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Functional and Serological Analysis of Type II Immunoglobulin G-Binding Proteins Expressed by Pathogenic Group A Streptococci

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Bacterial immunoglobulin-binding proteins expressed on the surface of group A streptococci represent a heterogeneous family of functionally related proteins. In this report, we describe efficient methods for extracting immunoglobulin-binding proteins and classifying them functionally and antigenically. A common characteristic of immunoglobulin-binding proteins expressed by group A streptococci appears to be the absence of internal methionine residues in the binding protein. This has enabled development of a rapid, efficient, cyanogen bromide-based extraction procedure for solubilizing these molecules from intact bacteria. Studies carried out with a series of monospecific polyclonal antibodies prepared in chickens have identified two major antigenic classes of immunoglobulin-binding proteins. The methods described in this report facilitate a rapid functional and serological screening of immunoglobulin-binding proteins that should now enable detailed epidemiological studies of the importance of these molecules in group A streptococcal infections and their relationship to other surface proteins, in particular, the antiphagocytic M protein.

The ability to establish infections in humans requires a variety of factors produced by bacteria that enable them to colonize the host successfully, invade beyond mucosal or skin surfaces, and elude the host immune defenses. Each step of this process may be influenced by a different combination of bacterial factors, and accumulating evidence suggests that many of these molecules can be coordinately regulated as part of a virulence locus (20). The presence of immunoglobulin-binding proteins on the surfaces of a variety of different human pathogens recently led to the suggestion that these molecules provide pathogens with the potential to evade or elude the host defense mechanisms (1, 5, 8, 35). Studies in our laboratory have demonstrated the existence of five functionally distinct forms of immunoglobulin G (IgG)-binding proteins associated with group A streptococcal isolates (27). Two group A streptococcal IgG-binding proteins have been cloned and sequenced and found to display significant sequence homology to antiphagocytic M proteins (13, 16). In studies of a virulence locus in group A streptococci, the M protein, IgG-binding proteins, C5a peptidase, and, in some strains, IgA binding proteins may be constituents of a coordinately regulated virulence locus; evidence for a transacting regulatory protein has also been obtained (3, 10, 12, 24, 31). To date, the importance of the expression of different functional forms of immunoglobulin-binding proteins for the virulence of group A streptococci has not been established. However, a recent study by Bessen and Fischetti, who measured the ability of intact organisms to bind to human IgG in a nonimmune fashion, did suggest an association between the expression of these functional molecules and the ability of group A organisms to colonize the skin (2). These studies did not distinguish among the different functional forms of immunoglobulin-binding protein.

The type II IgG-binding proteins associated with group A streptococci represent the most diverse group of IgG-binding molecules within a single family of bacteria. These molecules have been classified according to their nonimmune functional reactivities with human IgG subclasses and other species of IgG (Table 1). In our laboratory we have identified extensive functional diversity among immunoglobulin-binding proteins expressed by different group A isolates (27, 37, 38), and we also recently demonstrated the existence of at least two antigenically distinct classes of type IIa IgG-binding proteins (23). These findings suggest that within the family of type II IgG-binding proteins associated with group A streptococci, there may be as much diversity as that seen among the binding proteins of *Staphylococcus aureus*, protein A, and the protein G molecules of human group C and G streptococcal isolates (6, 7). This heterogeneity makes it difficult to relate the potential of expression of immunoglobulin-binding activity by group A strains to any potential role in pathogenesis. The purpose of this study was (i) to develop rapid, reproducible methods for extracting IgG-binding proteins from fresh clinical isolates, (ii) to identify the functional activities associated with individual proteins bands, and (iii) to classify these binding proteins antigenically. A detailed classification of IgG-binding proteins expressed by group A streptococci is a necessary requirement before any meaningful analysis of the relationship of IgG-binding proteins to disease-causing phenotype can be made.

MATERIALS AND METHODS

Bacteria. Group A streptococcal isolates were grown overnight at 37°C as stationary cultures in Todd-Hewitt broth. Approximately 2 g (wet weight) of bacteria per liter was recovered. Strain 64/14 was a group A isolate passaged sequentially in mice on 14 occasions (28). Group A strain

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TABLE 1. IgG-binding profiles of different type II immunoglobulin-binding proteins expressed by group A streptococci[a]

Strain	Functional designation	Reactivity with:						
		Human immunoglobulins				Rabbit IgG	Pig IgG	Horse IgG
		Prototype IgG1	IgG2	IgG3	IgG4			
A928	IIo	+	+	+	+	+	+	+
A207	II'o	+	+	+	+	+	−	−
64/14	IIa	+	+	−	+	+	+	+
	IIb	−	−	+	−	−	−	−
A992	IIc	+	−	−	+	+	+	+

[a] Adapted from the previously presented data (26) based on the reactivities of heat extracts.

64/23bp was strain 64 that had been sequentially passaged through human blood on 23 occasions. Clinical group A isolates were obtained from the Ohio Department of Health, the Centers for Disease Control, the University of Florida, the University of Minnesota, the Lancefield Collection, and the University of Lund. All isolates were pure cultures of group A streptococci.

Immunoglobulin. Human IgG was a gift from the Sandoz Corp., East Hanover, N.J. Human IgG myeloma proteins of each subclass were obtained from the World Health Organization/International Union of Immunologic Societies Immunoglobulin committee.

Labeling of proteins. Proteins were radiolabeled with ^{125}I (Amersham Corp., Chicago, Ill.) by the lactoperoxidase method with Enzymobeads (Bio-Rad, Richmond, Calif.). A PD-10 desalting column (Pharmacia Fine Chemicals, Piscataway, N.J.) was used to separate labeled proteins from free iodine. The specific activities of all radiolabeled proteins were approximately 0.3 mCi/mg.

Cyanogen bromide extraction of type II IgG-binding proteins. Group A streptococcal isolates were grown overnight at 37°C in Todd-Hewitt broth and treated with CNBr as described previously (22). Briefly, approximately 2 g (wet weight) of washed bacteria was resuspended to a final volume of 5 ml in phosphate-buffered saline (PBS). An equal volume of a CNBr solution (30 mg/ml in 0.2 M HCl) was then added to the bacterial suspension to yield a final CNBr concentration of 15 mg/ml; the pH of the reaction mixture was ~2.0. The mixture was rotated for 8 to 15 h at the ambient temperature and then centrifuged at 10,000 × *g* for 15 min. The resulting supernatant was dialyzed against four or five changes of 0.1 M HCl. The CNBr-free extracts were neutralized by adding 1.5 M Tris (pH 8.8) and filtered through a 0.2-μm-pore-size filter. Previous studies in which purified recombinant protein G (22) or recombinant type IIa proteins (23) were treated with CNBr under these conditions showed selective cleavage of only methionine residues.

Generation of anti-type II antibodies in chickens. In this study, four monospecific polyclonal chicken antibodies to different type II IgG-binding proteins were used.

The anti-type IIa antibody (αIIa) against the wild-type, high-molecular-weight, type IIa protein extracted from strain 64/14 by heat treatment was prepared as described previously (38). The anti-type IIb antibody (αIIb) against the type IIb binding protein extracted by heat treatment from strain 64/14 was prepared as described previously (38). The αpLOH antibody to an affinity-purified form of the recombinant low-molecular-weight type IIa IgG-binding protein was prepared as described previously (23). This protein is encoded by plasmid pLOH and is expressed under the control of its own promoter in *Escherichia coli* (23). The anti-type IIo antibody (αIIo) was prepared by immunization with a band (M_r, ~47,000) present in the CNBr extract of strain A928 that bound all four human IgG subclasses (i.e., type IIo). The CNBr extract of A928 was separated on sodium dodecyl sulfate (SDS)–10% polyacrylamide gels under reducing conditions and transferred to nitrocellulose by Western immunoblotting. The functionally active type IIo IgG-binding protein was identified by probing a parallel lane of separated proteins with an appropriately labeled human IgG subclass. The active band was cut from the blot and solubilized by treatment with dimethyl sulfoxide followed by selective precipitation with sodium bicarbonate (pH 9.3). The precipitate was resuspended in PBS (pH 7.3), mixed with an equal volume of adjuvant, and used as the immunogen in the protocol described below.

The immunization protocol was that used previously to prepare antibodies to other bacterial binding proteins (29). Essentially, each chicken was injected intramuscularly or subcutaneously with an immunogen containing approximately 50 μg of affinity-purified type II protein emulsified in complete Freund's adjuvant. Three weeks later, each chicken was injected with approximately 50 μg of the same immunogen emulsified in incomplete Freund's adjuvant. Eggs were collected from the chickens and immunoglobulins were extracted from egg yolks as described previously (29). The production of antibody was monitored by the ability of yolk extracts to inhibit binding of ^{125}I-labeled affinity-purified type II protein to immobilized human IgG as described previously (29).

Affinity purification of antibodies. For studies in which affinity-purified antibodies were prepared to be radiolabeled and used as probes in Western blot analysis, the chicken antibody was passed over a column of immobilized type II binding protein covalently coupled to Affi-Prep 10 (Bio-Rad) as described previously (23). For preparation of the antibody specific to recombinant pLOH (αpLOH), the column was covalently coupled with a purified recombinant type IIa binding protein isolated as described previously (23). For preparation of the affinity-purified antibody to the IIo protein (αIIo), a CNBr extract of strain A928 was prepared as described above. After neutralization and extensive dialysis, this extract was coupled to Affi-Prep 10. Affinity purification was carried out by applying the respective chicken antibodies to the corresponding immobilized type II binding protein column in PBS (pH 7.3). Unbound proteins were removed by washing with this buffer, and bound antibody molecules were eluted selectively with 0.1 M glycine-HCl (pH 2). The recovered proteins were neutralized, dialyzed into PBS, and radioiodinated for use as specific probes.

Polyacrylamide gel electrophoresis and Western blotting techniques. Protein samples were denatured by boiling for 5 min in 0.5 M Tris-HCl (pH 6.8) containing 2% (wt/vol) SDS, 5% (vol/vol) β-mercaptoethanol, 10% (vol/vol) glycerol, and 0.01% (wt/vol) bromphenol blue. Denatured proteins were electrophoresed on 10% polyacrylamide slab gels at 50 V for 16 h by the method of Laemmli (18). Prestained molecular weight standards (Bio-Rad) containing phosphorylase *b* (110,000), bovine serum albumin (84,000), ovalbumin (47,000), carbonic anhydrase (33,000), soybean trypsin in-

hibitor (24,000), and lysozyme (16,000) were included in each SDS-polyacrylamide gel assay.

The proteins were electrophoretically transferred to nitrocellulose (Bio-Rad) by a modification of the method of Towbin et al. (36). Briefly, SDS slab gels were presoaked for 30 min in 25 mM Tris–192 mM glycine–20% methanol (pH 8.3), assembled into a high-field-intensity transblot system (Bio-Rad), and electrophoresed in that buffer at 70 V for 3 h. The nitrocellulose blots were washed four times with 250 ml of 50 mM Veronal buffer (pH 7.4; VBS) containing 0.15 M NaCl, 0.25% gelatin, and 0.25% Tween 20 (VBS-gel-Tween) to saturate remaining protein binding sites on the nitrocellulose. Each wash was carried out for 15 min at ambient temperature with agitation. The nitrocellulose membranes were incubated for 3 h at ambient temperature in 25 ml of VBS-gel-Tween containing 3×10^5 cpm of the appropriate ^{125}I-labeled probe per ml. The nitrocellulose membranes were then washed four times (for 15 min each time) with 250 ml of VBS containing 0.01 M EDTA (pH 7.2), 1 M NaCl, 0.25% gelatin, and 0.25% Tween 20 on a rocking platform at the ambient temperature. The membranes were dried and autoradiographed with Kodak XAR-5 film and Kodak X-Omatic intensifying screens at −70°C for 1 to 3 days.

RESULTS

Comparison of extraction techniques. Previous studies from our laboratory demonstrated that heat treatment of group A strains at neutral pH leads to solubilization of the most homogeneous population of bacterial IgG-binding proteins from group A streptococci (37, 38). Recently, we found that not all bacterial immunoglobulin-binding proteins expressed by mouse-passaged group A strain 64/14 were represented in neutral heat extracts (23). Studies of protein G-positive human group C and G streptococci had demonstrated that CNBr treatment was an efficient method for solubilizing protein G (22). This finding, coupled with analysis of the predicted distribution of methionine residues in the two type II IgG-binding protein genes thus far cloned and sequenced (13, 16), suggested that the distribution of methionine residues within IgG-binding proteins might be a conserved feature of streptococcal IgG-binding proteins and that CNBr treatment may represent an efficient approach to solubilization of type II proteins. When group A strain 64/14 was treated with CNBr, all forms of IgG-binding protein known to be associated with this strain were recovered (23).

Our studies documented the existence of five functionally distinct forms of type II IgG-binding proteins associated with group A streptococcal isolates (27). These proteins were classified (Table 1) solely on the basis of their profiles of functional binding with subclasses of human IgG and other species of IgG. In the initial studies, we tested whether extraction of intact bacteria with CNBr expressing each functional form of type II IgG-binding protein resulted in efficient solubilization of these proteins. Selected strains were treated with CNBr as described in Materials and Methods. The extracts were separated on SDS–10% polyacrylamide gels and stained with Coomassie blue to identify the number of proteins and polypeptides solubilized. CNBr treatment of these group A strains resulted in the solubilization of a limited number of proteins or polypeptides (Fig. 1). As expected, a large number of low-molecular-weight peptides migrated with the dye front. The CNBr patterns for each strain were quite reproducible from experiment to experiment.

When the separated CNBr-extracted fragments were Western blotted and probed with each human IgG subclass, differences in subclass reactivity profiles could be identified (Fig. 2). Immunoglobulin-binding activity was associated with the majority of CNBr-extracted proteins or polypeptides with M_rs of >25,000. The functional reactivity pattern for proteins extracted from the representative strains (Table 2) was similar to that previously defined for the same strains on the basis of heat-extracted proteins (Table 1). A comparison of heat extracts and CNBr extracts of the same strain indicated that the M_rs of the CNBr fragments were slightly lower (usually <5,000) than those observed for the corresponding functional activities in heat extracts. In all cases the quantity of functional activity solubilized by heat treatment was significantly lower than that recovered in the CNBr extracts of the same isolate, and in no instance was any activity present in heat extracts that was not also present in the CNBr extract of the same isolate (data not shown). However, in agreement with previous results (23), certain IgG-binding proteins not extractable by heat treatment could be solubilized by treatment with CNBr. Taken together, the results of these studies suggest that the distribution of methionine residues in type II IgG-binding proteins is a conserved feature and that CNBr extraction is an efficient and reliable method for solubilizing type II IgG-binding proteins. It is possible that any type II binding protein with a methionine residue within or near the immunoglobulin-binding region could be destroyed by CNBr treatment; however, in our studies of over 100 group A isolates we have not found any strain that can bind immunoglobulin to its surface that does not contain a functional binding protein in the corresponding CNBr extract.

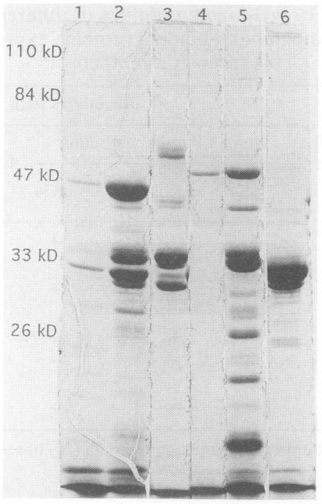

FIG. 1. Identification of the major proteins and polypeptides solubilized by CNBr treatment of representative group A streptococci. Group A strains expressing the five functionally distinct forms of type II IgG-binding proteins previously reported in heat extracts (27) were extracted with CNBr as described in Materials and Methods. The extracts were separated on SDS–10% polyacrylamide gels under reducing conditions, and the gels were stained with Coomassie blue. Lanes: 1, 64/14, a mouse-passaged derivative of strain 64; 2, 64/23bp, a human blood-passaged derivative of strain 64; 3, strain A207; 4, strain 11434; 5, strain A928A1; 6, strain A992.

Antigenic analysis of immunoglobulin-binding proteins. Specific antibodies to bacterial immunoglobulin-binding proteins can be generated (6, 7, 29). It is optimal to generate these serological reagents in chickens, whose nonimmune

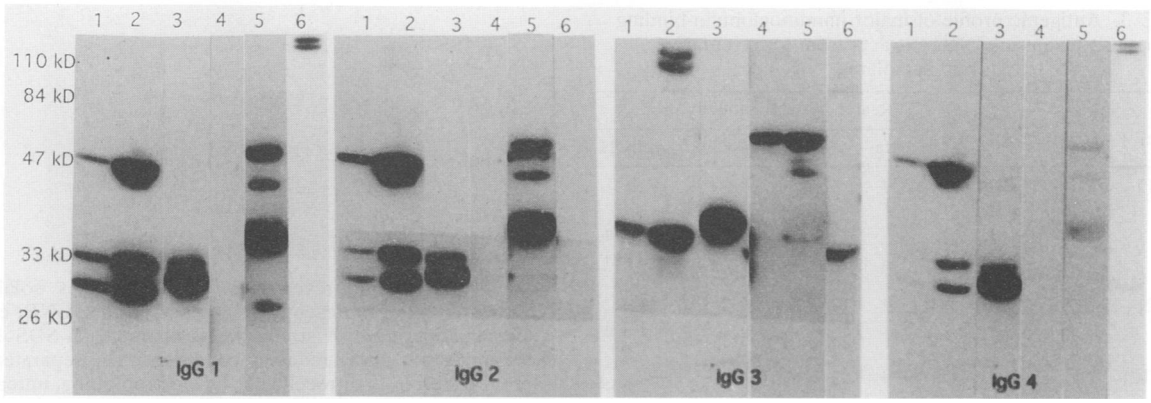

FIG. 2. Functional binding profiles of proteins in CNBr extracts of group A streptococci. CNBr extracts of the group A strains shown in Fig. 1 were run on four parallel SDS–10% polyacrylamide gels under reducing conditions. The separated proteins were transferred to a nitrocellulose membrane by electroblotting. Unreacted sites on the membranes were blocked, and the membranes were probed with the indicated radioiodinated human immunoglobulin subclasses. The probed membrane was washed and exposed to X-ray film with an intensifying screen for 18 h at −70°C. Lanes are as in Fig. 1.

immunoglobulins display no reactivity toward any bacterial immunoglobulin-binding protein thus far identified. This property enables chickens to be immunized without concern for the generation of anaphylactic reactions, which can occur when bacterial binding proteins are injected into an animal species whose nonimmune immunoglobulins can react directly with the binding protein (29). Furthermore, the use of chicken immunoglobulin facilitates serological screening with immunoglobulin fractions or antisera without the need to generate $F(ab')_2$ fragments. In our laboratory, we have successfully generated, in chickens, high-titer monospecific antibodies to type I and type III IgG-binding proteins as well as antibodies to a variety of different forms of type II IgG-binding proteins (7, 38).

In a series of preliminary studies, a variety of anti-type II antibody preparations were tested for reactivity with different representative functional forms of type II IgG-binding protein forms present in CNBr extracts. The initial antigenic analysis of the representative strains (Table 3) demonstrates that all of the functional binding proteins were identified by one or the other antibody (αpLOH or αIIo) but not both, with a single exception. The type IIb protein, which binds only IgG3, present in extracts of mouse-passaged strain 64/14, failed to react with either antiserum (Table 3). However, this protein was immunogenic, since a specific antiserum raised to this protein could identify this protein antigenically and detect certain shared epitopes on other type II functional proteins that were cross-reactive with the αIIo

TABLE 2. IgG-binding profile of major immunoglobulin-binding proteins present in CNBr extracts of representative group A isolates

Strain (M type)	Size (kDa)	Quantity[a]	Reactivity[b] with:							Functional designation
			Human immunoglobulins				Rabbit IgG	Horse IgG	Pig IgG	
			IgG1	IgG2	IgG3	IgG4				
64/23 (NT)[c]	47	++++	++++	++++	−	++++	++++	++++	++++	IIa
	38	++++	−	−	++++	−	−	−	−	IIb
	35	++++	++++	++++	−	++	+	+++	+	IIa
	30	++++	++++	++++	−	++	+	+++	+	IIa
A207 (M2)	38	++++	++++	++++	++++	++++	+++	++++	++++	IIo[d]
	35	++++	++++	++++	−	++++	+	++++	++++	IIa
11434 (M12)	50	+++	−	−	+++	−	−	−	−	IIb
928A1 (M55)	50	++++	++++	++++	++++	++++	++++	++++	++++	IIo
	45	+++	+++	+++	+++	+++	+++	+++	+++	IIo
	38	++++	++++	++++	−	++++	++++	++++	++++	IIa
	36	++++	++++	++++	−	++++	++++	++++	++++	IIa
992S (M18)	116	+	++	−	−	++	+	+++	+	IIc
	35	++++	−	−	++++	−	−	−	−	IIb

[a] Based on the intensity of Coomassie blue-stained bands on polyacrylamide gels. Pluses represent visual estimates of the intensities of the stained protein bands.

[b] The reactivity data are based on the intensities of bands on autoradiographs: ++++, strong reactivity visible within 18 h of autoradiography; +, requires extended exposure (48 to 72 h) to detect a reactive band; −, no reactivity.

[c] NT, not typable.

[d] Heat extracts of strain A207 failed to bind horse or pig IgG and were previously designated type II'o (Table 1).

TABLE 3. Antigenic profile of major immunoglobulin-binding proteins present in CNBr extracts from representative group A isolates

Strain (M type)	Size (kDa)	Functional designation	Reactivity with antibody probe			
			αIIa	αIIb	αIIo	αpLOH
64/14 (NT)	47	IIa	++++	++++	++++	−
	38	IIb	−	++++	−	−
	35	IIa	−	−	−	++++
	30	IIa	−	−	−	++++
A207 (M2)	38	IIo	−	+++	−	+++
	35	IIa	−	−	−	++++
11434 (M12)	50	IIb	+++	+++	+++	−
928A1 (M55)	50	IIo	++++	++++	++++	−
	45	IIo	+++	+++	+++	−
	38	IIa	++++	−	++++	−
	36	IIa	++++	−	++++	−
992S (M18)	116	IIc	+/−	+/−	+/−	−
	35	IIb	+++	+++	+++	−

[a] The reactivity data are based on intensities of bands on autoradiographs: ++++, strong reactivity visible within 18 h of autoradiography; +/−, requires extended exposure (48 to 72 h) to detect a reactive band; −, no reactivity. αIIa, chicken antibody to wild-type high-molecular-weight type IIa binding protein from strain 64/14 (33); αIIb, chicken antibody to wild-type IgG3 only binding type IIb binding protein from strain 64/14 (33); αIIo, chicken antibody to the purified type IIo protein from strain A928 (23); αpLOH, chicken antibody to the recombinant protein expressed by plasmid pLOH-1 (23).

antibodies (Table 3). Comparison of the antigenic reactivities of distinct type II binding proteins indicated that the αIIo antibody recognized an overlapping population of proteins recognized by either the IIa antibody or the αIIb antibody. Since the αIIo reagent had the highest titer of these antibodies, it was used in all further studies to identify members of one antigenic family of IgG-binding molecules. The results in Table 3 also indicated that the αpLOH reagent and the αIIo antibody recognize nonoverlapping groups of type II IgG-binding molecules. Consequently, in the subsequent experiments, both antibody probes were included. For the serological analysis of IgG-binding proteins expressed by clinical group A isolates, these antibodies were first affinity purified. An aliquot of either the αpLOH or the αIIo antiserum was applied to a column of the appropriate form of immobilized antigen and eluted selectively at a low pH. The eluted proteins were neutralized, dialyzed against PBS, and radioiodinated. The labeled probes were then used to compare CNBr-extracted proteins from various clinical isolates. The results of a series of representative strains are shown in Fig. 3. In each Western blot assay, a CNBr extract of strain 64/14 was included as a positive control, since this strain is known to express representative binding proteins from each antigenic class (23).

Analysis of IgG-binding proteins extracted from clinical group A isolates. The CNBr extraction procedure was applied to 33 clinical isolates obtained from the Ohio Department of Health and 17 additional laboratory isolates. The clinical isolates had been passaged in the laboratory fewer than five times before they were analyzed. The results for a series of representative isolates are shown in Fig. 4 and 5. Figure 4 demonstrates the limited number of polypeptides that are solubilized by CNBr treatment, and Fig. 5 documents the human IgG subclass binding reactivities of the

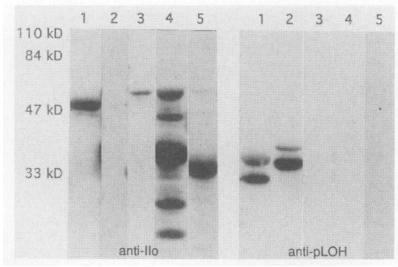

FIG. 3. Antigenic reactivities of polypeptides solubilized in CNBr extracts of representative group A strains. CNBr extracts of representative group A strains were separated on SDS–10% polyacrylamide gels under reducing conditions. The separated proteins were transferred to nitrocellulose by electroblotting, unreacted sites on the membranes were blocked, and the membranes were probed with either an affinity-purified radioiodinated αIIo antibody or an αpLOH antibody probe as indicated. The probed membranes were washed and exposed to X-ray film with intensifying screens for 24 h at −70°C. Lanes: 1, strain 64/14; 2, strain A207; 3, strain 11434; 4, strain A928A1; 5, strain A992.

extracted proteins. The functional binding properties of each isolate could be accounted for on the basis of the previous classification of binding profiles with human IgG subclasses (Table 1). The distribution of functional type II proteins in extracts from these strains is summarized in Table 4.

Each extract was also analyzed serologically for reactivity with the αpLOH and αIIo affinity-purified antibody probes. Of 50 clinical isolates tested, 46 expressed immunoglobulin-binding proteins; of these, 45 isolates were reactive with either the αpLOH or αIIo antibody probe. Only one strain expressed an IgG-binding protein that was not reactive, and two strains expressed antigenically related molecules without IgG-binding properties (Table 4).

No absolute correlation between the reactivity with a given antibody and the functional activities of the protein was observed, except that strains expressing type IIa proteins were all associated with molecules that were reactive with the αpLOH probe and those expressing type IIb pro-

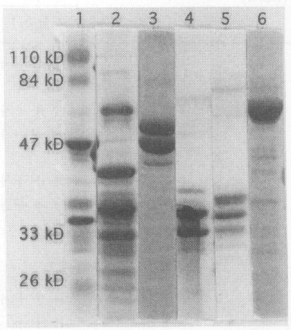

FIG. 4. Profile of polypeptides solubilized by CNBr extraction of selected fresh clinical group A isolates. Group A isolates were selected at random from isolates collected by the Ohio Department of Health. Each strain was extracted with CNBr as described in Materials and Methods. These extracts were separated on SDS–10% polyacrylamide gels under reducing conditions, and the gels were stained with Coomassie blue. Lanes: 1, CNBr extract of strain 64/14 mixed with prestained molecular weight markers (this extract is included as a control on all gels); 2, blood isolate 137051; 3, blood isolate 231021; 4, wound isolate 224042; 5, blood isolate 230051; 6, throat isolate 171032.

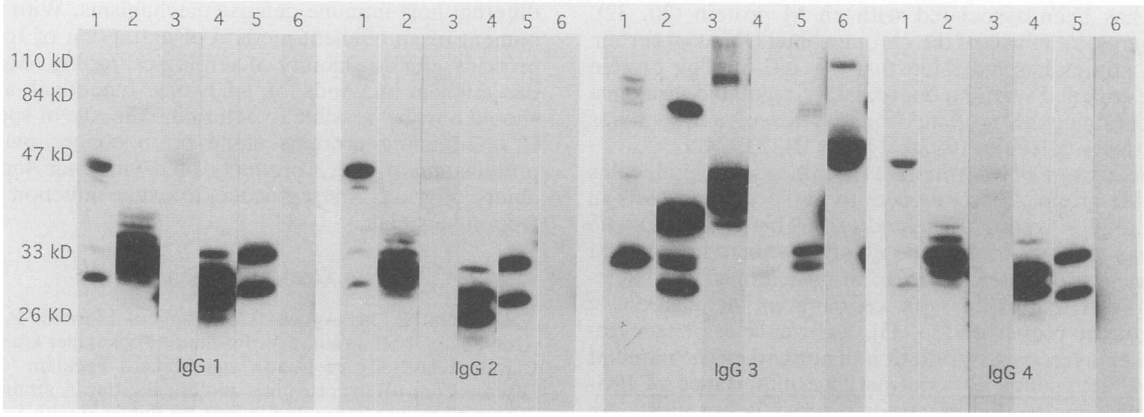

FIG. 5. Functional binding profile of polypeptides present in CNBr extracts of selected fresh clinical group A isolates. CNBr extracts of the selected bacteria described in the legend to Fig. 4 were run on four parallel gels. Separated proteins were transferred to nitrocellulose by electroblotting and probed with each ^{125}I-labeled human IgG subclass. The probed membranes were washed and exposed to X-ray film with intensifying screens for 18 h at −70°C. Lanes are as in Fig. 4.

teins, with the exception of the type IIb protein from strain 64/14, were all associated with molecules reactive with the αIIo probe (Table 4). Heterogeneity in immunoglobulin-binding activity for immunoglobulin-binding proteins belonging to either antigenic class was observed. Evidence for functional variation among type IIb IgG-binding proteins on the basis of their ability to be inhibited from binding to human IgG3 by streptococcal protein G was also noted (data not shown).

The distribution of immunoglobulin-binding proteins that were reactive with αpLOH and αIIo observed for the Ohio Department of Health strains differed from the distributions of other strains obtained from the Lancefield Collection (Rockefeller University, New York, N.Y.), the University of Florida (College of Medicine, Gainesville), the University of Lund (Lund, Sweden), the Centers for Disease Control (Atlanta, Ga.), and the University of Minnesota (Minneapolis). Analysis of 50 group A strains from various geographical locations other than Ohio indicated a lower percentage of strains expressing IgG-binding proteins that reacted with αpLOH. In this group, ~25% reacted with αpLOH and ~75% reacted with αIIo, compared with an approximate 50:50 distribution observed for strains collected by the Ohio Department of Health (data not shown).

Association of type II IgG-binding protein classes and M protein classes. Because of the reported similarities between M proteins and IgG-binding proteins (11, 13, 16), the antigenic classes of immunoglobulin-binding proteins on isolates for which M serotypes were available were analyzed. Strains that expressed M protein serotypes belonging to the antigenic class I defined by Bessen and colleagues (4) expressed immunoglobulin-binding proteins that reacted with the αIIo antibody probe. Similarly, although they were fewer, isolates from M-typable strains corresponding to the class II M protein family expressed immunoglobulin-binding proteins that reacted with the αpLOH antibody probe. Sixteen isolates that were M protein untypable expressed immunoglobulin-binding proteins that reacted with the αpLOH probe, whereas only two of the nontypable strains expressed immunoglobulin-binding proteins that reacted with the αIIo probe. The ability to divide streptococci into two major families is also consistent with results from recent studies of Musser et al. (21), Haanes et al. (14, 15), Bessen and colleagues (3, 4), and Podbielski et al. (25), suggesting the existence of two major lineages of group A streptococci.

TABLE 4. Antigenic and functional analysis of immunoglobulin-binding proteins solubilized by CNBr extraction of 50 group A strains

Antigenic reactivity with:	No. of strains (%) with the indicated reactivity and the following functional designation:					
	IIo	IIa	IIb	IIa + IIb	None	Total
αpLOH	9	9	0	1[a]	1	20 (40)
αIIo	5	0	18	0	1	24 (48)
αpLOH + αIIo	2	0	0	1[b]	0	3 (6)
Neither	0	1	0	0	2	3 (6)
Total	16 (32)	10 (20)	18 (36)	2 (4)	4 (8)	50 (100)

[a] The IIb binding protein from this isolate did not react with either antibody.

[b] This strain (64/14) possesses two antigenically distinct IIa receptors and a type IIb protein that did not react with either antibody (23).

DISCUSSION

The studies presented in this paper demonstrate that CNBr treatment is an effective way of solubilizing immunoglobulin-binding proteins from the surface of group A streptococci. In addition, we have demonstrated that the vast majority, greater than 90%, of isolates expressing IgG-binding activity do so via proteins that can be divided into one of two antigenic classes, based on their reactivities with αpLOH or αIIo antibodies (Table 4). Functional IgG-binding heterogeneity was associated with both antigenic classes. These findings have many parallels to those of studies of M protein expression by group A streptococci. Bessen and colleagues identified two antigenic classes of M proteins (4). Haanes et al. provided evidence for two lineages of group A streptococci from studies of the organization of M protein and linked genes on chromosomal DNA (14, 15), and Podbielski et al., who used polymerase chain reaction approaches, also identified two classes of putative M protein-like genes (25). Although previous immunochemical studies and genetic studies have demonstrated that for many strains the M protein and the IgG-binding protein are distinct molecules (6), there are strains in which an IgG-binding

activity has been associated with an M protein (30, 32). Recent genetic analysis of the chromosomal DNAs of certain group A strains has indicated that the IgG-binding protein could be encoded within a coordinately regulated virulence locus containing M protein, C5a peptidase, and perhaps other surface molecules (9, 10, 14, 15, 24, 31, 34).

The importance of M proteins as antiphagocytic molecules that enable group A streptococci to avoid phagocytosis in human blood is well documented (11). This antiphagocytic effect was further confirmed by studies in which an M protein gene was inserted into an M protein-negative bacterial host and expressed with concomitant acquisition of antiphagocytic properties (26, 33). Resistance to phagocytosis could be overcome by addition of antibodies to unique M protein determinants. By contrast, the importance of IgG-binding proteins to the infectious process is not clear. An association between the expression of IgG-binding proteins and skin isolates has been suggested by Bessen and Fischetti (2). These studies were carried out with laboratory isolates of group A strains in which the percentage of strains expressing IgG-binding proteins was lower than we observed with fresh clinical isolates in this study or that reported by Lindahl and Stenberg in another study of fresh clinical group A isolates (19). It should also be noted that Bessen and Fischetti measured IgG binding to intact bacteria, whereas in this study functional proteins in bacterial extracts were analyzed. It is important in analyzing IgG-binding proteins to use fresh clinical isolates, since IgG-binding proteins can be lost by sequential laboratory passage (27). We have also found that expression of surface proteins can be altered by passage of laboratory isolates through human blood or in mice; for example, compare the levels of expression of IgG-binding proteins in extracts of group A strain 64 after blood or mouse passage (Fig. 1 and 2).

It is clear that the importance of bacterial IgG-binding proteins in the pathogenic process or their usefulness as a marker for epidemiological studies will not emerge until a detailed classification system for these molecules is available. The studies presented in this report indicate that we now have efficient methods for solubilizing these proteins, in high yield, and serological reagents that can classify type II IgG-binding proteins into one of two major antigenic classes. Within each antigenic subgroup, we have evidence for a number of functional variants (Table 3). This pattern of subtle variation within antigenically related molecules is again reminiscent of the M protein; it is possible to delineate M proteins serologically into class I and class II and to classify strains further into serotypes on the basis of the expression of unique epitopes associated with the N-terminal portion on the protein (17). The findings that M protein genes and IgG-binding protein genes display significant sequence homology (13, 16) and may be coordinately regulated as part of a group A streptococcal virulence locus (9, 10) raise additional questions as to the role these molecules play alone or together in the pathogenesis of group A streptococcal infections.

The study of type II IgG-binding proteins associated with group A streptococci is at an early stage by comparison with the extensive detailed structural and antigenic analysis of M proteins (for a review, see reference 11). However, the parallels between these two groups of molecules suggest that they are both members of a larger M protein supergene family, which may also include IgA-binding molecules (3, 12). These molecules may have evolved from a common ancestral gene to provide the organism with complementary mechanisms to influence the interaction of bacteria with different host immune defense mechanisms. With the development of an efficient method of extraction of IgG-binding proteins, the availability of serological typing reagents, and the efficient methods of analyzing functional activity, it should now be possible to determine the role of specific type II IgG-binding proteins alone or in concert with the M protein and/or other products of a virulence locus in the ability of group A streptococci to cause infection or postinfection sequelae.

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