INFECTION AND IMMUNITY, Aug. 1999, p. 3915–3920 Vol. 67, No. 8
0019-9567/99/$04.00+0

Selective Distribution of a High-Affinity Plasminogen-Binding Site among Group A Streptococci Associated with Impetigo

MIKAEL D. SVENSSON,[1] ULF SJÖBRING,[1]* AND DEBRA E. BESSEN[2]*

Department of Laboratory Medicine, Lund University, Lund, Sweden,[1] and Department of Epidemiology and Public Health, Yale University School of Medicine, New Haven, Connecticut[2]

Received 14 January 1999/Returned for modification 8 April 1999/Accepted 28 May 1999

Group A streptococci can be classified according to their tendency to cause either impetigo, pharyngitis, or both types of infection. Genotypic markers for tissue site preference lie within *emm* genes, which encode fibrillar surface proteins that play a key role in virulence. *emm* gene products (M and M-like proteins) display an extensive array of binding activities for tissue and plasma proteins of the human host. In a previous study, a high-affinity binding site for human plasmin(ogen) was mapped to the *emm53* gene product. In this report, a structurally similar plasminogen-binding domain is found to be widely and selectively distributed among group A streptococci harboring the *emm* gene marker for the skin as the preferred tissue site for infection. The findings are highly suggestive of a central role for bacterial modulation of host plasmin(ogen) during localized infection at the epidermis.

Group A streptococci (GAS) are important human pathogens that can cause severe morbidity and mortality, as in cases of toxic shock syndrome and necrotizing fasciitis and during autoimmune sequelae such as rheumatic fever. However, the vast majority of GAS infections result in only mild disease, specifically pharyngitis and impetigo. The mucosal epithelium of the throat and the epidermal layer of the skin serve as the primary tissue reservoirs for the maintenance of this organism. GAS display a complex array of binding activities for human tissue and plasma components on their cell surfaces. For some host products, such as fibronectin and plasmin(ogen), there exists a multitude of distinct streptococcal structures capable of mediating these interactions. For example, human plasminogen can be bound by at least four distinct streptococcal cell surface proteins that differ in their binding affinities and are differentially expressed (4, 20, 25, 33). The precise reason for this redundancy is not well understood. However, GAS-bound plasminogen can be converted to plasmin—its active form—by secreted streptokinase (12, 27). Cell-bound plasmin functions as a broad-spectrum proteinase and therefore may act to modulate the bacterium's microenvironment and movement through host tissue. Precisely how each of the distinct plasmin(ogen) binding activities function during streptococcal infection remains unclear.

Many host tissue and plasma proteins are specifically recognized by GAS through structurally discrete binding domains which comprise a major part of surface fibrils that are collectively known as M and M-like proteins. Included among the M-protein-bound host proteins are regulators of the complement cascade, major components of both the coagulation system and the fibrinolytic pathway, and immunoglobulins (reviewed in references 9 and 18). The different binding domain combinations give rise to mosaic-like arrays that impart a unique spectrum of biological activities to the streptococcal cell. The product of the *emm* gene of M serotype 53 streptococci binds both human plasminogen and plasmin with high affinity (designated PAM, for plasminogen-binding group A streptococcal M protein) (4). The plasmin(ogen) binding site of PAM has been localized to a 13-amino-acid repeated domain (11) (Fig. 1A).

The M protein fibrils display extensive antigenic heterogeneity and provide the basis for a serological typing scheme, for which >80 serotypes have been defined. While decades of epidemiological studies have shown that some M serotypes have a strong tendency to be associated with only certain streptococcal diseases, more recent work has identified *emm*-related genetic markers for the so-called throat and skin types (8). In this report, by taking an epidemiological approach, we demonstrate a selective distribution of the PAM phenotype and genotype among a subpopulation of GAS strains that have a strong tendency to cause impetigo rather than pharyngitis.

MATERIALS AND METHODS

Bacterial strains. Of the 83 GAS strains used in this study, all but three (AP52, AP53, and Manfredo) represent a broad selection from among those previously described (6, 7). Bacteria were isolated from infected humans between the years 1941 and 1989 at several locations throughout the world: 29 strains from New York; 16 from Trinidad; 7 from Alabama; 6 from the Czech Republic; 3 each from Illinois, Minnesota, Ohio, and Egypt; 1 each from Missouri, North Carolina, Nebraska, Utah, Chile, the former Yugoslavia, Japan, Kuwait, and United Kingdom; and 4 from unknown places. Serotyping was performed by the laboratory providing the strain. The *emm* sequence type is established based on 160 bp encoding for part of the leader peptide and NH_2-terminal end of the mature M protein (Fig. 1B) (2, 3, 16a).

Measurement of the PAM genotype. Chromosomal DNA purified from each of the streptococcal strains was used as a template in a PCR-based mapping strategy; *emm* chromosomal patterns A through E (Fig. 1B) were established by using *emm* subfamily (SF)-specific primers (6–8). To ascertain the presence or absence of the PAM genotype, PAM-specific oligonucleotide primers corresponding to the portion of the *emm53* gene that encodes the binding site for human plasminogen (Fig. 1A) (11) were paired with *emm* SF-specific primers. The PAM primers are PAM-F (forward) (5′-GAGTTG[A/G]AACGACTTAAAA[A/G]CGAGAGACATG-3′) and its complement, PAM-R (reverse); they are degenerate in two positions in order to account for differences between the a1 and a2 repeat regions. For *emm* pattern A and B strains, PAM-F was paired with SF-specific primer SF1-R (5′-GTGCTTGACCTTTACCTGGAACAGCTT-3′). For *emm* pattern C strains, PAM-F was paired with SF-specific primers SF1-R and SF3-R (5′-GCTGTTTGAGCAGCTCTACC-3′). In *emm* pattern D and E strains, PAM-F was paired with SF3-R, whereas PAM-R was paired with SF-specific primer SF4-A (5′-CTCCTAGGTTCAGCTAAGCGTGAGTTG-3′) and/or SF4-L (5′-GAAATCCAAACAAGCACTACCTACTG-3′). For *emm*

* Corresponding author. Mailing address for Ulf Sjöbring: Lund University, Department of Laboratory Medicine, Solvegatan 23, 223 62 Lund, Sweden. Phone: 46-46-173238. Fax: 46-46-189117. E-mail: Ulf.Sjobring@mig.lu.se. Mailing address for Debra E. Bessen: Yale University School of Medicine, Department of Epidemiology and Public Health, 60 College St., Box 208034, New Haven, CT 06520. Phone: (203) 785-4480. Fax: (203) 737-4285. E-mail: debra.bessen@yale.edu.

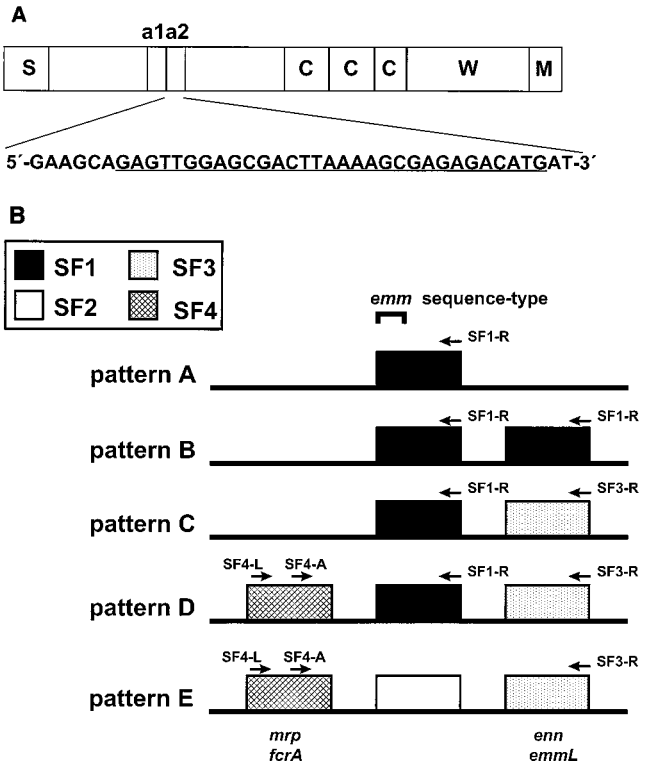

FIG. 1. Structural organization of *emm* genes. (A) Location of the high-affinity plasminogen-binding site and corresponding nucleotide coding sequence within the Emm53 (PAM or M53) protein. Schematic organization of the Emm53 protein: S, signal sequence; C, conserved (C repeat region) repeats; W, cell wall (peptidoglycan)-spanning domain; M, membrane anchor. The a repeats (a1 and a2) are 13 amino acids in length, and each a repeat binds plasminogen with high affinity. The nucleotide sequence of the a2 repeat is given. An oligonucleotide (PAM-F) corresponding to the underlined sequence or its complimentary sequence (PAM-R) was paired with a SF-specific primer for PCR-based genotype mapping. (B) Arrangement of *emm* SF genes and position of SF-specific oligonucleotide hybridization sites. The *emm* and *emm*-like genes are represented by four *emm* gene SF forms that are based on nucleotide sequence differences in the 3′-end portion encoding for the peptidoglycan-spanning domain (8). Most GAS strains have one of the five *emm* patterns (designated A through E), which are defined by the number of *emm* genes, their SF content, and their relative arrangement on the chromosome. For strains with multiple *emm* or *emm*-like genes, each gene differs at its 5′ end, and genes are usually separated from one another by 0.2 to 0.3 kb. The centrally positioned gene is used for *emm* sequence typing (3). Alternative nomenclature for *emm* and *emm*-like genes is indicated (bottom). Arrows depict the hybridization sites for oligonucleotide primers. The *emm53* gene, depicted in panel A, represents the central *emm* gene (SF1) of an *emm* pattern D strain.

pattern D strains, PAM-F was also paired with SF1-R. All primer pairs were used at annealing temperatures of 55°C. Isolates giving PCRs that consistently provided moderate-to-high yields of a DNA fragment of the expected approximate size with one or more PAM-SF primer pair(s) were scored as positive for the PAM genotype.

Binding of radiolabeled plasminogen to streptococci. Human plasminogen was purified from human plasma by affinity chromatography using lysine-Sepharose 4B (Pharmacia). Bound material was eluted with 0.1 M glycine (pH 2.0), and fractions containing at least 95% pure plasminogen were identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 2A, inset). Using a monoclonal antibody (MAb) specific for the NH_2-terminal peptide found only in the unprocessed Glu form of the zymogen (MAb n3641; American Diagnostica Inc.), Western blot analysis revealed that the purified preparation was largely the Glu form of plasminogen, and little or no plasmin was present (Fig. 2A, inset). Purified plasminogen was radiolabeled with ^{125}I (Amersham) by using the chloramine-T method to a specific activity of 100,000 cpm/ng. For the whole-bacterial-cell adsorption assay, streptococci were cultured for 16 h on blood agar plates or in Todd-Hewitt broth (Difco) at 37°C with 5% CO_2. Bacteria adjusted to the appropriate concentration were incubated with ^{125}I-labeled plasminogen in a total volume of 250 μl of phosphate-buffered saline (PBS) containing 0.02% NaN_3 and 0.1% Tween 20. Following incubation for 1 h at 20°C, an additional 2 ml of buffer was added, and the bacteria were centrifuged at 4,000 × *g* for 10 min. The supernatant was discarded, and radioactivity associated with the pellet was measured in a γ counter; measurements were performed in triplicate. Binding of ≥25% of ^{125}I-labeled plasminogen is indicative of an "M53-like" plasminogen-binding activity (positive PAM phenotype).

Analysis of plasminogen binding to rEmm proteins. The cloning and expression of recombinant Emm52 (rEmm52) from strain AP52 and rM5 from strain Manfredo has been previously described (11, 17). Western blot overlay with ^{125}I-labeled plasminogen was used to detect binding activity by rEmm proteins. In addition, rEmm proteins were tested for plasminogen binding in a microtiter assay. Recombinant proteins were immobilized in microtiter plates (4°C for 16 h) over a range of concentrations, and wells were blocked with PBS containing 2% bovine serum albumin for 4 h at 20°C, washed with PBS containing 0.05% Tween 20, and incubated with 0.5 ng of ^{125}I-labeled plasminogen. Following incubation (20°C for 2 h), plates were extensively washed, and bound radioactivity was measured in a γ counter.

Statistics. Statistical significance was calculated by χ^2 analysis with Yates' correction for sample size (Epi Info version 6.04b; Centers for Disease Control and Prevention, Atlanta, Ga.).

RESULTS

Distribution of the PAM genotype among GAS. The mapping of a plasminogen-binding motif to within the surface-exposed portion of M or M-like proteins (11) suggested that its distribution among members of the GAS species may be restricted. To ascertain whether PAM is tightly linked to genetic markers for principal tissue reservoir (i.e., *emm* patterns), PAM-specific oligonucleotides were designed for PCR-based mapping of the *emm* chromosomal region of 83 *emm* pattern-defined GAS strains (Fig. 1A and 1B). The GAS isolates under study represent all five of the *emm* patterns (A through E) and >40 distinct M serotypes or *emm* sequence types (Table 1). The wide range of M and *emm* types, combined with large spatial and temporal distances separating their isolation from human hosts, indicates that the GAS selected for this study are a biologically diverse sample set.

Previous studies demonstrate that the majority of *emm* pattern A, B, and C (A–C) strains are oropharynx associated, whereas most pattern D strains are isolated from impetigo lesions; pattern E strains are readily found at both tissue sites (6, 8). Thus, *emm* patterns A–C and D can be regarded as genetic markers for the throat and skin, respectively, as the principal tissue reservoir in the human host. Of the 35 *emm* pattern A–C strains examined, representing 15 distinct M- or *emm*-sequence types, only four displayed the PAM genotype (11.4%; Fig. 3). Among the 20 *emm* pattern E isolates, representing 13 distinct M- or *emm*-sequence types, all tested negative for PCR-based detection of the PAM genotype.

In sharp contrast to patterns A–C and E organisms, 24 of the 28 *emm* pattern D isolates tested positive with the PAM-specific oligonucleotides (85.7%; Fig. 3). Based on the generation of DNA products from multiple PCR amplifications, the PAM-specific priming site mapped to the central *emm* gene at a position that is upstream from the SF1 site (Fig. 1B). The difference between pattern D strains and either pattern A–C or E isolates, in terms of the PAM genotype, is highly significant ($P < 0.001$, df = 1). Of the 17 unique *emm* sequence types represented among the 28 pattern D strains, the positive PAM genotype was found among 13 *emm* types (76.5%), whereas the negative PAM genotype was limited to four *emm* types (23.5%) (Table 2). For *emm* types where multiple isolates were tested (*emm33*, *emm52*, *emm53*, *emmpotter41*, *emmpt2110*, and *emmstD633*), all were homogeneous in their display of the PAM genotype. The results indicate that the PAM genotype is largely restricted to *emm* pattern D strains.

Binding activity for plasminogen among GAS. To establish the association between the PAM genotype and the plasminogen-binding phenotype, GAS were analyzed for their capacity to bind human plasminogen. A detailed analysis of plasmino-

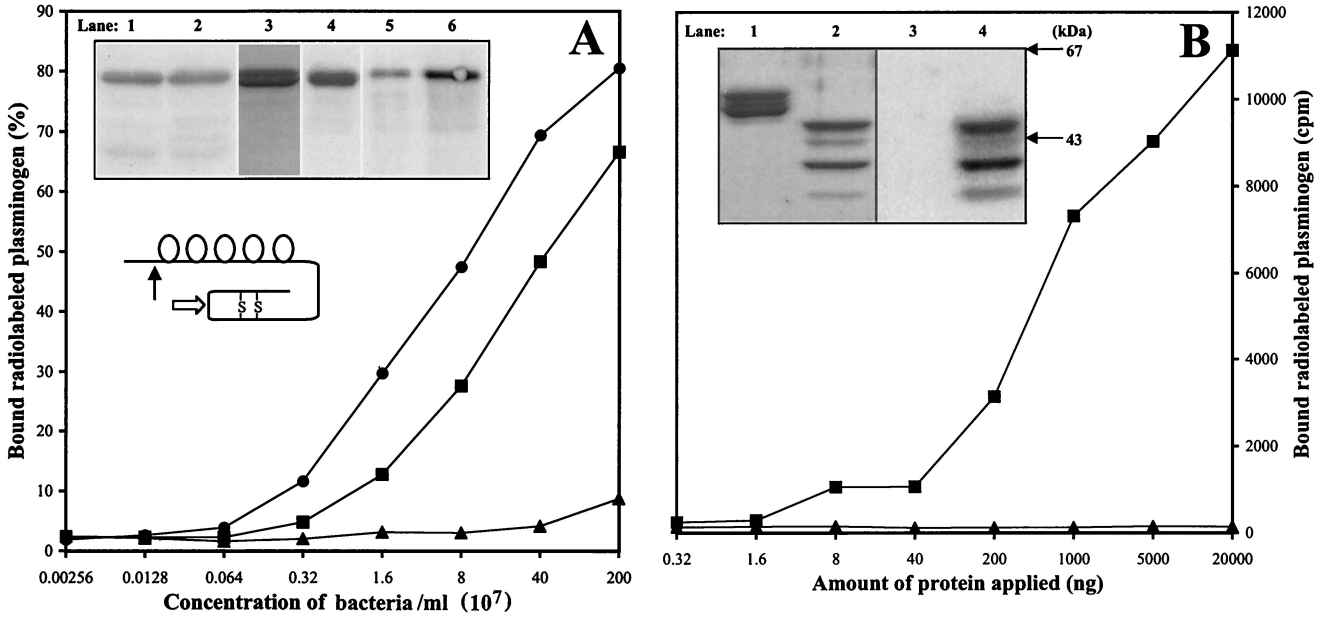

FIG. 2. Binding of Glu plasminogen to select strains and recombinant Emm proteins. (A) Inset, top: Human plasma (0.5 ml) was mixed with approximately 10^{10} CFUs of GAS (lane 1, strain AP53; lane 2, strain AP52), cells were washed, and bound proteins were eluted with 0.1 M glycine-HCl (pH 2.0). Blots of eluate were incubated with MAb directed to Glu plasminogen. Purified human plasminogen was analyzed by SDS-PAGE and Western blotting. The gel was stained with Coomassie brilliant blue (lane 3), and the blot was incubated with a MAb directed to the NH_2-terminal peptide specific for Glu plasminogen (lane 4). Purified, ^{125}I-labeled human plasminogen was analyzed by autoradiography (lane 5). Purified ^{125}I-labeled human plasminogen (0.25 ng) was mixed with approximately 10^{10} CFUs of strain AP53 in 0.5 ml of PBS containing 2% bovine serum albumin. Following incubation, cells were washed extensively, and bound material was eluted with glycine and subjected to autoradiography (lane 6). (A) Inset, middle: A schematic representation of plasminogen. The five kringle domains and the activation cleavage sites for tissue-type plasminogen activator and urokinase in the serine protease domain (open arrow) are indicated. Also depicted is the cleavage site for plasmin, resulting in a 77-amino-acid residue NH_2-terminal peptide and conversion of plasminogen from the Glu to the Lys form (closed arrow). (A) Bottom: Bacteria-bound ^{125}I-labeled plasminogen was measured by an adsorption assay, except that 0.1 ng per 0.25 ml of ^{125}I-labeled plasminogen was incubated with GAS present over a wide range of concentrations, expressed as CFU/ml. Shown are strain 1RP144 (M5, *emm* pattern A–C; ▲) and *emm* pattern D strains D617 (■) and AP53 (●) (Table 2). (B) Inset, top: Recombinant *emm* gene products (rM5, derived from M5 Manfredo, *emm* pattern A–C, lanes 1 and 3; rEmm52, derived from AP52, lanes 2 and 4) were separated by SDS-PAGE and stained with Coomassie blue (lanes 1 and 2); a replica of the gel was blotted and incubated with ^{125}I-labeled human plasminogen (lanes 3 and 4). (B) Bottom: Purified rM5 (▲) and rEmm52 (■) proteins were immobilized in microtiter plate wells and incubated with ^{125}I-labeled plasminogen. The amount of radio-iodinated material bound to various concentrations of recombinant proteins was measured in a γ counter.

gen binding by a few select strains is summarized in Fig. 2. Using a human plasminogen source of either purified ^{125}I-labeled plasminogen or unfractionated plasma, two *emm* pattern D strains (AP52 and AP53) were found to bind specifically to the unprocessed Glu form of the zymogen (Fig. 2A, inset). At concentrations of bacteria up to 2×10^9 CFU/ml, >50% of radiolabeled plasminogen was bound by two *emm* pattern D strains (D617 and AP53), whereas <10% of total plasminogen was bound by the *emm* pattern A–C strain (1RP144) (panel A). To demonstrate that the *emm* gene product represents the GAS component responsible for the observed binding of plasminogen, recombinant M proteins derived from two GAS strains were analyzed in direct binding experiments. As expected, the rEmm52 protein derived from strain AP52 bound Glu plasminogen efficiently, whereas the rM5 protein from *emm* pattern A–C strain Manfredo showed little, if any, affinity for the zymogen (Fig. 2B).

Using the whole-bacterial-cell adsorption assay, binding of human plasminogen was measured for all 83 GAS strains un-

TABLE 1. Epidemiologic features of group A streptococcal strains under study

emm pattern	No. of strains represented	No. of unique M or *emm* types	No. (%) isolated from:		
			Nasopharynx	Impetigo	Unknown site
A, B, or C	35	15	32 (91)	2 (6)	1 (3)
D	28	17	5 (18)	19 (68)	4 (14)
E	20	13	10 (50)	10 (50)	0 (0)

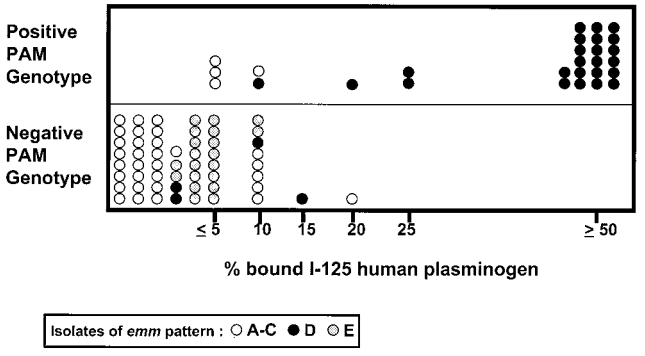

FIG. 3. Binding of human plasminogen by intact GAS. Each symbol represents a different GAS isolate (n = 83) that is defined according to its *emm* pattern. Intact GAS were measured for the percentage of ^{125}I-labeled human plasminogen bound in an adsorption assay. Approximately 2.0×10^9 CFUs were incubated with ^{125}I-labeled plasminogen (~10,000 cpm, corresponding to ~1 ng of plasminogen) in a total reaction volume of 0.25 ml. The values for the percentage of plasminogen bound are rounded to the nearest 5% interval. Also, shown is the PAM genotype for each isolate, as determined by PCR amplification of GAS-derived DNA using PAM-specific oligonucleotide primers.

TABLE 2. Properties associated with plasminogen-binding activity among *emm* pattern D strains

Strain	*emm* type[a]	Tissue site of isolation[b]	Isolation date	Place[b]	PCR product (PAM primers)	% of bound ^{125}I-labeled plasminogen
13RS60	33	NP	1941	New York	+	≥50
A910	33	NP	1966	ND	+	≥50
A982	33	Imp	1968	New York	+	≥50
3732-S	33	Imp	1969	Alabama	+	≥50
6010-S	33	Imp	1971	Alabama	+	≥50
D735	33	Imp	1973	New York	+	≥50
29487	33	Imp	1987	Czech Republic	+	≥50
AP52[c]	52	Imp	1967	Minnesota	+	≥50
D617	52	Imp	1972	Trinidad	+	≥50
AP53[c]	53	Imp	1967	Minnesota	+	≥50
ALAB49	53	Imp	1986	Alabama	+	≥50
29689	pt2110	Imp	1988	Czech Republic	+	≥50
ALAB55	pt2110	Imp	1986	Alabama	+	25
D964	pt2631	Imp	1976	Trinidad	+	≥50
D821	pt5757	Imp	1975	Trinidad	+	≥50
29486	st2370	Imp	1987	Czech Republic	+	≥50
D432	stD432	ND	1971	Egypt	+	≥50
D631	stD631	Imp	1972	Trinidad	+	≥50
D633	stD633	Imp	1972	Trinidad	+	≥50
D680	stD633	NP	1972	Trinidad	+	≥50
D641	stNS5	Imp	1972	Trinidad	+	≥50
D466	potter41	ND	1971	Egypt	+	25
D502	potter41	Imp	1971	Trinidad	+	10
D998	70	ND	1976	Japan	+	20
10RS101	32	NP	1942	New York	−	15
1RS79	42	NP	1941	New York	−	10
A457	36	ND	1961	Former Yugoslavia	−	≤5
D626	st88/31	Imp	1972	Trinidad	−	≤5

[a] For several isolates, previous reports (5, 8) indicated M serological types that are discordant with the *emm* sequence types presented here.
[b] Imp, impetigo; NP, nasopharyngeal site; ND, not determined.
[c] AP52 and AP53 are also designated CV686 and CV265, respectively (31). AP53 is the strain from which Emm53 (PAM) was originally derived (4).

der study, at a single near-saturating concentration of bacteria (approximately 8×10^9 CFU/ml). Of the *emm* pattern D strains, 22 of 28 (78.6%) displayed high levels of plasminogen binding (ranging from 25 to >50% of total ^{125}I-labeled plasminogen bound; Fig. 3). In contrast, the vast majority (83.6%) of *emm* patterns A–C and E strains bound ≤5% of ^{125}I-labeled plasminogen; binding of >20% plasminogen was not observed for any of the patterns A–C and E strains tested.

Correlations of PAM phenotype, PAM genotype, and tissue site of isolation. All 22 isolates binding ≥25% of human plasminogen in the whole-bacterial-cell adsorption assay also displayed the PAM genotype that is detected by PCR using PAM-specific oligonucleotide primers. Of the 22 strains that scored positive for plasminogen binding (i.e., those that had a positive PAM phenotype), 100% were *emm* pattern D (Fig. 3). Thus, the PAM phenotype positively correlates with the PAM genotype and furthermore, it is restricted to *emm* pattern D strains.

Of the 83 GAS isolates studied, 22 were positive for both the PAM phenotype and genotype, however, an additional six strains were positive for the PAM genotype only. Thus, six isolates hybridized with PAM oligonucleotides yet failed to exhibit strong plasminogen-binding activity. Of the four pattern A–C isolates that were positive for the PAM genotype but negative for the PAM phenotype (strains 87-214 [*emm1*], 1RP144 [*emm5*], 1GL217 [*emm17*], and 87-373 [*emm18*]), all were represented by *emm* types found among other strains that were clearly negative for the PAM genotype. Thus, it is possible that under the annealing conditions employed, the PAM-specific primers can hybridize to non-PAM-specific sequences whose distribution does not correlate well with *emm* type. For the two pattern D strains that were negative for the PAM phenotype (Table 2) but positive for the PAM genotype (strains D998 [*emm70*] and D502 [*emmpotter41*]), one displayed an *emm* type shared with a second isolate that is positive for the PAM phenotype (strain D466 [*emmpotter41*]). Since M and *emm* types are tightly linked to the 3′ end SF-specific regions that define *emm* pattern (Fig. 1B) (6), it seems likely that for strain D502, the PAM-specific oligonucleotides detected an *emm* gene whose product is not expressed at high levels or, alternatively, harbors a point mutation(s) that leads to a decrease in binding capacity.

The actual tissue site of isolation is known for 78 of the 83 GAS under study: 31 were isolated from impetigo lesions, whereas 47 were obtained from the nasopharyngeal mucosa and gave rise to either pharyngitis, clinically inapparent infections, or asymptomatic carriage. Of the 2 pattern A–C and 10 pattern E isolates derived from impetigo lesions, representing a total of nine distinct *emm* types, none displayed the PAM phenotype or genotype. In contrast, of the 18 pattern D impetigo isolates, representing 12 *emm* types, 16 scored positive for both the PAM phenotype and genotype, 1 had the PAM genotype only, and 1 was negative for both (Table 2). Of the five nasopharynx-derived pattern D organisms, three were positive for PAM (13RS60 [*emm33*], D680 [*emmstD633*], and A910 [*emm33*]), and two were negative for PAM (10RS101 [*emm32*] and 1RS79 [*emm42*]). Of the pattern D throat isolates, at least three (13RS60, D680, and 10RS101) were not known to be associated with clinical disease in their human hosts and most probably gave rise to an asymptomatic carrier state.

Of the 21 isolates associated with cases of acute rheumatic fever, four strains displayed the PAM genotype but bound ≤10% of human plasminogen in the whole-bacterial-cell ad-

sorption assay (all four are *emm* pattern A–C strains). Thus, rheumatic fever-associated strains tend to be deficient in PAM-mediated plasminogen binding activity. Of the seven GAS isolated from individuals with acute glomerulonephritis, three displayed both the PAM phenotype and genotype (all *emm* pattern D strains), whereas the remainder lacked both the PAM phenotype and genotype (*emm* pattern A–C and E strains).

Taken together, the data show a strong association between the PAM phenotype, PAM genotype and *emm* pattern D strains. All PAM-positive organisms segregate as a group with bacteria having a strong tendency to cause impetigo lesions. Furthermore, *emm* pattern E isolates and the occasional pattern A–C isolate that are obtained from impetigo lesions, are consistent in their lack of both the PAM phenotype and genotype, suggesting that alternative pathogenic mechanism(s) are used by these GAS subpopulations.

DISCUSSION

A recurring theme in the study of GAS is the existence of multiple gene products which bind various human tissue and plasma components. To better understand the biological role of bacterial-host binding activities during natural infection, one can test a hypothesis by comparing organisms that are genotypically and phenotypically well defined, using in vitro models consisting of human-derived components or alternatively, in vivo models that rely on animals. However, often the binding affinities are significantly higher for human components than for other mammalian forms, casting uncertainty on the true relevance of animal models for a disease that is uniquely human. For example, the molecule studied here (PAM) does not bind well to plasminogen of closely related species, such as the rhesus monkey, a nonhuman primate (10). An epidemiological approach provides a third general strategy and works by sorting a population of microorganisms according to their genotype, phenotype, and biological interactions with its natural host. Together, the three approaches can merge to form a more complete composite of the mechanisms underlying disease pathogenesis.

In the present investigation, we use a combined approach to further analyze the role of the GAS interaction with plasminogen, a zymogen found in high concentrations in plasma and tissue fluids. Cleavage of plasminogen can set off any one of several cascades of events that are part of important biological processes, such as dissolution of fibrin, wound healing, inflammatory cell migration, tumor cell metastasis, and the inhibition of angiogenesis (16, 28). Multiple pieces of evidence suggest that for some of these events, activation of plasminogen at the mammalian cell surface is required (14, 22, 24, 32). Therefore, the biological consequences of bacterial cell surface-bound plasmin activity are potentially quite numerous. GAS express surface proteins that allow for the capture of human plasmin(ogen) by at least four different mechanisms (4, 20, 25, 33). Furthermore, GAS produce at least two secreted proteins—streptokinase and cysteine protease—which can interact with plasmin(ogen) in a specific manner (26, 34). This report focuses on one of the plasminogen binding mechanisms exhibited by GAS, the high-affinity interaction that is mediated through PAM.

Recent evidence strongly suggests that surface-associated binding and activation of plasminogen enhances the invasion and dissemination of at least two bacterial pathogens, *Yersinia pestis* and *Borrelia burgdorferi* (13, 29). Invasion of normally sterile tissue by GAS is associated with high rates of morbidity and mortality; however, from an ecological standpoint, GAS invasive disease is a rare event. In contrast to the vector-borne pathogens *Y. pestis* and *B. burgdorferi*, which are transmitted to their mammalian hosts through insect bites, GAS are transmitted primarily by respiratory droplets or close contact. GAS colonize the mucosal epithelium of the upper respiratory tract or epidermis of the skin and, most often, cause only mild superficial infection. The strong epidemiological association demonstrated in this report narrows the focus for the most likely biological role of the PAM form of plasminogen-binding activity by GAS. Since *emm* pattern D strains display a strong tendency to cause impetigo and are much less often observed in association with pharyngitis (8), it seems most likely that PAM exerts its biological function within the epidermal tissue space.

The epidemiological associations uncovered in this study lead us to favor the hypothesis that PAM-directed plasmin(ogen) binding by GAS exerts its strongest biological effect during localized infection rather than during invasive disease. Population-based surveillance for invasive GAS disease during a 6-month time frame in Connecticut demonstrates that $<2\%$ of the isolates display *emm* pattern D (15). This finding is in further support of the idea that the key role of PAM is not linked to invasion of deep tissue. The concentration of plasminogen in tissue is highest in the circulation and interstitial fluids (1×10^{-6} to 2×10^{-6} M). However, the high affinity of PAM for human plasminogen (affinity constant, 8×10^{-8} M^{-1}) (4) suggests that PAM has undergone adaptive evolution in order to function ideally under conditions whereby the concentration of plasminogen is low.

During the development of an impetigo vesicle, GAS are largely confined to the surface of the outermost layer of granular keratinocytes, located just below the cornified layer (stratum corneum) of the epidermis (1). As a continuing stream of neutrophils migrate from the dermal vessels through the differentiated keratinocyte layers of the epidermis and into the subcorneal space where adherent GAS persist, a mild spongiosis (intraepidermal intercellular edema) often develops and extracellular tissue fluid gradually accumulates within the epidermis. The vesicle itself contains coagulated serum and neutrophils in addition to bacteria. As the infection progresses and the vesicle ruptures, the stratum corneum is no longer present and the remaining epidermal layer is covered by a crust composed of fibrin and cellular debris. An expanding inflammatory response can eventually lead to a deeper erosion of the epidermal layer or penetration of the dermis as observed in ecthyma (19, 23, 30).

In a normal response to damage at the dermal-epidermal junctional region, basal layer keratinocytes located at the margins of the "wound" will migrate laterally along a provisional matrix of fibrin and initiate the process of reepithelialization (21, 28). The migration of keratinocytes during wound healing, and possibly inflammatory cells responding to a chemotactic signal, is directed by pericellular proteolysis mediated by plasmin (16, 21). Conceivably, PAM acts to sequester plasmin(ogen) and thereby competes with the human cell receptors for its binding and, in doing so, circumvents the normal healing processes. Alternatively, plasminogen captured by PAM and subsequently activated by streptokinase might act directly on the fibrin network of either the overlying crust and/or dermal-epidermal junction, thereby allowing the bacteria to prolong their presence in superficial wounds. Finally, bacterial-surface-generated plasmin might function as a broad spectrum protease and modulate the bacterium's microenvironment in an undefined manner that ultimately favors its reproduction and transmission to a new host. However, the failure of PAM to exhibit high-affinity binding for plasminogen of nonhuman or-

igin may preclude the possibility of discerning among these possibilities by traditional in vivo models for infection.

ACKNOWLEDGMENTS

We thank Dominick Scaramuzzino and Jennifer McNiff for their feedback on the manuscript.

This work was supported by grants from the American Heart Association, the National Institutes of Health (AI-28944); the Swedish Medical Research Council (9926); and the Ax:son Johnson, Bergvall, Crafoord, Kock, and Österlund Foundations. M.D.S. is the recipient of a graduate scholarship from the Foundation for Strategic Research (Infection and Vaccinology program). D.E.B. is an Established Investigator of the American Heart Association.

REFERENCES

1. **Barnhill, R. L. (ed.).** 1998. Textbook of dermatopathology. McGraw-Hill, New York, N.Y.
2. **Beall, B., R. Facklam, J. Elliott, A. Franklin, T. Hoenes, D. Jackson, L. Laclaire, T. Thompson, and R. Viswanathan.** 1998. Streptococcal *emm* types associated with T-agglutination types and the use of conserved *emm* gene restriction fragment patterns for subtyping group A streptococci. J. Med. Microbiol. **47:**1–6.
3. **Beall, B., R. Facklam, and T. Thompson.** 1996. Sequencing *emm*-specific PCR products for routine and accurate typing of group A streptococci. J. Clin. Microbiol. **34:**953–958.
4. **Berge, A., and U. Sjobring.** 1993. PAM, a novel plasminogen-binding protein from *Streptococcus pyogenes*. J. Biol. Chem. **268:**25417–25424.
5. **Bessen, D., K. F. Jones, and V. A. Fischetti.** 1989. Evidence for two distinct classes of streptococcal M protein and their relationship to rheumatic fever. J. Exp. Med. **169:**269–283.
6. **Bessen, D. E., M. W. Izzo, T. R. Fiorentino, R. M. Caringal, S. K. Hollingshead, and B. Beall.** 1999. Genetic linkage of exotoxin alleles and *emm* gene markers for tissue tropism in group A streptococci. J. Infect. Dis. **179:**627–636.
7. **Bessen, D. E., M. W. Izzo, E. J. McCabe, and C. M. Sotir.** 1997. Two-domain motif for IgG-binding activity by group A streptococcal *emm* gene products. Gene **196:**75–82.
8. **Bessen, D. E., C. M. Sotir, T. L. Readdy, and S. K. Hollingshead.** 1996. Genetic correlates of throat and skin isolates of group A streptococci. J. Infect. Dis. **173:**896–900.
9. **Boyle, M. D. P.** 1995. Variation of multifunctional surface binding proteins: a virulence strategy for group A streptococci. J. Theor. Biol. **173:**415–426.
10. **Carlsson Wistedt, A., H. Kotarsky, D. Marti, U. Ringdahl, F. J. Castellino, J. Schaller, and U. Sjobring.** 1998. Kringle 2 mediates high affinity binding of plasminogen to an internal sequence in streptococcal surface protein PAM. J. Biol. Chem. **273:**24420–24424.
11. **Carlsson Wistedt, A. C., U. Ringdahl, W. Muller-Esterl, and U. Sjobring.** 1995. Identification of a plasminogen-binding motif in PAM, a bacterial surface protein. Mol. Microbiol. **18:**569–578.
12. **Christner, R., Z. Li, R. Raeder, A. Podbielski, and M. Boyle.** 1997. Identification of key gene products required for acquisition of plasmin-like enzymatic activity by group A streptococci. J. Infect. Dis. **175:**1115–1120.
13. **Coleman, J., J. Gebbia, J. Piesman, J. Degen, T. Bugge, and J. Benach.** 1997. Plasminogen is required for efficient dissemination of *B. burgdorferi* in ticks and for enhancement of spirochetemia in mice. Cell **89:**1111–1119.
14. **Edwards, D., and G. Murphy.** 1998. Proteases: invasion and more. Nature **394:**527–528.
15. **Fiorentino, T. R., B. Beall, P. Mshar, and D. E. Bessen.** 1997. A genetic-based evaluation of principal tissue reservoir for group A streptococci isolated from normally sterile sites. J. Infect. Dis. **176:**177–182.
16. **Heiple, J. M., and L. Ossowski.** 1986. Human neutrophil plasminogen activator is localized in specific granules and is translocated to the cell surface by exocytosis. J. Exp. Med. **164:**826–840.

16a. **Infotech in the Biotechnology Core Facility Branch Website.** 17 May 1999, revision date. [Online.] National Center for Infectious Diseases, Centers for Disease Control and Prevention. http://www.cdc.gov/ncidod/biotech/infotech_hp.html. [16 June 1999, last date accessed.]

17. **Johnsson, E., A. Thern, B. Dahlback, L. O. Heden, M. Wikstrom, and G. Lindahl.** 1996. A highly variable region in members of the streptococcal M protein family binds the human complement regulator C4BP. J. Immunol. **157:**3021–3029.
18. **Kehoe, M. A.** 1995. Cell wall-associated proteins in Gram-positive bacteria. New Compr. Biochem. **27:**217–261.
19. **Lever, W., and G. Schaumberg-Lever.** 1990. Histopathology of the skin, 7th ed. J. B. Lippincott Co., Philadelphia, Pa.
20. **Lottenberg, R., C. C. Broder, M. D. P. Boyle, S. J. Kain, B. L. Schroeder, and R. I. Curtiss.** 1992. Cloning, sequence analysis, and expression in *Escherichia coli* of a streptococcal plasmin receptor. J. Bacteriol. **174:**5204–5210.
21. **Martin, P.** 1997. Wound healing: aiming for perfect skin regeneration. Science **276:**75–81.
22. **Mignatti, P., E. Robbins, and D. Rifkin.** 1986. Tumor invasion through the human amniotic membrane: requirement for a protein cascade. Cell **47:**487–498.
23. **Montgomery, H.** 1967. Dermatopathology. Harper & Row, New York, N.Y.
24. **O'Reilly, M., L. Holmgren, Y. Shing, C. Chen, R. Rosenthal, M. Moses, W. Lane, Y. Cao, E. Sage, and J. Folkman.** 1994. Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. Cell **79:**315–328.
25. **Pancholi, V., and V. A. Fischetti.** 1998. Alpha-enolase, a novel strong plasmin(ogen) binding protein on the surface of pathogenic streptococci. J. Biol. Chem. **273:**14503–14515.
26. **Poon-King, R., J. Bannan, A. Viteri, G. Cu, and J. Zabriskie.** 1993. Identification of an extracellular plasmin binding protein from nephritogenic streptococci. J. Exp. Med. **178:**759–763.
27. **Ringdahl, U., M. Svensson, A. Wistedt, T. Renné, R. Kellner, W. Müller Esterl, and U. Sjöbring.** 1998. Molecular co-operation between protein PAM and streptokinase for plasmin acquisition by *Streptococcus pyogenes*. J. Biol. Chem. **273:**6424–6430.
28. **Romer, J., T. H. Bugge, C. Pyke, L. R. Lund, M. J. Flick, J. L. Degen, and K. Dano.** 1996. Impaired wound healing in mice with a disrupted plasminogen gene. Nat. Med. **2:**287–292.
29. **Sodeinde, O., Y. Subrahmanyam, K. Stark, T. Quan, Y. Bao, and J. Goguen.** 1992. A surface protease and the invasive character of plague. Science **258:**1004–1007.
30. **Swartz, M.** 1995. Cellulitis and subcutaneous tissue infections, p. 909–929. *In* G. Mandell, J. Bennett, and R. Dolin (ed.), Principles and practice of infectious diseases, 4th ed. Churchill Livingstone, New York, N.Y.
31. **Top, F. H., L. W. Wannamaker, W. R. Maxted, and B. F. Anthony.** 1967. M antigens among group A streptococci isolated from skin lesions. J. Exp. Med. **126:**667–685.
32. **Vassalli, J.-D., A.-P. Sappino, and D. Belin.** 1991. The plasminogen activator/plasmin system. J. Clin. Investig. **88:**1067–1072.
33. **Wang, H., R. Lottenberg, and M. D. Boyle.** 1995. A role for fibrinogen in the streptokinase-dependent acquisition of plasmin(ogen) by group A streptococci. J. Infect. Dis. **171:**85–92.
34. **Young, K., G. Shi, D. Wu, L. Chang, B. Chang, C. Ou, and H. Wu.** 1998. Plasminogen activation by streptokinase by a unique mechanism. J. Biol. Chem. **173:**3110–3116.

Editor: E. I. Tuomanen