

EVIDENCE FOR TWO DISTINCT CLASSES OF STREPTOCOCCAL M PROTEIN AND THEIR RELATIONSHIP TO RHEUMATIC FEVER

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Group A streptococci are human pathogens that infect primarily at the skin or nasopharyngeal mucosa. A major virulence factor present on the streptococcal surface is M protein, a molecule of which there exists more than 80 distinct serological types. M protein provides the streptococcus with the ability to resist phagocytosis by polymorphonuclear leukocytes, and only antibodies directed to type-specific determinants permit opsonophagocytosis in whole blood (1, 2). Structural studies reveal that M protein is composed of two α -helical polypeptide chains that give rise to a coiled-coil, fibrillar structure extending ~ 60 nm from the cell surface (3, 4). The antigenically variable determinants of type specificity are located in the NH₂-terminal portion of the molecule, distal to the cell wall (2, 5). Some epitopes of the M protein molecule are shared among different M serotypes, and the degree of relatedness increases at sites closer to the COOH terminus (6, 7).

Recent progress towards a group A streptococcal vaccine indicates that mucosal immunization with shared immunodeterminants of M protein leads to reduced pharyngeal colonization in a mouse model (8). To better define those epitopes for potential use in a non-type-specific-based vaccine, we sought to gain a more detailed understanding of the antigenic relatedness of surface-exposed portions of M protein among isolates of the same serotype, and between isolates of distinct serotypes. In this report, we describe an antigenically conserved domain within the surface-exposed portion of M protein of certain serological types, and provide evidence for two distinct classes of M protein molecules based on the presence or absence of this antigenic domain.

Acute rheumatic fever (ARF)¹ is one complication that can follow a nasopharyngeal streptococcal infection, and epidemiological studies point to a strong association between streptococci of certain serological M types and ARF attacks (9). Defining a particular serotype as "rheumatogenic" is not entirely clearcut since pharyngitis generally precedes the onset of ARF by 3–4 wk and thus, there is uncertainty as to whether the streptococcus isolated at the time of an ARF attack is of the same lineage as that which caused pharyngitis. Nevertheless, it is apparent that streptococci

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¹ *Abbreviations used in this paper:* ARF, acute rheumatic fever; IgGBP, IgG binding protein; k-ELISA, kinetic ELISA; MAP, M-associated protein; NT, nontypable; OF, opacity factor.

of certain M types are often associated with ARF outbreaks, whereas other serotypes are rarely seen in association with this disease. The following M types have been associated with one or more major outbreaks of ARF: M1, M3, M5, M6, M11, M12, M14, M17, M18, M19, M24, M27, M29, M30, M32, and M41 (9-12). The second finding of this report is that streptococcal serotypes expressing the conserved antigenic domain of M protein include those associated with outbreaks of ARF, suggesting that the two M protein classes of group A streptococci may differ in their virulence properties.

Correlations can be made between particular M serotypes and other physicochemical properties of group A streptococci. M-associated protein (MAP) was identified in the 1970s as a surface component of M protein-bearing organisms that co-purifies with the type-specific substance (10, 13, 14). Isolates of most M serotypes readily fall into one of two antigenic MAP groups (I and II), based on test antisera reactive with one group and not the other (10, 14). At the time MAP antigens were characterized, the definition of M protein was limited to that of a type-specific substance with antiphagocytic properties, and the existence of immunodeterminants shared among different M types was not known for certain. Therefore, crossreactive material that co-purified with M protein was usually considered to be distinct from the M protein molecule. Serotypes of the MAP I group include most M types associated with ARF plus several serotypes associated with pyoderma, and the serum of ARF patients contains elevated complement-fixing activity to MAP I antigen. MAP II serotypes include both pyoderma and nasopharyngeal serotypes, and typically produce opacity when cultivated in horse serum (the serum opacity reaction is a result of lipoprotein cleavage) (10). Thus, there is a strong correlation between MAP II antigens and opacity factor (OF) production, and between MAP I antigens and M serotypes associated with ARF. However, the biochemical composition of MAP antigens and OF remained largely undefined.

Using a panel of seven antibodies directed to well-defined antigenic sites within the M6 protein molecule, we analyzed the presence of surface-exposed epitopes on 138 streptococcal isolates representing more than 50 different M types. The data revealed a striking correlation between those serotypes possessing an antigenically conserved surface-exposed domain, and those serotypes previously described as having MAP I antigen (10). Included in the group that share a conserved domain with M6 protein are the ARF-associated serotypes. Furthermore, most isolates that lack the conserved domain on their surface give a positive serum opacity reaction, whereas nearly all isolates possessing the conserved domain fail to produce OF. Based on these fundamental differences, we propose that most group A streptococcal serotypes fall into one of two major classes of M protein, which parallel the MAP I and II antigenic types.

Materials and Methods

Bacterial Strains. Streptococci were from The Rockefeller University collection. With few exceptions, organisms used for experiments had been passaged in the laboratory no more than five times after the original isolation. All strains were previously characterized according to group carbohydrate and M type by Dr. R. C. Lancefield and co-workers (1).

Heat-killed Streptococci. Overnight streptococcal cultures grown in Todd-Hewitt broth were centrifuged, washed in saline, heat killed for 30 min at 56°C, and allowed to cool slowly to room temperature. Cultures were centrifuged, pellets were concentrated in PBS containing 1% acidified BSA to an OD₆₅₀ equivalent to 5.0, and they were stored at 4°C.

Antibody Preparation and Titration. Antisera were raised in rabbits to synthetic peptides corresponding to residues 121–145, 140–158, 216–235, and 248–269 of M6 protein from strain D471 (Fig. 1, Table I) (2). Affinity-purified antibody was prepared by absorption of antisera to the corresponding peptide covalently linked to glutardialdehyde glass beads (2, 8), and were stored in 1% BSA at -80°C . mAbs 10A11, 10B6, and 10F5 have been previously described (6, 15).

The concentration of monospecific antipeptide antibody and mAb giving half-maximal immunoreactivity to ColiM6 antigen (the product of the *emm-6.1* gene cloned in *Escherichia coli*) (16) was established by kinetic ELISA (k-ELISA) (17). Microtiter wells were coated with antigen (5 $\mu\text{g}/\text{ml}$), antibody was diluted twofold and incubated for 3 h at 25°C in 20 mM phosphate, pH 7.2/0.5 M NaCl/0.25% Brij, wells were washed, and they were incubated for 2 h with alkaline phosphatase-conjugated secondary antibody. Absorbance (405 nm) was recorded at 4-min intervals at room temperature with intermittent shaking on an ELIDA-5 reader (Physica Inc., New York, NY) programmed to calculate the kinetics of the enzyme reaction in individual wells.

Antibody Absorption Assay. For antibody absorption to whole streptococci, 80 μl of heat-killed organisms were mixed with 20 μl of affinity-purified antibody or mAb diluted in 1% BSA-PBS in preblocked V-bottomed microtiter wells to a final antibody concentration giving half-maximal immunoreactivity to ColiM6 antigen. Plates were sealed, mixed, and rotated end-over-end overnight at 4°C . Microtiter plates were centrifuged, the supernatants were removed and mixed 1:1 with 0.5% Brij/20 mM phosphate/1.0 M NaCl, and were transferred to flat-bottomed microtiter plates that had been precoated with ColiM6 antigen and blocked with PBS-Brij. Immunoreactivity was measured by k-ELISA (described above), and all measurements were performed in triplicate. The percentage of antibody bound to heat-killed streptococci was calculated based on control samples devoid of streptococci during absorption. The proportion of a given antibody that bound to the surface of heat-killed organisms was scored as follows: 0, <20% bound; +/-, 20–29.9%; +, 30–59.9%; ++, 60–84.9%; and + + +, 85–100%.

To measure nonimmune binding of IgG by Ig binding proteins present on the surface of many group A streptococcal isolates (18), heat-killed organisms were incubated with either purified mouse myeloma IgG (Sigma Chemical Co., St. Louis, MO) or rabbit serum IgG (Cappel Laboratories, Malvern, PA) as described above. The amount of unabsorbed antibody was measured by capture k-ELISA using wells coated with anti-mouse IgG (Cappel Laboratories) or anti-rabbit IgG (Pel-Freez Biologicals, Rogers, AR) affinity-purified antibody. Two streptococcal isolates tested bound 15% or more of mouse IgG by a nonimmune mechanism and therefore were excluded from these studies. Streptococci that bound 15% or more of rabbit IgG were scored positive (+) for IgG binding protein (IgGBP). Absorption of anti-M protein mAbs was measured for all streptococcal isolates. Anti-M protein rabbit antibody was tested only for those isolates that bound <15% of rabbit IgG by a nonimmune mechanism.

Serum Opacity Reaction. Heat-inactivated horse serum (Sigma Chemical Co.) was mixed 3:1 with Todd-Hewitt broth and inoculated with a loopful of streptococcal stock cultures (19). Organisms were incubated 18–24 h at 37°C , centrifuged, and the OD_{475} of the supernatant was measured. Cultures giving $\text{OD}_{475} > 0.500$ were considered to be positive (+) for serum opacity reaction; values < 0.200 were considered negative (-); cultures giving values between 0.200 and 0.500 were scored as +/-.

M Protein Extraction and Western Blot Analysis. M protein extracts were prepared from overnight broth cultures of streptococci using bacteriophage lysis as previously described (20). Lysin extracts were subjected to SDS-PAGE, electrotransferred to nitrocellulose, incubated with mAbs or anti-ColiM6 human Ig followed by alkaline phosphatase-conjugated secondary antibodies, and developed (20). Anti-ColiM6 Ig was prepared from human serum that had been affinity purified to ColiM6 linked to glutardialdehyde beads (2).

Results

Antibody Probes. The M6 protein molecule of strain D471 contains three major regions (A, B, and C) of repeated sequence segments (4, 21) (Fig. 1). High frequency,

intragenic recombinational events between reiterated DNA sequences within the structural gene encoding M protein can lead to deletion or duplication of one or more repeat blocks, and recombination between inexact repeats within the A, B, or C repeat regions can give rise to mutations in the amino acid sequence and antigenic structure (21, 22). The NH₂-terminal nonrepeat region and the A repeat block of M6 protein contain determinants of type specificity (2, 7). Surface-exposed immunodeterminants located in the B and C repeat regions of M6 protein are shared among different M serotypes (2, 6).

The antibody probes used for antigenic analysis are directed to antigenic sites within the B and C repeat regions of the M6 protein molecule of strain D471 (Fig. 1, Table I). Anti-peptide rabbit serum was affinity purified to synthetic peptides corresponding in sequence to B repeat residues 121-145 (probe IIIB) and 140-158 (IIB), residues 216-235 which flank the pepsin-susceptible site (PS), and C repeat residues 248-269 (IC) of the D471 M6 molecule. Epitopes of the mAb probes have been previously localized to B repeat residues 134-139 (IB; 10A11), and to C repeat residues 275-289 (IIC and IIIC; 10F5 and 10B6, respectively) (15). Complete and partial repeated segments of six of the seven epitopes found elsewhere in the D471 M6 molecule are indicated.

Surface-exposed Epitopes and Opacity Factor Production. Between one and six streptococcal isolates of most serological types among types M1 through M67, plus several nontypable (NT) isolates, were examined by the antibody absorption assay for the presence of surface-exposed antigenic epitopes shared with M6 strain D471 (Table II). Rabbit antibody probes (IIB, IIIB, PS, IC) were not tested for isolates that bound $\geq 15\%$ of rabbit IgG by a nonimmune mechanism. One or more B repeat probes gave strong immunoreactivity (++ or +++) to all isolates of serotypes M6, M5, M14, M19, and M36 (Table II A). At least one of the three C repeat probes tested reacted strongly with isolates representing approximately half of the serotypes ex-

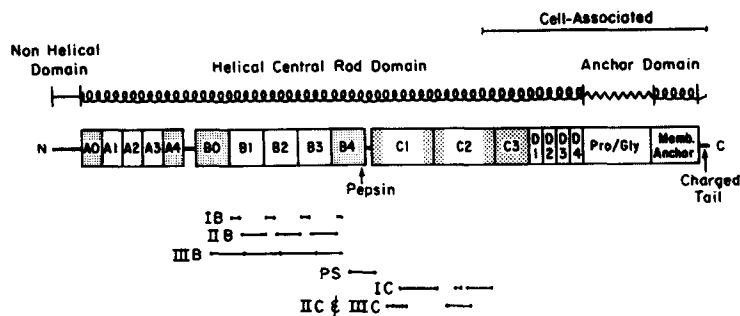


FIGURE 1. Region and domain assignments of the M6 protein of strain D471. Repeat regions A, B, C, and D are composed of tandemly repeating sequence sub-blocks that differ in sequence and length for each region. Region A is composed of 5 direct tandem repeats of 14 amino acids each, region B contains 5 tandem repeats of 25 residues each, and region C contains 2.5 repeats of 42 amino acids. Repeat blocks slightly divergent from the consensus repeat blocks are indicated by shading. The non-surface-exposed, cell-associated region begins at residue 298 (30). Pro/Gly is the proline and glycine-rich region located in the cell wall, and the membrane anchor is composed of 19 amino acids adjacent to a short charged tail. Pepsin designates the position of the pepsin sensitive site between residues 228 and 229. The molecular weight of the D471 M6 protein monomer is calculated to be $\sim 49,000$, and the molecule is 441 amino acids in length. Locations of antibody probes (Table I), and complete and partial repeats are indicated.

TABLE I
Antigenic Sites of Antibody Probes

Probe	Source	Amino acid sequence*	Repeat positions†
IB	mAb 10A11 (134-139)	K I A K E Q	159-164, 184-189, 209-211
II B	Anti-pep (140-158)	E S K E T I G T L K K T L D E T V K D	165-183, 190-208
III B	Anti-pep (121-145)	G T L K K T L D E T V K D K I A K E Q E S K E T I	146-170, 171-195, 196-211
PS	Anti-pep (216-235)	S K Q D I G A L K Q E L A K K D E G N K	280-283, 286-301
IC	Anti-pep (248-269)	L D A S R E A K K Q V E K D L A N L T A E L	239-241, 243-247, 249-252
II C	mAb 10F5 (275-289)	E K Q I S D A S R Q G L R R D §	239-241, 243-247, 249-252
III C	mAb 10B6 (275-289)	E K Q I S D A S R Q G L R R D	239-241, 243-247, 249-252

* Amino acid sequence corresponds to the mature M6 protein from strain D471 (27).

† Repeat positions define the locations within the molecule at which amino acid sequences are repeated in whole or part.

§ Despite the fact that mAbs 10F5 and 10B6 have been mapped to the same general region, they exhibit unique immunoreactivities (15).

TABLE II
Surface-exposed Antigenic Epitopes and Opacity Factor Production

Type	Strain	Site	Date	IgGBP	IB	IIB	IIIB	PS	IC	IIC	IIIC	OF
A. Isolates sharing B and C repeat region epitopes												
M6	D471	?	1971	-	+++	+++	+++	+++	+++	+++	+++	-
	1RP112	NP	1952	-	++	+	++	+++	++	+++	++	-
	1RP178	NP	1955	-	++	+	++	+++	+++	+++	+++	-
	MO1015	NP	1986	-	++	++	+	++	++	+++	++	-
	4RS103	NP	1942	-	++	+	++	+++	+++	+++	+++	-
M5	B788	NP	1982	-	+++	+++	++	++	+	+++	+++	-
	1RP144	NP	1953	-	+++	++	+	++	+	+++	+++	-
	2RP19T	NP	1946	-	+++	+	+	++	+	+++	+++	-
	2RP143	NP	1953	-	++	+	+	++	+	+++	+++	-
M14	D469	?	1971	-	++	++	+	+	+++	+++	+++	-
	4RP106	NP	1951	-	+	+++	+	++	+++	+++	+++	-
	25RS84	NP	1942	-	++	+++	+	++	+++	+++	+++	-
	D773	NP	1974	-	+++	+	+	+	++	+++	+++	-
M19	1GL205	NP	1946	-	++	+	0	+	++	+++	+++	-
	1RP43	NP	1948	-	++	+	0	+	++	+++	+++	-
	1RP97	NP	1951	-	++	+	0	+	++	+++	+++	-
	D709	NP	1973	-	++	+	0	+	++	+++	+++	-
M36	A457	?	1961	+	+++					+++	+++	-
NT	1RP257	NP	1962	-	++	+	0	+	++	+++	+++	-
B. Isolates sharing C repeat region epitopes only												
M1	D710	?	1973	+	0					+++	+++	-
	1GL100	NP	1946	+	0					+++	++	-
	3RP215	NP	1958	+	0					+++	+++	-
M3	D922	?	1975	-	0	0	0	0	++	+++	++	-
	1RP99	NP	1951	+	0					+++	++	-
	2GL215	NP	1946	-	0	0	0	0	+	+++	+	-
	979-88	NP	1988	+	0					+++	++	-
M4	D896	NP	1975	-	0	0	0	0	++	+++	+/-	-
	C240	NP	1942	-	0	0	0	0	+/-	+++	+	-
M8	D784	SK	1974	-	0	0	0	+/-	+	++	0	-
M12	A735	NP	1964	-	0	0	0	0	++	+++	++	-
	2RP196	NP	1956	-	0	0	0	0	+	+++	+	-
	A374	NP	1960	-	0	0	0	0	++	+++	++	-
M17	1GL12	NP	1946	-	0	0	0	0	+	+++	+/-	-
	J17E/165	NP	1932	-	0	0	0	0	+	+++	++	-
	1RSC150	NP	1943	-	0	0	0	0	+	+++	+/-	-
M18	1RP268	NP	1963	+	0					+++	+++	-
	1RP38	NP	1948	+	0					+++	+++	-
	1RP68	NP	1949	+	0					+++	+++	-
	1RP110	NP	1952	+	0					+++	+++	-
	986-88	NP	1988	-	0	0	0	0	+	+++	++	-
M23	1RP62	NP	1949	-	0	0	0	0	++	+++	+++	-
	19RS17	NP	1943	+	0					+++	+++	-
M24	22RS72	NP	1941	-	0	0	0	0	+	0	++	-
	1RP284	NP	1964	-	0	0	0	0	+	0	+++	-
	1RSC165	NP	1942	-	0	0	0	0	+	0	+++	-
M26	11RS100	NP	1942	-	0	0	0	0	++	+++	++	-
M27	A910	NP	1966	-	0	0	0	0	+++	+++	+++	-
M28R	D685	NP	1972	+	0					+++	++	-

continued

TABLE II (continued)

Type	Strain	Site	Date	IgGBP	IB	IIB	IIIB	PS	IC	IIC	IIIC	OF
M29	D470	?	1971	-	0	0	0	0	+	+++	+++	-
	3RP70	NP	1949	-	0	0	0	0	+	+++	+++	-
	4RS68	NP	1941	-	0	0	0	0	+	+++	++	-
M30	1GL120	NP	1946	-	0	0	0	0	+	+++	+++	-
	1RP31	NP	1947	-	0	0	0	0	+	+++	+++	-
	D617	SK	1972	+	0					+++	+++	-
M32	10RS101	NP	1942	+	0					+++	+++	-
	D641	SK	1972	+	0					+++	+++	-
M33	A984	NP	1968	+	0					+++	+++	-
	13RS60	NP	1941	-	0	0	0	0	+++	+++	+++	-
	D323	BL	1969	+	0					+++	+++	-
M37	D466	?	1971	-	0	0	+/-	0	+++	++	0	-
M38	2RSC3	NP	1942	+	0					+++	+++	-
M41	D463	?	1971	-	0	0	0	0	+	0	++	-
	D421	SK	1971	+	0					+++	++	+/-
M43	C506	NP	1943	+	0					+++	+++	-
	D821	SK	1975	+	0					+++	+++	-
M46	A837	SK	1965	+	0					++	0	-
M48	D493	?	1971	+	0					+++	+++	-
M52	A889	?	1966	+	0					+++	+++	-
	D680	NP	1972	+	0					+++	+++	-
M53	D948	SK	1976	-	0	0	0	0	+++	+++	+++	-
M54	D432	?	1971	+	0					+++	++	-
M55	D442	?	1971	+	0					+++	+++	-
M56	D633	SK	1972	+	0					+++	+++	-
	R67	SK	1967	+	0					+++	+++	-
M57	D306	?	1968	-	0	0	0	0	+++	+++	+++	-
M58	D998	?	1976	-	0	0	0	0	+++	+++	+++	-
M59	D997	?	1976	+	0					+++	0	-
NT	D735	SK	1973	-	0	0	0	0	+++	+++	+++	-
	3RP126	NP	1952	-	0	0	0	0	+	+++	++	-
C. Opacity factor producing isolates												
M2	D444	?	1971	+	0					++	0	+
	1RP256	NP	1962	+	0					+/-	0	+
	10RS57	?	1941	+	0					0	0	+
	60RS84	?	1947	+	0					0	0	+
	B344	NP	1950	+	0					0	0	+
	29RP112	NP	1952	+	0					0	0	+
M4	2RP113	SK	1952	-	0	+	+	+/-	+/-	+	0	+
	B967	SK	1958	+	0					+	0	+
	B512	UR	1953	-	0	0	+/-	0	0	0	0	+
	B775	?	1956	-	0	0	0	0	0	0	0	+
M9	D339	?	1970	+	0					0	0	+
	D995	?	1976	+	0					0	0	+
	1RP278	NP	1964	+	0					0	0	+
M11	A658	?	1963	+	0					0	0	+
	D691	NP	1972	-	0	0	0	0	0	+	0	+
	B887	?	1956	-	0	0	0	0	0	+	0	+
	A410	?	1960	-	0	0	0	0	0	+	0	+
	B518	?	1953	+	0					+	0	+

continued

TABLE II (continued)

Type	Strain	Site	Date	IgGBP	IB	IIB	IIIB	PS	IC	IIC	IIIC	OF
M13	D742	?	1973	-	0	0	+/-	0	0	+/-	0	+
	D474	?	1971	-	0	0	0	0	0	0	0	+
M15	D424	BL	1971	+	0					0	0	+
M22	F312	?	1976	+	0					0	0	+
	D734	?	1975	+	0					0	0	+
	B243	?	1947	+	0					0	0	+
	B401	NP	1951	+	0					0	0	+
	LO1174	NP	1962	+	0					0	0	+
M25	D316	SK	1969	+	0					0	0	+
M28R	T28/150A	?	1935	-	0	0	0	0	0	0	0	+
	D722	NP	1973	+	0					0	0	+
M35	C135	NP	1942	+	0					0	0	+
M40	D733	?	1973	+	0					0	0	+
M44	C848	NP	1945	+	0					0	0	+
M49	D938	SK	1976	-	0	+/-	0	+/-	0	+/-	0	+
	B915	NP	1958	+	0					+/-	0	+
	B737	SK	1957	-	0	+/-	0	0	+/-	0	0	+
	NZ131	SK		-	0	+	+	0	+	0	0	+
M51	D976	?	1976	+	0					0	0	+
M58	D774	SK	1974	+	0					0	0	+
M60	D398	?	1970	-	0	0	0	0	0	0	0	+
M61	D812	SK	1975	-	0	0	0	0	0	0	0	+
M62	A956	?	1967	+	0					0	0	+
M63	D459	?	1971	+	0					+	0	+
M66	D794	?	1975	+	0					0	0	+
NT	1RP18	SK	1946	+	0					0	0	+
	1RP66	NP	1949	+	0					++	0	+
	1RP190A	NP	1955	+	0					+	0	+
	3RP150	NP	1953	+	0					0	0	+
	1RP15	NP	1946	+	0					0	0	+
D. Isolates devoid of opacity factor and C repeat epitopes												
M1	1RP94	NP	1951	-	0	0	0	0	0	0	0	-
M31	F376	NP	1980	-	0	0	0	0	0	0	0	-
M34	C142	?	1942	-	0	0	0	0	0	0	0	-
M39	D869	NP	1975	+	0					0	0	-
	19RS14	NP	1941	-	0	0	0	0	0	+/-	0	-
M42	1RS79	NP	1979	-	0	0	0	0	0	0	0	-
M47	C716	?	1943	-	0	0	0	0	0	0	0	-
M50	A203	mo	1959	-	0	0	0	0	0	0	0	+/-
M51	A291	?	1960	+	0					0	0	-
M65	D793	?	1975	+	0					0	0	-
M67	D795	?	1975	-	0	0	0	0	0	0	0	-

The original site of isolation is indicated as follows: NP, nasopharyngeal; SK, skin (including wound infections); BL, blood; UR, urine; ?, site unknown. All isolates are derived from humans, with the exception of the M50 strain (mo, mouse). The date of isolation is indicated and all isolates of a given M type were isolated from different individuals at intervals exceeding one year, with few exceptions. The proportion of an antibody probe that bound to the streptococcal surface was scored: 0, <20% bound; +/-, 20-29.9%; +, 30-59.9%; ++, 60-84.9%; and +++, 85-100%. Rabbit antibody probes IIB, IIC, PS, and IC were not tested for isolates that bound $\geq 15\%$ of rabbit IgG by a nonimmune mechanism (IgGBP). Isolates were scored positive (+) or negative (-) for IgGBP and for OF production as described in Materials and Methods.

aminated (Table II B). Furthermore, the antibody probe directed to the pepsin site region reacted only with isolates that also strongly bound B repeat antibodies. The proximity of the antigenic epitope of probe PS to that of the C repeat region probes suggests the presence of an antigenic domain, which is delimited at one end in the area encompassing both the pepsin-susceptible site and the first C repeat block of the D471 M6 molecule (Fig. 1).

Many isolates of group A streptococci produce opalescence when cultivated in the presence of horse serum (19). Of the 77 isolates displaying strong reactivity with probe IIC, only two produced OF (Table III). Similarly, none of the 69 isolates bound strongly by probe IIIC were OF producers. Thus, isolates sharing a surface-exposed conserved domain with type 6 streptococci fail to produce OF. 11 of the 138 isolates studied lacked strong antibody reactivity with all probes and were OF nonproducers (Table II D). Conversely, all but two of the 48 OF-producing isolates lacked strong binding by any of the three C repeat probes tested (Table II C; Table III). The data strongly suggest that OF is produced almost exclusively by those isolates deficient in epitopes crossreactive with the C repeat domain of M6 protein.

Rabbit IgG was bound via a nonimmune mechanism by 67 of the 138 streptococcal isolates tested. Group A streptococci can express Ig binding proteins with specificity for a wide variety of isotypes (18). Of the OF-producing isolates, 34 (or 71%) expressed an IgGBP with specificity for rabbit IgG (Table II C). Of the streptococci that were bound strongly by C repeat region probes and failed to produce OF, 30 of 79 (38%) displayed IgGBP activity. Thus, OF-producing organisms were nearly twice as likely to bind rabbit IgG by an Fc receptor. We do not know whether isolates expressing IgG binding activity have antigenic epitopes reactive with probes IIB, IIIB, PS, and IC.

Antiphagocytic and Type-specific Properties of OF-producing Isolates. Since most OF-producing streptococci are devoid of immunoreactivity to the B and C repeat region probes (Table II C), we sought to determine whether these organisms produced a type-specific, antiphagocytic molecule by measuring survival after rotation in human blood (23). Of eight randomly selected OF-producing isolates lacking C repeat epitopes, five exhibited strong survival, two others were partially resistant to phagocy-

TABLE III
Correlation between C Repeat Epitopes and Opacity Factor Production

A	Probe	Immunoreactivity	Total isolates	OF production	
				OF ⁻ (%)	OF ⁺ (%)
	IIC	2+ to 3+	77	75 (97)	2 (3)
		1+ to 3+	85	75 (88)	10 (12)
		0 or +/-	53	15 (28)	38 (72)
	IIIC	2+ to 3+	69	69 (100)	0 (0)
		1+ to 3+	72	72 (100)	0 (0)
		0 or +/-	66	18 (27)	48 (73)
B	OF production	Total isolates	2+ to 3+ Immuno-reactivity (%)	2+ to 3+ Immuno-reactivity (%)	Probe
-	88	IIC	75 (85)	IIIC	69 (78)
+	48	IIC	2 (4)	IIIC	0 (0)

tosis, and one isolate was completely phagocytosed (data not shown). The addition of rabbit serum containing high titers of type-specific antibody to two surviving isolates led to neutralization of the antiphagocytic property of M protein, resulting in phagocytosis (data not shown). Thus, the ability of most OF-producing, C repeat epitope-negative isolates to survive completely or partially in human blood suggests that the majority express M protein on their surface in a functional form.

Extraction of M Protein and Western Blot Analysis. The possibility exists that OF-producing streptococci do in fact express M protein-containing C repeat region epitopes shared with M6, except that the M protein epitopes are present in a form inaccessible to antibody probes, perhaps due to masking by the cell wall or a noncovalently bound streptococcal product. To address this possibility, the entire M protein molecule was released from the streptococcal cell wall using a muralytic enzyme. C repeat epitope-positive, OF-nonproducer M6 strain D471 and OF-producing, C repeat epitope-deficient isolates were compared for the presence of M protein by Western blot analysis. When extracts were probed with a combination of antibodies IIC and IIIC, only the C repeat-bearing isolate D471 displayed M protein immunoreactivity (Fig. 2 A). In contrast, human antibody that had been affinity purified to the entire M6 protein molecule (ColiM6) detected material extracted from all C repeat epitope-deficient streptococci examined (Fig. 2 B). The immunoreactive material exhibited the multiple banding pattern characteristic of M protein (20). The data suggest that C repeat region epitopes common with M6 are absent from both the extracted and in situ forms of M protein expressed by OF-producing streptococci.

Discussion

The original objective of this study was to gain a more detailed understanding of the antigenic relatedness of M protein molecules expressed by different streptococcal isolates. This information is important for the design of a vaccine targeted to highly conserved antigenic sites of M protein (8). The results indicate that the majority of serotypes associated with ARF outbreaks share a surface-exposed antigenic domain that can be localized to the C repeat blocks of type 6 M protein. In addition,

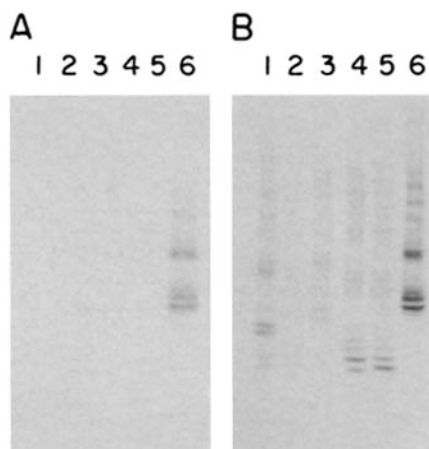


FIGURE 2. Western blot immunanalysis of lysin extracts of Class II streptococci. (Lane 1) Strain D398 (M60); (lane 2) D938 (M49); (lane 3) D316 (M25); (lane 4) D691 (M11); (lane 5) D339 (M9); and (lane 6) Class I control strain D471 (M6). Blots were probed with (A) a combination of mAbs 10F5 (probe IIC) and 10B6 (probe IIIC), or (B) human antibody affinity purified on a ColiM6 column. Incubation of control blots with nonimmune rabbit IgG failed to reveal nonimmunological binding of IgG by IgGBP (data not shown).

the data reveal that there are two major classes of M protein that differ by the presence or absence of this M6-like, C repeat region domain.

The conserved, C repeat region shared with the fibrillar M6 protein molecule appears to form an antigenic domain distinct from the adjacent B repeat region. Evidence in support of this antigenic domain includes the proximity of the highly conserved C repeat region epitopes to the weakly homologous B repeat region and pepsin-susceptible site (Fig. 1). The B repeat region through residue 230 forms a more flexible coiled-coil structure than the C repeat region beginning at residue 231 (4), suggesting that the C repeat region antigenic domain may have unique structural characteristics as well. Amino acid sequence identity between maximally aligned sequences of M6 and M12 (24) is <27% between residues 121 (first residue of B repeat region block of M6 from D471) and 231, but abruptly increases to 97% complete identity between residues 232 and 298 (extending from the beginning of the first C repeat sub-block to the perimeter of cell wall; Fig. 1) (data not shown). A similar pattern of homology to M6 protein is observed with the M24 protein (25). The location of the amino acid sequence homologies between M6 and other M types, combined with the antigenic analyses presented in this study, strongly suggests that the NH₂-terminal end of the conserved domain lies at the beginning of the first C repeat block.

The close parallel observed between those serotypes previously designated as having MAP I antigen (10), and those M types reacting strongly with C repeat region antibody probes, provided the impetus to measure OF production by the streptococcal isolates under study. Those serotypes that share surface-exposed antigenic epitopes with the C repeat region of M6 protein and fail to produce OF, and serotypes deficient in epitopes crossreactive with the C repeat region but produce OF are summarized in Table IV. We propose to designate the two major groups of M protein serotypes as Class I and Class II, respectively, and this classification closely parallels those serotypes previously designated MAP I and II, respectively (10) (Table IV). It is likely that the MAP I antigen represents an immunologically crossreactive portion

TABLE IV
*Comparison of Class I and Class II M Protein Serotypes
to MAP I and II Antigen Serotypes*

Class I Highly probable: 1, 3, 5, 6, 12, 14, 17, 18, 19, 24, 29, 30, 33
Probable: 8, 23, 26, 27, 32, 37, 38, 41, 43, 46, 48, 52, 53, 54, 55, 56, 57, 59
MAP I: 1, 3, 5, 6, 12, 14, 15, 17, 19, 24, 26, 30, 33, 52, 53, 54, 57
Class II Highly probable: 2, 9, 11, 22, 49
Probable: 4, 13, 15, 25, 28R, 35, 40, 44, 51, 60, 61, 62, 63, 66
MAP II: 2, 4, 9, 13, 22, 25, 28R, 48, 58, 60, 61, 62, 63
Class Variable: 58
MAP Variable: 11, 18, 41, 49, 55, 59

A serotype was classified as Class I if >50% of the isolates examined displayed strong binding to one or more C repeat region probes and failed to produce OF (probable). Classified serotypes were further designated as highly probable for that class if at least three isolates were tested and >80% fell into that class. M serotypes designated as having MAP I and MAP II antigen (or variable) are adapted from Widdowson (10).

of M protein, specifically the C repeat domain. While little is known of the antigenicity or structure of Class II M proteins or of MAP II antigen, it is apparent that some immunodeterminants are shared by the extracted forms of Class I and II M proteins (Fig. 2). Whether the shared antigenic epitopes reside in buried portions of the M protein molecule remains to be established. The partial amino acid sequence currently available for one Class II M protein (M49) reveals weak homology with M6, except for 78% identity to a 26-residue stretch within the C repeat region of M6 (26), possibly explaining the marginal immunoreactivity of M49 isolates with C repeat region probes (Table II). Of the M protein molecules whose complete DNA and amino acid sequence is known to date (M5, M6, M12, M24), all four can be categorized as Class I molecules, and in addition, all exhibit nearly complete identity to one another in the C repeat domain (24, 25, 27, 28). Perhaps the extensive *antigenic* evaluation of over 50 serotypes presented in this study was necessary in order to establish the existence of a second class of M protein molecules.

Nucleic acid hybridization using DNA probes derived from the *emm-6.1* gene has been performed on several isolates representing M serotypes (7), which can be classified as I or II according to Table IV. Using a DNA probe corresponding in position to a portion of the second C repeat sub-block of strain D471 and extending into the membrane anchor region (Fig. 1), a distinction can be made between Class I and II serotypes. Hybridization to M28R, M49, and M62 serotypes (Class II) was observed only under conditions of low stringency, whereas Class I serotypes (M1, M5, M6, M12, M19, M24, M30, M55) hybridized under high stringency conditions. Thus, the DNA hybridization findings suggest that there may be fundamental differences between Class I and II M protein molecules at the DNA level.

A major question regarding the molecular basis of ARF is concerned with identifying a unique feature common to ARF-associated streptococci. All M serotypes that appear to account for the majority of outbreaks of ARF (9-12) express Class I M protein, with the possible exception of M11. To date, heart crossreactive epitopes contained within M protein molecules have been localized to type-specific A and B repeat region immunodeterminants (29). However, the serum of ARF patients contains elevated levels of complement-fixing activity specific for the MAP I antigen, and MAP I antibody is absorbed by human heart tissue (10, 13). Thus, immunodeterminants within the conserved C repeat region of Class I M proteins should also be considered as possible candidates for initiating or contributing to the autoimmune response associated with rheumatic heart disease. Group A streptococci bearing Class II M protein types can be viewed as natural mutants that are avirulent in regards to their capacity to initiate ARF. Understanding the molecular properties of Class II M proteins, and the differences between Class I and II streptococci, may provide insight into the molecular basis for rheumatic fever.

Summary

The antigenic relatedness of surface-exposed portions of M protein molecules derived from group A streptococcal isolates representing more than 50 distinct serotypes was examined. The data indicate that the majority of serotypes fall into two major classes. Class I M protein molecules share a surface-exposed, antigenic domain comprising the C repeat region defined for M6 protein. The C repeat region of M6 protein is located adjacent to the COOH-terminal side of the pepsin-susceptible

site. In contrast, Class I M proteins display considerably less antigenic relatedness to the B repeat region of M6 protein, which lies immediately NH₂-terminal to the pepsin site. Surface-exposed portions of Class II M proteins lack antigenic epitopes that define the Class I molecules. Studies in the 1970s demonstrated that M protein serotypes can be divided into two groups based on both immunoreactivity directed to an unknown surface antigen (termed M-associated protein) and production of serum opacity factor. These two groups closely parallel our current definition of Class I and Class II serotypes. Both classes retain the antiphagocytic property characteristic of M protein, and Class II M proteins share some immunodeterminants with Class I M proteins, although the shared determinants do not appear to be exposed on the streptococcal surface. Nearly all streptococcal serotypes associated with outbreaks of acute rheumatic fever express M protein of a Class I serotype. Thus, the surface-exposed, conserved C repeat domain of Class I serotypes may be a virulence determinant for rheumatic fever.

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