Complement Activation by a B Cell Superantigen[1]

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Staphylococcal protein A (SpA), acting as a B cell superantigen, binds to the Fab region of human V_H3^+ Igs. Using SpA abrogated of its IgG Fc binding activity (Mod SpA) as a model B cell superantigen, we determined whether such an interaction causes complement activation. Addition of Mod SpA to human serum led to complement consumption and the generation of C3a. To determine whether this complement activation 1) was due to an interaction between V_H3^+ Igs and the Fab binding site of SpA and 2) proceeded via the classical complement pathway, we tested a panel of monoclonal IgM proteins for the ability to bind C1q following interaction with SpA. C1q binding was restricted to SpA-reactive, V_H3^+ IgM proteins. To formally determine whether the binding of SpA to the reactive V_H3^+ IgM proteins led to complement activation, we reconstituted the serum from a hypogammaglobulinemic patient with monoclonal IgM proteins and measured complement consumption and C3a generation following the addition of Mod SpA. We observed complement consumption and C3a production only in Mod SpA-treated serum reconstituted with a V_H3^+, SpA-binding, IgM protein. Taken together, these results provide compelling evidence that the interaction of the Fab binding site of SpA and V_H3^+ Igs can lead to complement activation via the classical pathway. This novel interaction may have significant implications for the in vivo properties of a B cell superantigen. ***The Journal of Immunology,*** **1996, 157: 1200–1206.**

Staphylococcal protein A (SpA),[6] a cell wall component of *Staphylococcus aureus*, binds to the Fc fragment of IgG. The site that binds the IgG Fc region is termed the classical binding site on SpA (1). In addition, an alternative site on SpA has been defined that binds to the Fab fragments of Igs independent of the heavy chain isotype (1–6). Studies have mapped the Fab determinants to framework regions (FR) 1 and 3 in the variable heavy chain (V_H) region (7–9), with a possible contribution of residues in CDR2 (8). Binding of this alternative site on SpA is restricted to human Igs using V_H3 heavy chains (9, 10). The alternative binding site endows SpA with the ability to cross-link membrane IgM on B cells and thereby induce their activation in what was previously considered to be a non-V_H-selective manner (6). These properties are reminiscent of those of a T cell superantigen and have led SpA to be characterized as a B cell superantigen (9–15). Recently, several other proteins have also been defined as B cell superantigens, including HIV gp120, protein Fv (a human liver sialoprotein), and protein L (a coat protein of *Peptostreptococcus magnus*) (16–18).

Unlike T cell superantigens, a B cell superantigen has the ability to react with potentially large amounts of its receptor (Ig) in the serum as well as on B cells. For example, the B cells reactive with HIV gp120 and the Fab binding site on SpA are restricted to those that express V_H3 gene family products (9, 10, 16). Since this family is the largest of seven human V_H gene families and is expressed by 30 to 60% of peripheral B cells (12, 19, 20), it is possible that a large proportion of human serum Igs use V_H3 heavy chains (10). The encounter of a B cell superantigen with such a large reservoir of reactive serum Igs could have deleterious effects on the host, particularly if this interaction leads to the formation of immune complexes with subsequent activation of the complement cascade. Such complement activation could result in prominent tissue inflammation and damage.

In earlier studies, the addition of SpA to serum was reported to activate the complement cascade (21, 22). This activation was attributed to interaction of the Fc fragment of IgG with the classical binding site on SpA. However, recent reports on the B cell superantigen properties of SpA have led us to postulate that the interaction of its alternative binding site with the Fabs of reactive (V_H3^+) Igs leads to activation of the complement cascade. We now report that SpA, abrogated of its IgG Fc binding activity by hyperiodination (Mod SpA), retains its ability to activate the complement cascade. Complement activation was dependent on the binding of Mod SpA to V_H3^+ Igs and proceeded via the classical complement pathway. These results provide the first direct evidence that the interaction of a B cell superantigen with the Fabs of reactive Igs leads to activation of the classical complement cascade.

Materials and Methods

Hyperiodination of SpA (Mod SpA) and human serum albumin (Mod HSA)

Recombinant SpA (Repligen Corp., Cambridge, MA) and HSA (Calbiochem, San Diego, CA) were hyperiodinated using a standard protocol (9).

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[6] Abbreviations used in this paper: SpA, Staphylococcal protein A; FR, framework region; V_H, variable heavy chain; Mod SpA, Staphylococcal protein A hyperiodinated to abrogate its immunoglobulin G Fc binding ability; HSA, human serum albumin; OPD, *o*-phenylenediamine; VB, veronal buffer; CH_{50}, hemolytic complement assay; PEG, polyethylene glycol; V_L, variable light chain.

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Serum

Serum was obtained from healthy human donors, 24 to 45 yr old, and a recently diagnosed 20-yr-old woman with common variable hypogammaglobulinemia whose serum IgG level was <80 mg/dl and whose serum IgA and IgM levels were <10 mg/dl.

IgM monoclonal proteins

Purified IgM monoclonal proteins, representative of several V_H gene families (13, 17, 23, 24), were kindly provided by Drs. Hans Spiegelberg (La Jolla, CA), Ralph Schrohenloher (Birmingham, AL), Marianna Newkirk (Montreal, Canada), and Denise Shaw (Birmingham, AL).

ELISA for determining Mod SpA binding to IgG Fc

The ability of Mod SpA to bind polyclonal IgG was assessed in an ELISA. Half-area microtiter plate wells (Costar, Cambridge, MA) were coated with 100 μl of Mod SpA, SpA, or BSA (Calbiochem, La Jolla, CA) at 10 μg/ml in PBS overnight at 4°C. The wells were then saturated with 100 μl of 1% BSA/PBS for 2 h at room temperature. The wells were washed three times with 0.05% Tween-20 in PBS and incubated with 100-μl aliquots of varying concentrations of peroxidase-conjugated polyclonal IgG (Dako Corp., Carpinteria, CA) for 1 h at room temperature. The plates were washed as described above, and the bound Ab was detected by the addition of *o*-phenylenediamine (OPD) substrate (Eastman Kodak, Rochester, NY) in 20 mM citrate buffer, pH 4.0. The OD was read spectrophotometrically at 450 nm.

ELISA for determining Mod SpA binding to IgM Fab

The ability of Mod SpA to bind the Fabs of Igs was evaluated in an IgM binding ELISA. Half-area microtiter plate wells were coated with 100 μl of Mod SpA, SpA, or BSA at 10 μg/ml in PBS overnight at 4°C. The wells were then saturated with 100 μl of 1% BSA/PBS for 2 h at room temperature. The wells were washed three times with 0.05% Tween-20 in PBS and incubated with 100-μl aliquots of varying concentrations of polyclonal IgM (Calbiochem) for 2 h at room temperature. The plates were washed as described above, and peroxidase-conjugated goat $F(ab')_2$ anti-human IgM Fc Ab (Jackson ImmunoResearch Laboratories, West Grove, PA) was added for 1 h at room temperature. The bound peroxidase-conjugated Ab was detected by the addition of OPD substrate in 20 mM citrate buffer, pH 4.0, and the OD was read spectrophotometrically at 450 nm. SpA- and Mod SpA-coated wells incubated without the IgM proteins consistently had absorbance readings below that in BSA-coated wells, demonstrating that SpA and Mod SpA were unable to bind the developing Ab (data not shown).

Hemolytic complement assay (CH_{50} assay)

We used the CH_{50} assay to measure the functional activity of complement in serum treated with SpA or Mod SpA. In this assay, the magnitude of lysis of Ab-coated SRBCs is a reflection of complement activity. Reduced serum complement activity (i.e., inhibition of SRBC hemolysis) is consistent with consumption of complement components by prior treatment of the serum.

SRBCs (Diamedix Corp., Miami, FL) were washed twice with a working solution of veronal buffer (VB; 1/5 dilution of VB with Ca^{2+} and Mg^{+}), and the concentration was adjusted to 1×10^9 cells/ml. To sensitize the SRBCs, 600 μl of SRBCs was mixed with 600 μl of IgM anti-SRBC Ab (1/60 in VB; Diamedix Corp.) and incubated for 30 min at 37°C. The cells were washed twice with VB, and the concentration was adjusted to 5×10^8 cells/ml. SpA and Mod SpA at various concentrations were preincubated with 100 μl of a 1/4 dilution in VB of normal human serum or serum from a severely hypogammaglobulinemic patient (reconstituted with monoclonal V_H3^+ IgM proteins at a concentration of 1 mg/ml) for 1 h at 37°C. Ten microliters of IgM-sensitized SRBCs plus 190 μl of VB were then added to 100-μl aliquots of serially diluted mixtures of untreated, SpA-treated, and Mod SpA-treated serum and incubated for 1 h at 37°C. The cells were centrifuged at 2000 rpm for 2 min, and supernatants were read spectrophotometrically at 405 nm. Controls included serum without SRBCs, SRBCs incubated in water (100% hemolysis), and SRBCs incubated in VB (0% hemolysis) (data not shown). CH_{50} units were determined by a standard equation (25).

Measurement of C3a

The ability of Mod SpA to activate the classical complement cascade following an interaction with serum Igs was assessed by determining the generation of the complement activation product, C3a. Microtiter plate wells were coated with 50 μl of polyclonal goat anti-human C3a Ab at 10 μg/ml in PBS overnight at 4°C. The wells were then saturated with 200 μl of 1% BSA/PBS for 30 min at room temperature. During this time period, varying concentrations (equivalent molar ratios) of SpA, Mod SpA, HSA, or Mod HSA were incubated with normal human serum or serum from a severely hypogammaglobulinemic patient (reconstituted with V_H3^+ IgM monoclonal proteins at a concentration of 1 mg/ml) for 30 min at 37°C. EDTA (20 mM) was added to stop the reactions. The reaction mixtures were centrifuged at 2000 rpm for 5 min, and 10-μl supernatant aliquots were added to 10 μl of 32% PEG (16% PEG final concentration) and allowed to precipitate for 30 min at 4°C with occasional mixing. These mixtures were centrifuged, and the supernatants were diluted in 1% BSA/PBS. The microtiter plate wells were washed with 0.05% Tween-20 in PBS and incubated with 50-μl aliquots of the supernatants at different dilutions for 30 min at room temperature. The plates were washed as described above, and mouse anti-human C3a Ab (1 μg/ml; kindly provided by Dr. Reinhard Burger, Bonn, Germany) was added for 30 min at room temperature. After washing, the wells were incubated with peroxidase-conjugated goat anti-mouse IgG Ab (Bio-Rad, Hercules, CA) for 30 min at room temperature. The bound Ab was detected by adding 2,2′-azinobis 3-ethyl benzothiazoline-6-sulfonic acid substrate in 0.1 M sodium citrate buffer, pH 4.2, and the OD was read spectrophotometrically at 405 nm following the addition of 50 μl of PBS/well. Negative controls included normal human serum incubated alone and the reagents incubated without normal human serum (data not shown).

Table I. *Abrogation of IgG Fc binding ability following hyperiodination of SpA*[a]

Polyclonal IgG (μg/ml)[b]	Mod SpA	SpA	BSA
2.6	0.042[c]	0.782	0.043
1.3	0.043	0.672	0.043
0.65	0.042	0.590	0.044
0.26	0.042	0.431	0.042

[a] Polyclonal IgG was used in a direct binding ELISA to determine the effect of hyperiodination of SpA on its binding to IgG Fc.
[b] Peroxidase-conjugated polyclonal IgG.
[c] Values represent means of duplicate determinations of absorbance readings at 450 nM for a representative experiment; similar results were obtained for every batch of Mod SpA produced.

ELISA for determining SpA/IgM binding to C1q

The ability of V_H3^+ Igs to bind C1q following their interaction with SpA was determined by an ELISA. Half-area microtiter plate wells were coated with 100 μl of C1q (Quidel, San Diego, CA) at 5 μg/ml in PBS (ionic strength, 9.7 mS/cm at room temperature) overnight at 4°C. The wells were then saturated with 100 μl of 1% BSA/PBS for 2 h at room temperature. During this time period, varying concentrations of polyclonal IgM or monoclonal IgM proteins were preincubated with 1 μg/ml biotinylated SpA (26) at 37°C. The wells were then washed three times with 0.05% Tween-20 in PBS and incubated with 100-μl aliquots of the IgM/biotinylated SpA mixtures for 2 h at 37°C. The plates were washed as described above, and peroxidase-conjugated streptavidin (Calbiochem) was added for 1 h at 37°C. The bound peroxidase-conjugated streptavidin was detected by the addition of OPD substrate in 20 mM citrate buffer, pH 4.0, and the OD was read spectrophotometrically at 450 nm. Dau is an SpA-nonreactive, V_H3^+ IgM monoclonal protein that was used as a negative control in these binding assays.

To circumvent the potential problem of the biotinylated SpA being dissociated from the IgM proteins during the washing steps, we altered the ELISA protocol described above by using the IgM proteins at a constant concentration of 8 μg/ml and developing the ELISA with peroxidase-conjugated goat $F(ab')_2$ anti-IgM Fc Ab, instead of peroxidase-conjugated streptavidin.

Results

SpA and Mod SpA cause complement consumption

After demonstrating that Mod SpA was unable to bind IgG Fc while it retained the ability to bind Ig Fabs (Tables I and II), we compared its ability with that of unmodified SpA to cause complement consumption when added to normal human serum. The treated sera were added to SRBCs sensitized with IgM anti-SRBC Abs in a hemolytic complement assay (CH_{50} assay) as described in *Materials and Methods*. As demonstrated in Table III, >60% inhibition of CH_{50} activity was observed in SpA-treated (mean ±

Table II. *Retention of Ig Fab binding ability following hyperiodination of SpA*[a]

Polyclonal IgM (μg/ml)[b]	Mod SpA	SpA	BSA
5.0	0.843[c]	0.863	0.052
2.5	0.720	0.771	0.047
1.25	0.664	0.685	0.046
0.625	0.493	0.518	0.045
0.313	0.418	0.368	0.044
0.156	0.270	0.299	0.045
0.0	0.045	0.046	0.045

[a] A sandwich ELISA was used to determine the ability of Mod SpA to bind Ig Fabs.

[b] Polyclonal IgM was added to protein-coated wells. Bound IgM was determined by using a peroxidase-conjugated goat $F(ab')_2$ anti-human IgM Fc antibody.

[c] Values represent means of duplicate determinations of absorbance readings at 450 nm for a representative experiment; similar results were obtained for every batch of Mod SpA produced.

Table III. *Effect of SpA and Mod SpA on the hemolytic complement activity (CH_{50}) of normal human serum*

Donor Number	Protein Added	CH_{50} Units[a]	% CH_{50}[b] Inhibition
1	None	164	
	SpA[c]	37	77%
	Mod SpA[c]	61	63%
2	None	305	
	SpA	142	53%
	Mod SpA	115	62%
3	None	294	
	SpA	127	57%
	Mod SpA	173	41%
4	None	147	
	SpA	48	67%
	Mod SpA	41	72%
5	None	185	
	SpA	46	75%
	Mod SpA	77	58%
6	None	142	
	SpA	81	43%
	Mod SpA	34	76%
			SpA, 62 ± 12% (Mean ± SD) Mod SpA, 62 ± 13% ($p > 0.1$, paired t test)

[a] CH_{50} units were calculated by a standard equation (25).

[b] Percentage of CH_{50} inhibition was calculated by the formula, (1 − [CH_{50} units with SpA or Mod SpA added/CH_{50} units with no protein added]) × 100%; results are averages of duplicate tubes.

[c] 240 μg/ml.

SD, 62 ± 12%) and Mod SpA-treated (62 ± 13%) serum ($p > 0.1$, by paired t test). These results are consistent with the interpretation that both agents can consume complement in normal human serum.

Complement consumption by SpA and Mod SpA is associated with C3a generation

To establish whether complement consumption was associated with complement activation, we investigated the ability of unmodified SpA and Mod SpA to generate the complement activation product, C3a, when added to normal human serum. Following incubation with SpA or Mod SpA, C3a generation in the treated serum was determined in an ELISA as described in *Materials and Methods*. As demonstrated in Figure 1, addition of either SpA or Mod SpA to normal human serum caused the generation of C3a in a dose-dependent manner. At low added protein concentrations,

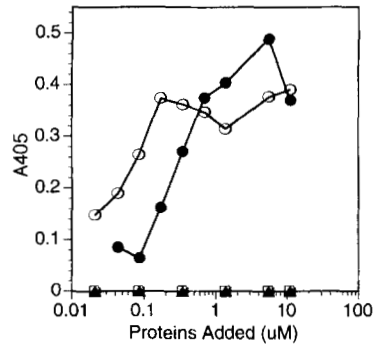

FIGURE 1. Generation of C3a in normal human serum by the addition of SpA, Mod SpA, HSA, or Mod HSA. The generation of C3a was analyzed in an ELISA as described in *Materials and Methods*. Various concentrations of SpA (open circle), Mod SpA (closed circle), HSA (open square), or Mod HSA (closed triangle) were incubated with normal human serum. The reaction mixtures were PEG precipitated, and 1/2000 dilutions of the supernatants were analyzed in anti-C3a-fixed ELISA plates. Background absorbance was determined in anti-C3a-coated wells to which no PEG-precipitated supernatants were added, and this absorbance was subtracted from the raw data. OD values are the results from a representative experiment; similar results were obtained in four separate experiments.

more C3a was generated by the addition of SpA than by that of Mod SpA to serum. To establish that the effect of Mod SpA was not a function of its hyperiodination, unmodified HSA and hyperiodinated HSA (Mod HSA) were incubated with normal human serum, and the reaction mixtures were analyzed for C3a generation. Neither Mod HSA nor HSA caused the generation of C3a (Fig. 1). These results demonstrate that the complement consumption caused by SpA and Mod SpA, as shown in Table III, was associated with the appearance of the complement activation product, C3a. The results clearly indicate that complement activation can be mediated by interaction of the Fab binding site of SpA with reactive Igs. In addition, the data suggest that the IgG Fc binding site of SpA contributes to the ability of unmodified SpA to activate complement.

IgM proteins bind C1q following their interaction with SpA

If Mod SpA-induced complement activation proceeded via a B cell superantigenic mechanism, then the Fab binding site on SpA must interact with V_H3^+ Igs, leading to C1q binding. To directly test this hypothesis, we assessed the ability of human polyclonal IgM and a panel of monoclonal IgM proteins (representative of several V_H gene families) to bind C1q following their interaction with SpA. In these experiments, we were able to use unmodified SpA, rather than Mod SpA, since IgM proteins bind SpA solely by determinants in their Fab region (1–6). First, by ELISA, we identified IgM proteins that bound SpA. We observed that polyclonal IgM and all but two of the V_H3^+ monoclonal IgMs from our panel, Dau and Berry, bound SpA (Fig. 2). These results are consistent with previous reports (10, 13, 14) that some V_H3^+ IgMs fail to bind SpA.

Following incubation with SpA, the IgM proteins were analyzed for their ability to bind C1q in an ELISA. As shown in Figure 3, the interaction of SpA with polyclonal IgM led to binding of C1q. This binding appeared to be restricted to V_H3^+ IgMs, since the only monoclonal IgM proteins that bound C1q following their interaction with SpA (Pom, HUAB 14–3, and Vincent) belonged to the V_H3 gene family. The other SpA-reactive, V_H3^+ IgM proteins (Riv, HUAB 2–3, and Lay) failed to bind C1q. Neither of the

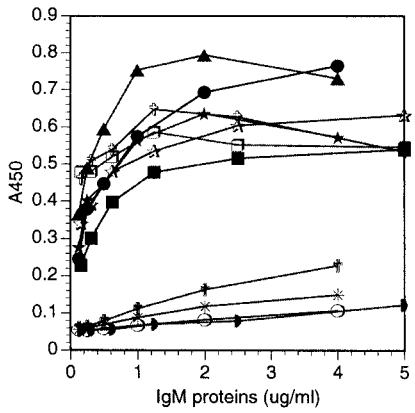

FIGURE 2. Binding of monoclonal IgM proteins to SpA. The binding of IgM proteins to SpA was analyzed in an ELISA as described in *Materials and Methods*. Various concentrations of polyclonal IgM (closed circle), monoclonal V_H3^+ IgM proteins, Pom (closed triangle), HUAB 14–3 (closed square), Vincent (open square), HUAB 2–3 (open star), Riv (open cross), Lay (closed star), Berry (closed half moon), and Dau (open circle), or monoclonal V_H4^+ IgM proteins, Cor (asterisk) and Lew (closed cross), were added to SpA-fixed ELISA plates. Background absorbance was determined in SpA-coated wells in which no IgM proteins were incubated, and this absorbance was subtracted from the raw data. ODs represent the means of duplicate determinations from a representative experiment; similar results were obtained in three separate experiments.

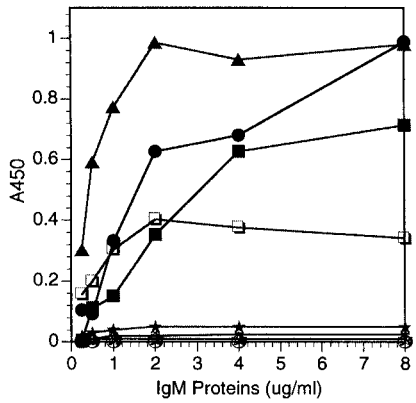

FIGURE 3. Binding of SpA-reactive IgM/SpA to C1q as detected by peroxidase-conjugated streptavidin. The binding of IgM/SpA reaction mixtures to C1q was analyzed in an ELISA as described in *Materials and Methods*. Various concentrations of polyclonal IgM (closed circle) or monoclonal V_H3^+ IgM proteins, Pom (closed triangle), HUAB 14–3 (closed square), Vincent (open square), HUAB 2–3 (open star), Riv (open cross), Lay (closed star), and Dau, an SpA-nonreactive IgM protein (open circle), preincubated with biotinylated SpA (1 μg/ml) were added to C1q-fixed ELISA plates. The background absorbance was determined in C1q-coated wells in which IgM proteins and biotinylated SpA were sequentially incubated, and the ELISA was developed by standard protocol. This absorbance was subtracted from the raw data. OD values represent the means of duplicate determinations from a representative experiment; similar results were obtained in three separate experiments.

SpA-nonreactive monoclonal IgM proteins, Dau and Berry (data not shown for Berry), bound C1q following its incubation with SpA.

We considered the possibility that the binding of the SpA-reactive, IgM proteins (Riv, HUAB 2–3, and Lay) to C1q might not be detected in our ELISA if the biotinylated SpA (our readout) was dissociated from the C1q-bound IgM proteins during the washing

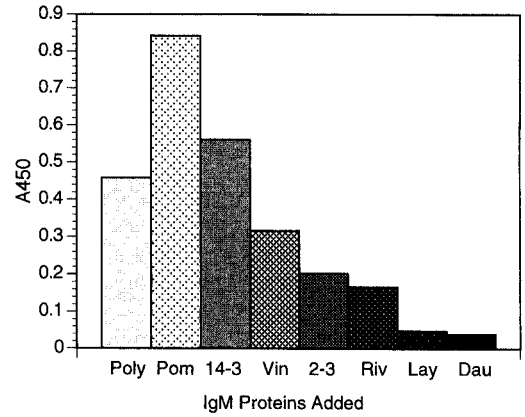

FIGURE 4. Binding of SpA-reactive IgM/SpA to C1q as detected by peroxidase-conjugated anti-IgM Fc Ab. Binding of IgM/SpA reaction mixtures to C1q was analyzed as described in *Materials and Methods*. Background absorbance was subtracted as described in Figure 4, except the wells were developed with peroxidase-conjugated goat $F(ab')_2$ anti-IgM Fc Ab. OD values represent the means of duplicate determinations from a representative experiment; similar results were obtained in three separate experiments.

steps (see *Materials and Methods*). To circumvent this possibility, we used an ELISA in which the wells were developed with peroxidase-conjugated goat $F(ab')_2$ anti-IgM Fc Ab to detect IgM proteins that bound to C1q following their interaction with SpA. As shown in Figure 4, five of the six SpA-reactive, V_H3^+ IgM proteins demonstrated binding to C1q following their incubation with SpA. As expected, SpA-nonreactive monoclonal IgM proteins, such as Dau, did not bind C1q following incubation with SpA. Results from binding studies of non-V_H3^+ IgM proteins were consistently negative (data not shown). None of the wells containing only monoclonal IgM proteins had absorbance readings above that observed in BSA-coated wells, thus demonstrating that the monoclonal IgM proteins were unable to bind C1q-coated wells unless they were first preincubated with SpA.

Mod SpA activation of complement is V_H3^+ Ig dependent

To determine whether the binding of SpA to V_H3^+ IgM proteins caused complement consumption, we used serum (reconstituted with selected monoclonal IgM proteins) from a severely hypogammaglobulinemic patient. Mod SpA was added to this serum, and the serum was analyzed for complement consumption. As demonstrated in Figure 5, the addition of Mod SpA to hypogammaglobulinemic serum reconstituted with Berry, an SpA-nonreactive, V_H3^+ IgM, did not alter the CH_{50} activity of the serum. Likewise, the addition of Mod SpA to this serum reconstituted with Lay, an SpA-reactive, C1q-nonbinding, V_H3^+ IgM, did not alter CH_{50} activity. By contrast, the addition of Mod SpA to hypogammaglobulinemic serum reconstituted with Pom, an SpA-reactive, C1q-binding, V_H3^+ IgM, did support complement consumption. In addition, incubation of Mod SpA alone with the hypogammaglobulinemic serum did not cause any complement consumption.

To demonstrate that this complement consumption was also associated with complement activation, we investigated whether the addition of Mod SpA to hypogammaglobulinemic serum reconstituted with Berry, the SpA-nonreactive, V_H3^+ IgM, or with Pom, the SpA-reactive, C1q-binding, V_H3^+ IgM, generated C3a. Figure 6 shows that the addition of Mod SpA to Berry-reconstituted hypogammaglobulinemic serum caused C3a generation equivalent to that observed in serum incubated with Berry alone. In contrast, the

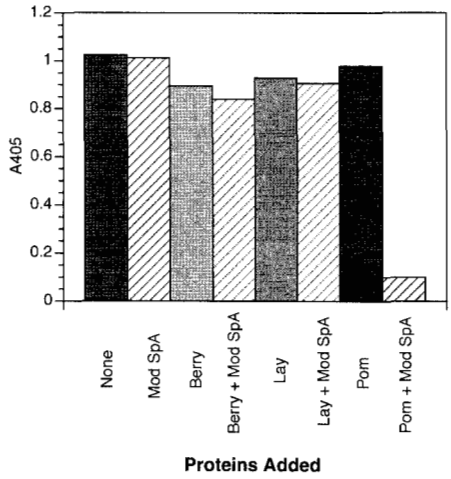

FIGURE 5. Mod SpA-mediated complement activation in IgM-reconstituted hypogammaglobulinemic serum. Monoclonal V_H3^+ IgM proteins were added to serum from a hypogammaglobulinemic patient at a concentration of 1 mg/ml (Berry was used as a negative control). The serum was then preincubated with 240 μg/ml Mod SpA for 1 h at 37°C. The remaining protocol followed the CH_{50} assay described in *Materials and Methods*. Solid bars represent experiments without Mod SpA added, and hatched bars represent experiments with Mod SpA added. OD readings at 405 nm correspond to the amount of hemoglobin in the supernatant following hemolysis of SRBCs. Results are averages of duplicate tubes from a representative experiment; similar results were obtained in two separate experiments.

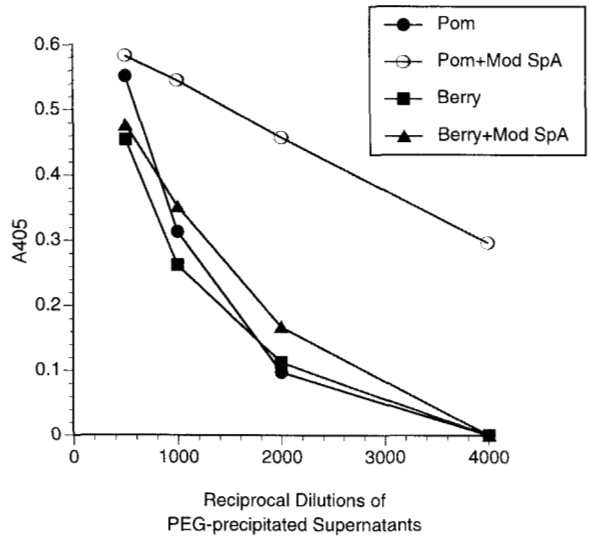

FIGURE 6. Mod SpA-mediated generation of C3a in IgM-reconstituted hypogammaglobulinemic serum. Monoclonal V_H3^+ IgM proteins were added to serum from a hypogammaglobulinemic patient at a concentration of 1 mg/ml. The serum was then preincubated alone or with 240 μg/ml Mod SpA for 30 min at 37°C. The reaction mixtures were PEG precipitated, and the supernatants were serially diluted starting at 1/500 and then analyzed in anti-C3a-fixed ELISA plates. Background absorbance was determined as described in Figure 1. OD values are the results from a representative experiment; similar results were obtained in two separate experiments.

addition of Mod SpA to Pom-reconstituted hypogammaglobulinemic serum led to the generation of at least 3 times the amount of C3a as observed in Pom-reconstituted serum incubated without Mod SpA. These results demonstrate that an interaction between Mod SpA and the Fabs of its reactive Igs is necessary for complement consumption and activation.

Discussion

The studies reported herein were undertaken to determine whether the interaction of a B cell superantigen and its reactive Igs leads to activation of the classical complement cascade. Because of our long standing interest in SpA (27–30) and the expanding body of evidence bearing on its B cell superantigenic properties (1–15), we used SpA or Mod SpA as a model B cell superantigen. Hyperiodination abrogates the IgG Fc binding activity of SpA (6) and thus allows us to focus solely on the Fab (alternative) binding site that endows SpA with its B cell superantigenic properties. This is a useful step when considering the impact of SpA on mixtures of Igs containing IgG molecules. It enables the investigator to determine whether the observed properties of SpA are due to its alternative or classical Ig binding site. Earlier studies indicated that SpA activates complement when added to serum (21, 22). However, this finding was observed before the Fab binding site on SpA was defined. Accordingly, this finding was attributed to an interaction between the Fc region of serum IgG molecules and what we now appreciate to be the classical Ig binding site on SpA. We hypothesized that the interaction between the alternative Ig binding site on SpA and Fabs of reactive Igs might also cause complement activation.

Using a hemolytic complement assay, we observed that both SpA and Mod SpA caused complement consumption when added to normal human serum (Table III). Complement consumption was due to activation of complement, since the addition of either SpA or Mod SpA to normal human serum led to generation of the complement activation product, C3a (Fig. 1). These results clearly indicate that although SpA activates complement, Mod SpA retains its ability to activate the complement cascade. Thus, the interaction of the Fab binding site of SpA with its reactive Igs in the absence of an intact IgG Fc binding site can lead to complement activation. The hyperiodination treatment of SpA was not responsible for the ability of Mod SpA to activate complement, since the addition of hyperiodinated HSA, a control Ag, to normal human serum did not lead to C3a generation. The data from the C3a ELISA suggest that at low doses of added protein, the IgG Fc binding site of SpA also contributes to the ability of SpA to activate complement in whole serum. Indeed, more C3a generation was observed with unmodified SpA than with Mod SpA. These results may reflect the ability of unmodified SpA to interact with the Fc regions of complement-binding IgGs (independent of their V_H usage) in addition to the Fabs of complement-binding V_H3^+ IgMs. This explanation is consistent with a prior study, which reported that the addition of preformed complexes of SpA and IgG Fc fragments to serum can activate complement via the classical pathway (31).

We determined whether complement activation was 1) dependent on an interaction between the Fab binding site on SpA and V_H3^+ Igs and 2) proceeded via the classical complement pathway by assessing the ability of IgM/SpA to bind C1q. In an ELISA, we used monoclonal IgM proteins and thus were able to use unmodified SpA, since it does not bind to the Fc region of IgM. We observed that polyclonal IgM and three of six V_H3^+, SpA-reactive IgM proteins from our panel (Pom, HUAB 14–3, and Vincent) bound C1q following their interaction with SpA (Fig. 3). We considered the possibility that the affinity with which these IgM proteins bind SpA might have influenced our ability to detect their subsequent binding to C1q. If the IgM proteins bound weakly to SpA, it was possible that the biotinylated SpA (our readout) was

dissociated from the C1q-bound IgM proteins. This would have prevented us from detecting the binding of IgM proteins to C1q following their interaction with SpA. Therefore, to circumvent this potential problem, we developed the wells in our C1q binding ELISA with peroxidase-conjugated goat $F(ab')_2$ anti-IgM Fc Ab (Fig. 4). With this modified assay, two of the three C1q non-binding IgM proteins (HUAB 2–3 and Riv) from Figure 3 were now able to bind C1q following their interaction with SpA. Thus, the affinity with which these IgM proteins bind SpA could have contributed to our ability to detect their subsequent binding to C1q.

However, the data available suggest that the affinity with which these IgM proteins bind SpA does not strongly correlate with their ability to bind C1q following an interaction with SpA. For example, in previous studies, polyclonal IgM (strong C1q binder) and Riv (weak C1q binder) were observed to have almost identical association constants for SpA binding (K_a = 2.24 × 10^6 and 2.23 × 10^6 M^{-1}, respectively) (14). Furthermore, HUAB 14–3 (strong C1q binder) and HUAB 2–3 (weak C1q binder) were found to have similar K_a values for SpA binding (3.2 × 10^7 and 2.1 × 10^7 M^{-1}, respectively) (31). Data on binding kinetics are not available for the other monoclonal IgM proteins in our panel due to the limited supply of these IgM paraproteins collected from Waldenstrom's patients. Thus, there are probably other factors involved in the binding of IgM proteins to C1q following their interaction with SpA, as discussed below. Nevertheless, taken together, the results shown in Figures 3 and 4 indicate that binding of the first component of complement and activation of the complement cascade represent a superantigenic property of SpA.

Of note, one SpA-reactive, V_H3^+ monoclonal IgM protein (Lay) inexplicably did not bind C1q. A possible explanation is that Lay lacks some of the residues in its Fc region necessary for C1q binding. The C1q binding site on IgM has been mapped to residues 340 to 440 in the Cμ3 domain of the Fc fragment (32). However, no sequence data are currently available for the Fc region of the monoclonal IgM, Lay, a paraprotein from a patient with Waldenstrom's macroglobulinemia.

Another possible explanation for the inability of Lay to bind C1q is that this IgM protein, after interacting with SpA, may not be able to assume a conformation necessary for binding of C1q. This would be in accordance with the finding that the conformational change that occurs in an IgM molecule following binding to an Ag is a prerequisite for C1q binding to the Ag/antibody complex (33). This conformational change may be influenced by residues in the V_H region. It has recently been reported that two chimeric IgG molecules, with similar binding affinities for the same peptide, but with different V_H region sequences, have markedly different C1q binding abilities (34, 35). Among our monoclonal IgM panel, Pom (an SpA-reactive, C1q binder) and Lay differ by one amino acid residue in FR1, one residue in CDR1, five residues in FR3 (four within the proposed SpA binding site), and one residue in FR4. It is therefore possible that one or more of these amino acid differences account for the inability of Lay to form the correct, stable conformation for C1q binding.

It is also possible that residues in the light chain may influence the heavy chain conformation needed for C1q binding. Although SpA does not bind directly to the variable light chain (V_L) region, the V_L used by the Ig molecule has been shown to affect the affinity of SpA binding (14). Among our panel of monoclonal IgM proteins, the products of several V_L gene families are used. However, only Lay uses the V_L gene family, Vκ1. Thus, it is possible that residues in the Vκ1 light chain as well as residues in the V_H3 heavy chain of Lay prevent the conformational change required for C1q binding.

Notwithstanding, we have demonstrated that incubation of SpA with most V_H3^+, SpA-reactive IgM proteins leads to C1q binding. These data, taken together with the results of our experiments on normal human serum treated with Mod SpA, strongly suggest that the interaction of the Fab binding site on SpA and V_H3^+ Igs causes complement activation via the classical pathway.

To formally determine whether the binding of the SpA-treated IgM proteins to C1q can lead to activation of the complement cascade, the interaction between Mod SpA and serum (reconstituted with our V_H3^+ IgM proteins) from a hypogammaglobulinemic patient was analyzed in a CH_{50} assay. Complement consumption was observed only when Mod SpA was added to serum that had been reconstituted with a V_H3^+, SpA-reactive monoclonal IgM protein, Pom (Fig. 5). Since the addition of Mod SpA alone to the serum did not cause complement consumption, we eliminated the possibility that direct activation of the alternative complement pathway contributed to the findings observed with the Mod SpA-treated, normal serum.

Complement consumption was associated with complement activation (Fig. 6). The addition of Mod SpA to Pom-reconstituted, hypogammaglobulinemic serum caused the generation of at least 3 times the amount of C3a that was generated by Pom-reconstituted serum incubated without Mod SpA. In contrast, the addition of Mod SpA to Berry (the SpA-nonreactive IgM protein)-reconstituted serum generated an amount of C3a equivalent to that detected in Berry-reconstituted serum incubated alone.

Taken together with the complement consumption, C3a generation, and C1q binding data, the above results provide formal proof that the interaction of V_H3^+ Igs with the Fab binding site on SpA leads to binding of C1q and activation of the classical complement cascade. Complement activation by protein Fv and HIV gp120, two newly defined B cell superantigens (16, 17), has recently been described (36, 37). However, the results reported herein provide direct evidence for the first time that the interaction of a model B cell superantigen, Mod SpA, with its reactive (V_H3^+) Igs leads to activation of the classical complement cascade.

Complement activation may contribute to the B cell activation induced by a B cell superantigen (6). Complement activation can cause enhanced humoral immune responses by several mechanisms that involve membrane IgM and the CR2/CD19 complex on the B cell surface (38–40). One mechanism involves the ability of a polyvalent Ag/antibody/complement complex (formed in the fluid phase) to bind to the B cell surface via both membrane IgM and CR2/CD19 (39, 40). Alternatively, C1q can bind to membrane IgM that has been cross-linked by a polyvalent Ag and activate complement on the B cell surface. The complement degradation products C3dg and C3d can then simultaneously bind to membrane CR2/CD19 molecules (38). Cross-linking of membrane IgM and CR2/CD19 by either mechanism could then lead to activation of the B cell. It is possible that the interaction of a B cell superantigen, such as Mod SpA, with membrane IgM leads to complement activation, as does its interaction with secreted Igs. Such complement activation by either of these mechanisms could, therefore, enhance the B cell activation induced by a B cell superantigen (6).

The interaction of a B cell superantigen with the Fabs of reactive Igs in vivo could lead to the formation of complement-activating immune complexes, which may have profound clinical significance. For example, patients with SpA^+, *Staphylococcus aureus*-induced endocarditis (41) and patients treated with autologous plasma perfused through an SpA-immunoabsorbent column (42) develop immune complex-mediated glomerulonephritis and vasculitis, respectively. These manifestations may be the result of an

interaction between SpA and V_H3^+ Igs with the formation of immune complexes and resultant complement activation. This hypothesis is supported by the observation that C3a is generated in the serum of patients who develop complications following treatment with autologous plasma perfused through an SpA-immunoabsorbent column (43). Moreover, some cases of glomerulonephritis, arthritis, and thrombocytopenia in HIV-infected patients (44–46) may be caused by deposition of complexes containing HIV gp120 and V_H3^+ Igs. Unlike a conventional Ag, a B cell superantigen can react with a large fraction of serum Igs. This interaction can lead to activation of the classical complement cascade and, thus, has the potential to elicit prominent tissue inflammation in a host. Further studies will be required to correlate these in vivo manifestations with the B cell superantigens proposed to be involved.

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