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Expression of both M protein and hyaluronic acid capsule by group A streptococcal strains results in a high virulence for chicken embryos

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Abstract The human pathogenic microorganism *Streptococcus pyogenes* can resist against phagocytic attack of human granulocytes. Streptococcal M protein and hyaluronic acid were identified as virulence factors involved in this protection. So far, no experiments have been reported which describe the contribution of both components together in one system. We used the chicken embryo as an in vivo phagocytosis model to investigate the role of both components on the virulence of streptococci. For this, isogeneic mutants of group A streptococcal strains (GAS) which lack hyaluronic acid capsule (cap^-) or M protein (M^-) expression were used for infection and their virulence was compared with laboratory strains which had lost their ability to produce one or both virulence factors after longtime laboratory passages on blood agar. The experiments revealed that strains producing both M protein and hyaluronic capsule were highly virulent. Only 1–10 colony-forming units were enough to cause a 50% lethality of 12-day-old chicken embryos. Those strains lacking one of these components showed a significant decrease in virulence. Finally, strains which failed to express either hyaluronic acid or M protein showed an additional tenfold decrease in virulence. This indicates a partial contribution of both M protein and hyaluronic acid to the virulence of GAS in the chicken embryo.

Key words *Streptococcus pyogenes* · Isogeneic mutants · M protein · Hyaluronic acid · Virulence

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Abbreviations cap^+ or cap^- hyaluronic acid capsule positive or negative strains · *FBG* fibrinogen · *FN* fibronectin · *GAS* group A streptococci · *HA* hyaluronic acid · *HSA* human serum albumin · M^+ or M^- M protein expressing or not expressing strains

Introduction

Human pathogenic group A streptococci (GAS) are the etiological agents for a broad spectrum of different infections including streptococcal pharyngitis, impetigo, soft tissue infections which may develop into the more serious forms necrotizing fasciitis, and/or streptococcal toxic shock [1–3]. These latter diseases are associated with a high morbidity and mortality. In addition, GAS are often associated with the postinfectious autoimmune syndromes of acute rheumatic fever and poststreptococcal glomerulonephritis [4–6].

GAS produce a variety of surface components and extracellular products contributing to the pathogenicity. The components protecting the bacteria against phagocytic attack play an important role in the pathogenic process. Streptococcal M proteins are considered to be the major surface components responsible for resistance of GAS against phagocytosis [7] and have been also described to be involved in adhesion [8, 9]. They belong to a family of surface proteins with regions of close sequence homologies and show a broad binding spectrum to different plasma and tissue proteins [10–16]. The adjacent genes in the chromosome encoding M-like proteins are regulated by the virR gene. The whole gene segment is called vir-regulon and can be divided in three types which differ in the number of encoding genes for M-like proteins [17]. However, the question of which of the M-like protein(s) expressed by one strain is/are antiphagocytic and how these proteins interfere in the phagocytic process have not been answered so far. The affinity of M-like proteins to plasma proteins seems to contribute to the protection of streptococci against phagocytosis. The coating of GAS with fibrinogen resulted

in a decreased phagocytosis by granulocytes [18, 19], whereas cells with an albumin layer were increasingly ingested [19]. The albumin coat, however, seemed to prevent intracellular killing of phagocytized streptococci by the granulocytes [19].

Recent results have shown that hyaluronic acid (HA) can also impair phagocytosis of mucoid GAS by human granulocytes. Using genetically defined acapsular mutants of *Streptococcus pyogenes*, an increase of phagocytized bacteria by granulocytes and a decrease in virulence for mice were reported [20, 21]. Moreover, HA of GAS has been shown to play a critical role in pharyngeal colonization and infection in mice [22]. One antiphagocytic effect of HA is its ability to prevent streptococcal attachment to phagocytic cells [23].

The aim of this study was to investigate how both M protein and HA, separately and together, influence the virulence of streptococci. We used the chicken embryo model as an in vivo system, since we were recently able to demonstrate that strains expressing proteins of the M family, with affinity for fibrinogen, albumin and/or IgG, showed an increased virulence for 12-day-old chicken embryos [24]. Genetically characterized GAS mutants which lack expression of HA capsule (cap^-) or M protein (M^-) were compared in this study with the appropriate wild-type strains. Furthermore, selected laboratory strains differing in expression of M protein or HA were also examined. The experiments revealed that strains producing both M protein and HA were extremely virulent, and those strains lacking M protein or HA showed a significantly decreased virulence.

Materials and methods

Streptococcal strains and growth

Streptococcal strains are listed in Table 1. Streptococci were grown for 18 h at 37C in 20 ml Todd-Hewitt broth. For the chicken embryo assay 2-ml aliquots of the cultures were separated and the bacteria were diluted in Todd-Hewitt broth. For bacterial binding assay, cells from the large volumes were sedimented by centrifugation and washed twice with saline.

Chemicals and techniques

Human serum albumin (HSA), and peroxidase were purchased from Sigma Chemical Co. (St. Louis, Mo.). Human IgG and IgA came from Serva (Heidelberg, Germany) and human fibrinogen was obtained from A. B. Kabi (Stockholm, Sweden). A number of proteins were conjugated with peroxidase as previously described [24] using the periodate oxidation method [25]. The production of HA by the streptococcal strains was estimated by the turbidimetric method of DiFerrante [26]. The range of turbidity was qualitatively estimated in the range from negative (–) to positive (+++) using the cap^- strain ATCC 12344 as a negative control and the cap^+ strain ATCC 12363 as a positive control.

Dot binding of plasma proteins to streptococci

This assay was used to measure the expression of M and M-like receptor proteins on the streptococcal cell surface. Proteins of the M family can bind to different human plasma proteins like albumin, fibrinogen and different subclasses of IgG [10, 12, 16, 19, 27]. We measured the binding by immobilizing the whole bacteria onto nitrocellulose membranes and then incubating the membranes with different peroxidase-labeled plasma proteins as described recently [24]. Briefly, streptococci were washed twice with saline and the bacterial number was adjusted to 10^8 cells/ml. From each suspension 100 µl, in serial dilutions, were applied to nitrocellulose membrane using a dot blot apparatus. Membranes were air dried and blocking was performed with 5% skimmed milk in phosphate-buffered saline-Tween 20. The binding was assayed by incubation of strips with peroxidase-labeled human proteins as described previously [14].

Chicken embryo assay

Fertile White Leghorn hens eggs were set in a humidified, self-turning incubator maintained at 37 °C. Dose-lethality curves were established by i.v. inoculation (usually through the chorioallantoic vein) of 12-day-old chicken embryos with increasing numbers of GAS in 100 µl Todd-Hewitt broth by the method of Tiefenberg et al. [28]. Colony-forming units (CFU) were determined using a counting chamber; within long chains in each case three cells were counted as one CFU. After inoculation appropriate dilutions of the remaining bacteria were spread on blood agar plates to verify the number of applied bacteria. At least twelve eggs were infected in parallel with each streptococcal dilution. The LD_{50} and LD_{90} were determined by probit analysis utilizing the course of lethality. Viability was checked for the subsequent 5 days by daily candling. From eggs in which the embryos had been died after infection, bacteria were reisolated from the allantoic fluid and plated on blood agar. Such passaged cap^- mutants were tested to determine whether revertant bac-

Table 1 Streptococcal strains. Strain M1 40/58 is the strain SF 130/13 Griffith from the Public Health Reference Laboratory London and is filed in the WHO Laboratory Prague under the number 40/58. This strain is the parent strain of AT1 SF 130, which lost its ability to express M protein by passages on blood agar plates in our laboratory over more than 20 years

Strain	Relevant features	Source/reference
AM1 40/58[a]	M type 1, (M^+), grows in human blood, (cap^-)	WHO Laboratory, Prague
AT1 SF 130[a, b]	T type 1, (M^-) does not grow in human blood, (cap^-)	German Streptococcal Reference Center, Jena
NY5[b]	T type 10, does not grow in human blood, (cap^+; M^-)	Statens Serum Institute Copenhagen
87-282	M type 18, mucoid (cap^+; M^+)	[20]
Tx4[c]	M type 18, nonmucoid (cap^-; M^+)	[20]
T24	M type 24, mucoid (cap^+; M^+)	[21]
T24-4[c]	M type 24, nonmucoid (cap^-; M^+)	[21]
T24Ω[c]	M type 24, mucoid (cap^+; M^+)	[8]

[a] In a screening of a number of strains these strains did not show hyaluronic acid production (Günther and Ozegowski, unpublished)
[b] Spontaneous diminution of M protein expression during laboratory passages
[c] Genetically manipulated "knock-out" mutants

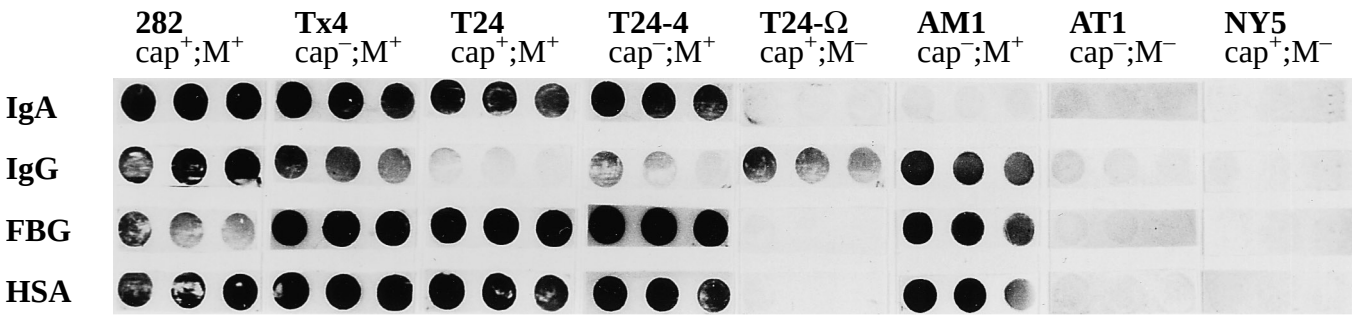

Fig. 1 Binding of the peroxidase-labeled plasma proteins human serum albumin (*HSA*), fibrinogen (*FBG*), IgG, and IgA to streptococcal cells blotted onto nitrocellulose membrane. The hyaluronic acid capsule did not interfere the binding. Most M^- strains lost binding to the applied plasma proteins, with the exception of the mutant T24-Ω, which seems to express a second, not knocked-out M-like protein with affinity for IgG. The cells were applied in three serial dilutions starting from the left with 10^8 cells/ml

Table 2 Hyaluronic acid production of the investigated strains before and after infection of fertile eggs

Strain	Before	After passage
87-282	++	++
Tx4	–	–
T24	++	+++
T24-4	–	–
T24-Ω	+	++
AM1	–	–
AT1	–	–
NY5	+	+

teria had developed, by estimating the production of HA by the method of DiFerrante [26]. The M^- mutant T24-Ω, after reisolation, was grown under selective conditions with 500 µg/ml kanamycin and the bacteria were tested for changes in expression of plasma protein-binding proteins (see above).

Results

Expression of proteins of the M family

Members of the M protein family are known to bind to different plasma proteins. In our experiments we investigated the binding of peroxidase-labeled HSA, fibrinogen, IgG and IgA directly at the cell surface of streptococci by the bacterial dot binding assay (Fig. 1). This assay confirmed recent results [20] that the capsulated strains 87-282 and T24 and the noncapsulated isogenic mutants T×4 and T24-4 maintained expression of M protein and M-like proteins to the same degree. As illustrated in Fig. 1, these strains showed the same binding pattern independently of whether they produced HA or not. On the other hand, the lack of M24 protein in the M^- mutant T24-Ω abolished the binding to fibrinogen, albumin and IgA seen in the wild-type strain T24. However, the IgG binding of the M^- mutant T24-Ω was preserved. The IgG-binding protein seemed to be more accessible at the cell surface of the M^- mutant than at the cells of wild-type strain T24. Perhaps the loss of M protein increases access to other surface proteins.

We also included type 1 strains AM1 and AT1 in our experiments. Both these strains lack the ability to produce HA in vitro. The parent strain AM1, which has been previously shown to express M1 protein and protein H at the cell surface [10, 27] showed a high expression of M1 protein, indicated by affinity to fibrinogen (Fig. 1 and [14]). Protein H does not bind to fibrinogen [10, 27]. The blood agar passaged strain AT1 failed to bind any of the plasma proteins, indicating that neither M1 protein nor protein H is expressed, although the DNA of strain AT1 hybridized with a probe (Schmidt and Gubbe, unpublished) encoding for the consensus sequences of the three C class I repeats of M1 protein [10].

The strain NY5 (cap^+; M^-) did not express plasma protein-binding proteins at the cell surface (Fig. 1), but did produce HA acid (Table 2).

Virulence in chicken embryo

Isogenic mutants

Table 3 shows the results of the chicken embryo virulence test. The wild-type strains T24 and 87-282 producing both HA capsule and plasma protein-binding proteins of the M family were highly virulent for chicken embryos. Only 1 CFU of strain T24 and 12 CFU of strain 87–282 were enough to cause a 50% death rate. The appropriate cap^- isogenic mutants T24-4 and T×4, however, were 100 times less virulent, as indicated by their LD_{50} and LD_{90}. The M^- isogenic mutant T24-Ω, which still produce HA (cap^+), also showed a significant decreased virulence.

Laboratory strains

The laboratory strain AM1, which lacks the ability to produce HA, exhibited a decreased virulence similar to the cap^- mutants T24-4 and T×4. The M^- variant AT1, also cap^-, was 10 times less virulent than strain AM1. Strain NY5, which was found to produce HA but not M or M-like proteins at the cell surface, also showed decreased virulence. However, using this strain, we did not obtain a LD_{100} with up to 10^5 CFU.

Thus, the stepwise decrease of virulence, found with the mutant strains after inactivation of HA production or of M

Table 3 Virulence in chicken embryos of group A streptococcal (GAS) strains with hyaluronic acid capsule (cap^+), without capsule (cap^-), expressing M protein (M^+) or not (M^-). (*CFU* Colony-forming unit, *n.t.* not tested)

CFU	Strains							
	87–282 cap^+; M^+	Tx4 cap^-; M^+	T24 cap^+; M^+	T24-4 cap^-; M^+	T24-Ω cap^+, M^-	AM1 cap^-; M^+	AT1 cap^-; M^-	NY5 cap^+; M^-
1×10^5	n.t.	n.t.	n.t.	n.t.	23/23	13/13	10/10	11/12
1×10^4	n.t.	n.t.	n.t.	n.t.	20/20	10/14	14/20	9/13
1×10^3	16/16	21/32	n.t.	25/41	18/24	5/12	3/11	12/24
1×10^2	22/26	11/27	17/17	15/39	16/24	4/15	3/11	10/24
1×10^1	10/28	3/12	18/18	1/22	4/12	4/14	1/12	9/24
1×10^0	3/12	n.t.	12/18	n.t.	1/12	n.t.	n.t.	4/12
Control TH[a]	2/15	2/15	1/15	0/8	1/14	2/18	2/18	2/12
LD_{50} (CFU)	1.2×10^1	2.1×10^2	$<1\times 10^0$	4×10^2	7×10^1	3.5×10^2	1.5×10^3	3.5×10^2
LD_{90} (CFU)	2.1×10^2	4×10^4	1.8×10^1	1.2×10^4	2×10^3	2.8×10^4	8.9×10^4	1.5×10^6

[a] Todd-Hewitt (TH) broth was injected

protein expression, was also confirmed with the laboratory isolates. Although, with the laboratory strains we did not screen for deficiencies other than that for M protein and HA, the experiments with these strains fully supported the results obtained with the isogenic mutants that both M protein and HA contribute to the virulence of GAS.

Stability of HA and M protein production

All strains were tested before and after infection of the chicken embryos for production of HA and M protein. None of the mutant or variant strains changed their ability to produce M protein after two passages in the developing embryo. The strains isolated from the allantoic fluid after infection showed the same binding pattern to plasma proteins as seen before injection (Fig. 1). The M^- mutant T24-Ω maintained its ability to grow in the presence of kanamycin. The same was true for the production of HA. None of the cap^- mutants or the cap^- laboratory strains reverted to HA production after multiplication in the infected chicken embryo (Table 2). All of the cap^+ strains still produced HA.

Discussion

The current studies show that both M protein and the HA capsule of GAS are important antiphagocytic surface components that supplement each other to make a strain highly virulent. The loss of expression of one of these components led to a significant stepwise decrease in the virulence of the tested GAS strains in chicken embryos. Until now only the separate contribution of the HA capsule [20, 21] or M protein [7] to phagocytosis of GAS has been investigated. A decreased virulence in mice described recently with HA-negative mutants [20, 21] could also be confirmed in the chicken embryo. Earlier studies revealed that the antiphagocytic activity of the HA capsule could be demonstrated more clearly using animal phagocytes rather than human granulocytes [20, 23, 29].

The antiphagocytic role of M protein(s) has been investigated extensively and the results have been recently reviewed [30]. Human granulocytes were found to be best suited for investigation of the protective contribution of M protein [7]. Animal models only functioned with a few strains of GAS.

Recently, we showed that an i.v. infection of chicken embryos of about 12 days old with GAS could be used to investigate of phagocytosis of GAS and to study their virulence in vivo [24]. In the 12-day-old chicken embryo the phagocytic system is fully developed [29, 30]; however, lymphocytes as effector cells of immune response are not detected until shortly before hatching [30]. We, therefore, used this model to investigate the contribution of both HA and M protein to the antiphagocytic activity of GAS.

The results showed a graduated virulence between the tested strains. Only a few CFU were required to cause lethality of the chicken embryos if the cap^+ M^+ wild-types strains 87-282 and T24 were used for infection. The lack of expression of HA and/or M protein significantly decreased the virulence of the strains, demonstrated using the isogenic mutants cap^- M^+ T×4 and T24-4 as well as the cap^+ M^- mutant T24-Ω. Infections with the laboratory strains cap^- M^+ AM1, cap^- M^- AT1, and cap^+ M^- NY5 led to the same results seen with the isogenic insertion mutants, e.g., strain AM1 (cap^- M^+) showed the same virulence as the cap^- M^+ mutants T×4 and T24-4. The two strains AT1 and NY5, which did not express plasma-binding M-like proteins at the cell surface, showed the weakest virulence. The chicken embryo model proved to be a suitable in vivo test system for investigating the influence of different streptococcal components on the virulence of these bacteria.

In the chicken embryo model such strains lacking either M protein or HA or both of these components did not lose their virulence completely, suggesting that additional factors can influence their virulence for chicken embryos. One such factor could be the production of streptolysins O

and S, which can destroy the nearly unprotected blood cells in the developing chicken embryo. This is the subject of an investigation now underway.

Bacteria can often use more than one mechanism to survive in the macroorganism. This study demonstrates that the protection of GAS against phagocytic attack is multifunctional, mediated in part by two biochemically different components, M protein(s) and the carbohydrate HA. The expression and action of these surface components in concert afford increased resistance to phagocytosis and enhance the virulence of the microorganism.

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