that of wild type Mac-1 (Fig. 1f) suggesting that the enzymatic activity is separate from its substrate binding. The presence of the endopeptidase activity potentially exacerbates the virulence of Mac-1 compared with other microbial-originated antibody binding proteins, such as protein A and protein G. When Mac-1 was used to inhibit the IgG-Fc binding to immobilized FcγRIII, the results show that, instead of stoichiometric inhibition, a concentration of 2.3 nM Mac-1 was sufficient to completely block the binding of 3 μM Fc to its receptor (Fig. 2b). Thus, the endopeptidase activity of Mac-1 results in 1000-fold increase in its IgG blocking efficiency.

**The Endopeptidase Activity of Mac-1 Is Inhibited by Cysteine and Aspartic Protease Inhibitors**—Mac-1 has been recently reported as a new class of cysteine protease with no sequence homology to other known cysteine proteases (13). Using the SDS-PAGE endopeptidase cleavage assay, various protease inhibitors were screened for their ability to block Mac-1 degradation of IgG. Although Mac-1 was characterized as a cysteine protease, not all cysteine protease inhibitors inhibit Mac-1.

Among the serine and/or cysteine protease inhibitors, TPCK, TLCK, and iodoacetamide inhibited Mac-1 activity, whereas E64, leupeptin, cystatin, and PMSF did not (Fig. 5). Interestingly, pepstatin, an aspartic protease inhibitor, also inhibited Mac-1 activity indicating a potential involvement of an aspartate either as a catalytic residue or near the catalytic cysteine of Mac-1, which is consistent with the previous mutational studies that identified Asp286 and Asp288 as important residues for enzymatic activity or structure of Mac-1 (17).

**Mac-1 Has Comparable Enzyme Kinetic Parameters for IgGp, IgGγ, IgGγ, and IgGγ**—To estimate the Mac-1 binding affinity to all four IgG isotypes, solution binding experiments were performed with varying concentrations of IgG1, IgG2, IgG3, and IgG4 (Table II). Mac-1 bound IgG1, IgG2, and IgG3 with similar affinity, 2.6, 1.5, and 3.3 μM, respectively, but recognized IgG2 with lower affinity of 32 μM. The enzyme kinetic parameters of Mac-1 for substrates IgG1, IgG2, IgG3, and IgG4 were determined by a kinetic assay using immobilized C94A Mac-1, protein G, and protein A as reporters for residual intact immunoglobulins. In

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**Table II**

| Immobilized reporter | Analyte mixed with Mac-1 | kD (μM) | Km (μM) | Vmax (s⁻¹) | kcat (s⁻¹) |
|----------------------|-------------------------|---------|---------|------------|------------|
| Mac C94A             | IgG1                    | 2.6     | 6.8     | 0.13       | 0.12       |
|                      | IgG2                    | 32      | 18.9    | 0.09       | 0.02       |
|                      | IgG3                    | 1.5     | 13.9    | 0.09       | 0.08       |
|                      | IgG4                    | 3       | 11.9    | 0.12       | 0.1        |
| Protein G            | IgG1                    | 2       | 7.8     | 0.13       | 0.12       |
|                      | IgG2                    | 3.1     | 10.1    | 0.19       | 0.03       |
|                      | IgG3                    | 6.1     | 13      | 0.11       | 0.09       |
|                      | IgG4                    | 4.7     | 10.9    | 0.13       | 0.12       |
| Protein A            | IgG1                    | 1.3     | 9.1     | 0.16       | 0.13       |
|                      | IgG2                    | 2.1     | 11.2    | 0.16       | 0.03       |
|                      | IgG3 ND                 | ND      | ND      | ND         | ND         |
|                      | IgG4                    | 3.4     | 11.4    | 0.14       | 0.12       |

ND, not detectable.
these experiments, a 2 μM concentration each of the substrate immunoglobulin was incubated with fixed amount of Mac-1, and the SPR response was measured over a period of time (Fig. 6, a, c, and e). The kinetic constants for all the substrates were determined by plotting the reciprocal of the reaction velocity versus reciprocal of the substrate concentration (Lineweaver-Burk plot) (Fig. 6, b, d, and f). In the case of protein A measurements, kinetic constants of Mac-1 were calculated for IgG1, IgG2, and IgG4 only as protein A exhibited no detectable binding to IgG3. The rate constants calculated from C94A Mac-1, protein G, and protein A measurements are similar for all the four IgG isotypes. The apparent $K_m$ values of Mac-1 varied from 6.8 to 18.9 μM and follow an order of IgG1 > IgG3 > IgG2 > IgG4 (Table II). The larger $K_m$ of 18.9 μM calculated for the IgG2 substrate from C94A Mac-1 measurement is possibly due to weaker binding between C94A Mac-1 and IgG2 as confirmed by the 10-fold lower $K_D$ of Mac-1 to IgG2 when compared with IgG1, IgG3, and IgG4. $V_{max}$ of Mac-1 determined from the intercept on the y axis of the Lineweaver-Burk plots varied from 0.09 to 0.19 nmol min$^{-1}$ with $k_{cat}$ ranging from 0.02 to 0.13 s$^{-1}$ that follows an order of IgG1, IgG4 > IgG3 > IgG2. The $K_m$ and $k_{cat}$ values of Mac-1 lie within the range of other cysteine proteases like papain (8 μM to 320 mM and 0.06–52 s$^{-1}$), chymopapain (0.1 μM to 5 mM and 0.003–16 s$^{-1}$), actinidain (20 μM to 89 mM and 0.03–33 s$^{-1}$), and cathepsin L (0.7 μM to 0.1 mM and 0.002–50 s$^{-1}$) as reported in the BRENDA, the comprehensive enzyme information system (www.brenda.uni-koeln.de).

Mac-2 Recognition of FcγR Prevents the Binding of IgG to Its Receptor—Unlike Mac-1, Mac-2 recognizes Fc receptors on the effector cells. To determine whether binding of Mac-2 to FcR precluded antibodies from binding to the receptors, a mixture of 13 μM FcγRII with various concentration of IgG was used in
a solution competition binding experiment on a Mac-2 immobilized sensor chip. The result showed that FcγRII binding to Mac-2 decreased as the concentration of IgG increased, suggesting that Mac-2 and IgG share a closely related binding site on FcγRII (Fig. 7). This result implied that Mac-2 may contribute to the pathogenesis of GAS infection by blocking immune complex-mediated activation of effector function.

**DISCUSSION**

GAS is an important human bacterial pathogen that causes substantial morbidity and mortality worldwide. The pathogen has evolved several complex mechanisms to evade host defenses, proliferate, and cause diseases in human. A recent study (14) has identified two major allele families of *mac* genes designated as *mac-1* and *mac-2* with considerable divergence in the middle one-third of the protein. Our studies confirmed that Mac-1 is a cysteine/aspatic protease that recognizes and cleaves the lower hinge region of human IgG. Mac-2, which cleaves IgG at a much slower rate, is capable of binding to immunoglobulin receptors, FcγRII and FcγRIII. The Fc region of IgG molecule has multiple recognition sites for different components of the immune system, including Fcγ receptors, neonatal Fc receptors, rheumatoid factors, and bacterial proteins like staphylococcal proteins A and G (16, 18–20). The Fcγ receptor binding site on Fc is distinct from those of other binding proteins as it binds to the lower hinge region between the CΔ1 and CΔ1,2 domains of Fc, while other ligands bind to the joint region between the CΔ1,2 and CΔ1,3 domain. It has been shown that Fcγ receptors recognize Fc in an asymmetric fashion with one receptor bound to both the chains of Fc (16, 21–23). SPR measurement showed that Mac-1 recognizes competitively the same region of IgG as Fcγ receptors.

Recently, Mac-1 was reported to have endopeptidase activity on human IgG (13). The results indicated that in addition to human IgG, Mac-1 also cleaved monkey and rabbit IgG, which suggests that any IgG with a conserved lower hinge sequence of PELLGG at positions 232–237 can be cleaved. Interestingly, these lower hinge residues are also involved in the Fcγ receptor recognition of Fc. The search for the inhibitors of Mac-1 revealed that not all cysteine protease inhibitors inhibit its endopeptidase activity. Cysteine protease inhibitors like E64, leupeptin, cystatin, and PMSF did not inhibit Mac-1. To our surprise, pepstatin, an aspartic and human immunodeficiency virus protease inhibitor, also inhibited Mac-1. This could be interpreted as the presence of an aspartate residue either directly involved in the catalysis or close to the active site, consistent with data from site-specific mutagenesis studies (17). Mac-1 binds and cleaves all the four subclasses of IgG isotypes. The *K*~m~ and *k*~cat~ values calculated from Lineweaver-Burk plots show that the order of Mac-1 cleavage follows IgG~1~ > IgG~4~, IgG~2~ > IgG~3~, and IgG~1,3~ > IgG~2~, respectively. The *K*~m~ and *k*~cat~ values of Mac-1 for IgG isotypes fall within the range of other known cysteine proteases like papain, chymopapain, actinadian, and cathepsin.

To address whether the cysteine protease activity of Mac proteins is important for their biological function, we showed using a catalytic inactive mutant Mac-1 that the binding of Mac-1 to IgG is independent of its enzymatic activity. The presence of endopeptidase activity of Mac-1, however, increases its ability to remove immunoglobulins by 1000-fold *in vitro* and thus could greatly enhance the ability of GAS to interfere with phagocytosis, endocytosis of IgG-opsonized particles, and NK cell-mediated ADCC (Fig. 8). Reduced Mac-2 had weaker IgG endopeptidase activity than Mac-1. However, it retains the capacity to bind Fcγ receptors. The Fc receptor binding of Mac-2 competes with that of IgG and thus should inhibit the Fcγ receptor-mediated phagocytosis and ADCC (Fig. 8).

Our results bear on the two models that have been proposed to explain the functional mechanism of Mac. Lei et al. (12) proposed that binding of Mac-1 to Fcγ receptors blocked their interaction (blocking mechanism). It was subsequently reported (13) that Mac-1 has IgG endopeptidase activity, resulting in the proposal that the enzymatic activity of Mac was...
essential for its function (endopeptidase-essential mechanism). Our data suggest that Mac-2 functions mainly through the blocking mechanism. In contrast, Mac-1 did not bind to recombinant Fcγ receptors, and treatment of IgG and Fc fragment with Mac-1 diminished their binding to Fcγ receptors, consistent with the endopeptidase-essential mechanism. However, a Cys\textsuperscript{94}→Ala mutant Mac-1 protein that is unable to cleave IgG retains the ability to inhibit IgG-mediated phagocytosis by human PMNs (14); this can be explained by our solution binding results that the mutant retained its ability bind to IgG with similar affinity as wildtype. Although no direct interaction between Mac-1 and Fcγ receptors was detected in our solution binding assays, it is possible that Mac-1 binds to native Fcγ receptors present on PMNs. The observations that Mac-1 binds to the surface of human PMNs and that the binding is blocked by mouse monoclonal antibody specific to FcγRIII is consistent with this idea (12). Clearly, further studies are required to determine the extent to which Mac-1 uses the blocking mechanism in the complex environment of GAS-human interactions in vivo.

In summary, our data suggest that secreted Mac proteins made by GAS mediate host immune evasion by two different molecular mechanisms, each detrimentally altering antibody-mediated activities of the human immune system. These proteins would result in either degradation of immunoglobulins or blockade of their receptors, thus inhibiting normal host immune function and enhancing pathogen survival.

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