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Restricted Ability of Group B Streptococcal C5a-ase To Inactivate C5a Prepared from Different Animal Species

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Most strains of group B streptococci (GBS) elaborate a cell surface-associated enzyme that rapidly inactivates the human complement-derived chemoattractants C5a and $C5a_{desarg}$ by cleaving the His-Lys bond at positions 67 and 68 in the C5a molecule. We have suggested that rapid inactivation of C5a and $C5a_{desarg}$ by this enzyme, called C5a-ase, can hinder the inflammatory response at sites of GBS infection. We tested the ability of GBS C5a-ase to inactivate C5a preparations from various animal species to determine the proper species for studying the role of GBS C5a-ase in the pathogenesis of GBS infections. Exposure of C5a preparations from humans, monkeys, and cows to GBS caused inhibition of C5a functional activity as measured by the ability of C5a to stimulate human polymorphonuclear leukocyte (PMN) adherence and human PMN chemotaxis. Bovine PMN chemotaxis to bovine C5a was also abolished after exposure of bovine C5a to GBS. In contrast, mouse, rat, guinea pig, rabbit, pig, and sheep C5a preparations retained full functional activity after exposure to GBS as measured by chemotaxis of human PMNs, PMNs from the same animal species, or both. These data suggest that there are structural differences between C5a proteins from different species which alter their susceptibility to GBS C5a-ase and indicate that most commonly used animal models of human GBS infection are inadequate for detection of a contribution of GBS C5a-ase to GBS virulence.

Group B streptococci (GBS) are important pathogens in human neonates and immunocompromised hosts (1). Infections in neonates can be overwhelming and are characterized by poor polymorphonuclear leukocyte (PMN) influx into infected tissues (14). The reason for this poor PMN response is not completely understood.

The PMN chemoattractant C5a is a cleavage product of complement component C5 that is produced when the complement cascade is activated. Human C5a is a 74-amino-acid peptide that is rapidly converted to $C5a_{desarg}$ in serum by the action of carboxypeptidase N, which removes the carboxy-terminal Arg from the molecule (18). C5a and $C5a_{desarg}$ are the major chemoattractants for PMNs produced after serum complement is activated (9). We have previously reported that many clinical isolates of GBS elaborate a cell-associated enzyme that rapidly inactivates C5a and $C5a_{desarg}$ (15). This enzyme, which we have have named C5a-ase, cleaves human C5a between the His and Lys present at amino acid positions 67 and 68 near the carboxy terminus of the C5a molecule. This cleavage drastically reduces the ability of the C5a molecule to bind to its PMN receptor and destroys the agonist activity of C5a for human PMN adherence and chemotaxis (3). This loss of activity following truncation of the carboxy terminus of C5a is consistent with previous experimental evidence demonstrating the critical importance of the carboxy terminus of C5a for complete agonist activity (18, 21).

C5a-ase is associated with the bacterial surface, but the enzyme can be released from the bacteria by enzymatic digestion of the cell wall. The soluble enzyme has been purified and is a protein of approximately 120,000 Da (5). Neutralizing antibodies directed against the soluble enzyme are frequently found in sera of normal adults, but C5a-ase on the surface of encapsulated type III GBS is not neutralized by these antibodies. This resistance to neutralization appears to be due to a protective effect of the capsule, since C5a-ase associated with the surface of unencapsulated type III GBS is neutralized by serum antibodies (4). The capsule is a major virulence factor for GBS. While the increased virulence of encapsulated strains is felt to result from the ability of the capsule to prevent opsonization of the bacteria by complement (19), the ability of C5a-ase to evade antibody neutralization on encapsulated GBS could further contribute to virulence.

We have postulated that this enzyme plays a role in the pathogenesis of human GBS infections by blunting the C5a-mediated proinflammatory response. To test this hypothesis, it is necessary to examine the contribution of C5a-ase to the virulence of GBS infections in experimental animals. It is not known, however, whether GBS C5a-ase also inactivates C5a from animals. Rats, mice, pigs, sheep, rabbits, and monkeys have been used as animal models of human GBS infection (8, 13, 23–25). In addition, GBS are an important cause of bovine mastitis (20). We therefore examined the ability of GBS C5a-ase to inactive C5a preparations from these and other animals.

(Portions of this work were submitted as part of a thesis for a Master of Science degree in Medical Laboratory Science at the University of Utah by Ju-Kay Chang.)

MATERIALS AND METHODS

Preparation of C5a. Recombinant human C5a (rhC5a) was purchased (Sigma, St. Louis, Mo.). Native C5a was partially purified by the method of Gerard and Hugli (12) from sera obtained from humans or from one of the following animal species: monkey (*Macaca nemestrina*), pig, cow, sheep, rabbit, rat, mouse, and guinea pig. Sera were treated with 2 mM 2-mercaptomethyl-3-guanidinoethylthiopropanoic acid (Plummer's Inhibitor; Calbiochem, La Jolla, Calif.) to inactivate carboxypeptidase N, the enzyme which removes the

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carboxy-terminal Arg from the C5a molecule, and then mixed with 10 mg of zymosan (Schwartz Biotech, Cleveland, Ohio) per ml of serum for 60 min at 37°C to activate complement. Zymosan was removed by centrifugation, and most of the protein was precipitated by bringing the supernatant to 1 N HCl. The precipitates were removed by centrifugation at 40,000 × *g* for 25 min, and the supernatants, which contain C5a, were dialyzed exhaustively against phosphate-buffered saline (PBS), pH 7.4. These preparations are hereafter referred to as C5a.

Treatment of C5a with GBS C5a-ase. GBS strains 7360, COH-1, and GW are type III clinical isolates of GBS. Strains 7360 and COH-1 express C5a-ase, whereas GW does not (4). Z223 is a transposon-induced mutant strain of COH-1 which lacks detectable C5a-ase activity (16). C5a was exposed to GBS that express C5a-ase or GBS that do not express C5a-ase (as a control) as previously described (3). Briefly, frozen aliquots of GBS were thawed, inoculated into Todd-Hewitt broth, grown overnight at 37°C, pelleted, washed with PBS, and adjusted to an A_{620} of 0.9 (5×10^8 CFU/ml). GBS were pelleted, the supernatant was removed, and the pellet was suspended in rhC5a at 1 μg/ml or a 1:10 dilution of C5a in Hanks buffered salt solution (HBSS) containing 1% human serum albumin at a ratio of 1 ml of C5a to 10^9 CFU of GBS. The C5a and GBS were incubated together with mixing for 30 min at 37°C for 30 min, and the GBS were removed by centrifugation. The C5a was then frozen until being tested for activity.

Isolation of PMNs. Human PMNs were isolated from heparinized peripheral blood of normal adult donors by dextran sedimentation, centrifugation through Ficoll-Paque (Pharmacia, Piscataway, N.J.), and hypotonic lysis, as previously described (3). Pig PMNs were isolated from heparinized pig blood by the same procedure used for human PMNs (7). Bovine PMNs were prepared by centrifuging acid citrate glucose-anticoagulated blood at 1,000 × *g* for 30 min at room temperature. The buffy coat was removed and pelleted, and the erythrocytes were hypotonically lysed (0.2% NaCl followed by 1.6% NaCl). The leukocytes were then pelleted, washed, and resuspended for use in the chemotaxis assay (6). To prepare sheep PMNs (22), heparinized sheep blood was centrifuged at 1,000 × *g* for 15 min and the buffy coat and the lowest 25% of the erythrocyte pellet were pooled, following which the contaminating erythrocytes in these leukocyte fractions were lysed by hypotonic lysis. Rat PMNs were prepared by dextran sedimentation of 5 ml of heparinized whole blood with 1 ml of 2% dextran for 20 min at 37°C. The leukocyte-rich plasma was removed and then pooled with the erythrocyte pellet after the contaminating erythrocytes were removed by hypotonic lysis. Mouse PMNs were isolated from a mouse peritoneum 4 h after injection of 1% glycogen in PBS.

The percentages of PMNs in the preparations, as determined by 1% gentian violet stain in 1.5% acetic acid, were as follows: human, >95%; pig, 70 to 98%; cow, 70 to 90%; sheep, 40 to 50%; rat, 30 to 40%; mouse, 40 to 94%.

PMN adherence assay. Functional C5a activity was determined by the ability of C5a to stimulate PMN adherence of ^{111}In-labeled human PMNs to gelatin-coated plastic dishes as previously described in detail (3). Briefly, isolated PMNs were labeled with [^{111}In]oxine and suspended in HBSS containing 0.5% human serum albumin at 5.5×10^6/ml. PMNs (225 μl per well) were incubated with 25 μl of the appropriate agonist in gelatin-coated 16-mm-diameter tissue culture wells for 5 min at 37°C. Nonadherent cells were collected by aspiration and a single wash with HBSS. Adherent cells were lysed with 1 M NH_4OH and counted in a gamma counter. The percentage of adherent cells was calculated by dividing the counts per minute of the adherent fraction by the sum of the counts per minute of the adherent and nonadherent fractions. Each experiment was done with a single point for the indicated condition.

PMN chemotaxis. PMN chemotaxis to C5a was determined in 48-well microchemotaxis chambers (Neuroprobe, Cabin John, Md.) with 5 μm-pore-size Micropore filters (Millipore Corp., Bedford, Mass.) between the upper and lower wells, as previously described (3, 15). Portions (50 μl) of PMNs (2×10^6/ml in HBSS containing 10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid] and 1% human serum albumin) were added to the upper well of the chamber, and the C5a preparations, diluted in HBSS–1% human serum albumin, were added to the lower well. The chambers were incubated at 37°C for 2 h, and the filters were removed and stained with hematoxylin. The number of PMNs that had migrated completely through 10 random ×400 fields per filter (the chemotactic index) was determined by microscopy with a photographic reticle in the eyepiece. Each experiment was performed by using two to four replicates for each condition.

Immunoblot of rat C5a. Rat C5a was partially purified from zymosan-activated rat serum by 1 N HCl precipitation and treated with GBS strains that either did or did not express C5a-ase as described above. C5a preparations were then subjected to electrophoresis on sodium dodecyl sulfate (SDS)–18% polyacrylamide gel electrophoresis (PAGE) under reducing conditions as previously described (5) and transferred to nitrocellulose paper by the method of Towbin et al. (27). The nitrocellulose paper was blocked with 5% nonfat dry milk in 0.01 M Tris–0.145 M NaCl, pH 8, containing 0.5% Tween (TBST). The paper was then incubated for 1 h with a 1:1,000 dilution of polyclonal rabbit antiserum raised against purified rat C5a (a kind gift of Tony Hugli). After being washed three times with TBST, the paper was incubated with a 1:1,000 dilution of alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (Sigma) for 1 h at room temperature and washed with TBST three times. Bands that bound alkaline phosphatase-conjugated antibody were visualized with nitroblue tetrazolium and 5-bromo-4-chloroindoxylphosphate (Sigma) as the substrate (2).

Statistics. Differences between conditions were statistically determined by using Student's *t* test.

RESULTS

We previously showed that stimulation of PMN adherence to gelatin-coated plastic is a very sensitive assay for determining rhC5a activity (3). In the studies reported here, we tested the ability of C5a prepared from various animal species to stimulate human PMN adherence. As shown in Fig. 1, increases in PMN adhesion are directly related to rhC5a concentration, with a detectable increase in PMN adherence occurring after addition of as little as 1 ng of rhC5a per ml. As expected, C5a partially purified from the zymosan-activated sera of humans, monkeys, cows, rats, sheep, and rabbits also stimulated rapid PMN adhesion in vitro in a concentration-dependent manner, with an apparent plateau in PMN response occurring with increasing concentrations of human, monkey, cow, and rat C5a proteins.

As expected, treatment of rhC5a with a strain of GBS which expresses the C5a-ase enzyme abolished the PMN adhesion response to C5a (Table 1). Likewise, native C5a

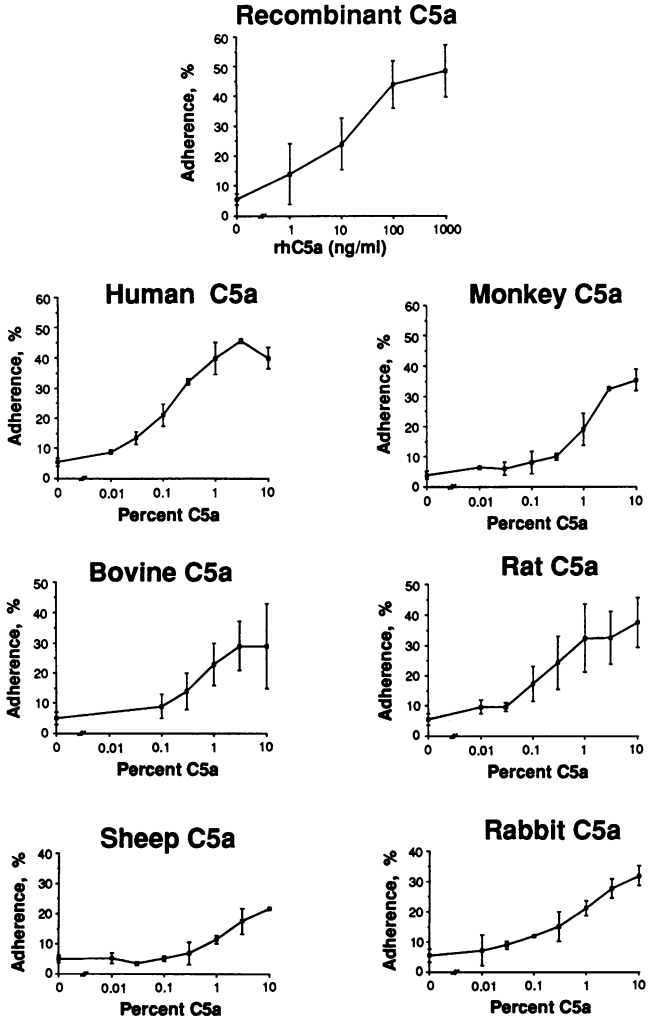

FIG. 1. Response of human PMN adherence to increasing concentrations of rhC5a or partially purified human, monkey, bovine, rat, sheep, or rabbit C5a. Data are expressed as the means ± the standard deviations of the following numbers of experiments: rhC5a, $n = 5$; human, $n = 5$; monkey, $n = 3$; bovine, $n = 4$; rat, $n = 7$; sheep, $n = 2$; rabbit, $n = 3$. The numbers on the abscissa refer to the final concentrations of the C5a preparations present in the adherence assay.

proteins prepared from human, monkey, and cow sera were also inactivated by exposure to a C5a-ase-bearing strain of GBS, as determined by the ability of the treated C5a to activate human PMN adherence (Table 1). In contrast, exposure of sheep, rabbit, or rat C5a to GBS C5a-ase did not result in a significant loss of the ability of the C5a preparations to stimulate human PMN adherence (Table 1). Exposure of C5a to GBS that do not express C5a-ase did not significantly reduce the activity of all of the C5a preparations (Table 1).

These experiments suggested that sheep, rabbit, and rat C5a proteins are resistant to GBS C5a-ase. A hypothetical problem, however, was that other factors present in these C5a preparations also stimulate PMN adherence, and if so, these other agonists could mask loss of C5a activity. We therefore utilized the PMN chemotactic response to measure C5a activity more specifically, since most of the chemotactic

TABLE 1. Ability of GBS C5a-ase to inactivate C5a with human PMN adherence as an indicator

C5a species (concn; n)	Mean % adherence[a] ± SD			
	Buffer	C5a	C5a + C5a-ase-positive GBS	C5a + C5a-ase-negative GBS
Recombinant human (100 ng/ml; 5)	5 ± 2	44 ± 8	7 ± 3[b]	36 ± 10[c]
Human (1%; 5)	5 ± 2	40 ± 9	10 ± 4[b]	34 ± 9[c]
Monkey (1%; 3)	4 ± 1	19 ± 5	6 ± 1[b]	15 ± 3[c]
Cow (1%; 4)	5 ± 3	22 ± 6	9 ± 7[b]	20 ± 8[c]
Sheep (1%; 2)	5 ± 1	12 ± 1	11 ± 2[c]	12 + 5[c]
Rabbit (1%; 3)	6 ± 2	21 ± 2	16 ± 4[c]	24 ± 7[c]
Rat (1%; 7)	5 ± 2	32 ± 11	29 ± 7[c]	38 ± 5[c]

[a] rhC5a or C5a prepared from the indicated species was incubated with either a strain of GBS that expresses C5a-ase (7360) or a strain of GBS that does not express C5a-ase (GW) (as a control). Treated C5a and untreated C5a were then tested for the ability to stimulate human PMN adherence as described in Materials and Methods.

[b] $P < 0.01$ compared with untreated C5a.

[c] $P > 0.1$ compared with untreated C5a.

activity present in serum after complement activation is derived from C5a and $C5a_{desarg}$. In addition to the C5a preparations used in the adherence experiments, C5a preparations from pig, guinea pig, and mouse sera were tested for the ability to stimulate human PMN chemotaxis in a modified Boyden chamber. All of the preparations of C5a were found to induce PMN chemotaxis in a concentration-dependent manner (Fig. 2). The C5a preparations were treated with C5a-ase-positive and -negative strains of GBS and tested for the ability to induce human PMN chemotaxis by using concentrations of C5a below that which caused a plateau in the PMN response. As previously reported (3), the ability of rhC5a to induce chemotaxis was significantly reduced by treatment with GBS C5a-ase (Table 2). Consistent with the results of the PMN adherence assay, the chemoattractant activity of native human, monkey, and cow C5a preparations for human PMNs was significantly reduced by exposure to strains of GBS that express C5a-ase but not by exposure to strains that do not express C5a-ase (Table 2). In contrast, GBS C5a-ase did not inactivate the chemoattractant activity of the other C5a preparations (sheep, pig, guinea pig, rabbit, rat, or mouse) for human PMNs (Table 2).

We also tested the ability of GBS C5a-ase to inactivate C5a prepared from five of these animal species as measured by the chemotactic response of PMNs isolated from the same animal species. As shown in Table 3, PMNs prepared from cows, pigs, mice, rats, or sheep exhibited chemotaxis in response to C5a prepared from the same animals. Consistent with the results obtained with human PMNs, treatment of bovine C5a GBS C5a-ase completely abolished the bovine PMN chemotactic response to bovine C5a, while treatment of pig, sheep, rat, and mouse C5a preparations with C5a-ase did not affect their ability to act as chemoattractants for PMNs prepared from the same species.

The inability of GBS C5a-ase to inactivate rat C5a is interesting since rat C5a has a His-Lys bond in the same position as human C5a (Fig. 3) (17). This raises the possibility that rat C5a is cleaved by C5a-ase at this His-Lys bond but retains full functional activity in its truncated form. However, rat C5a that had been exposed to GBS C5a-ase showed no discernible decrease in molecular mass on SDS-PAGE, as analyzed by immunoblotting (Fig. 4). This result is

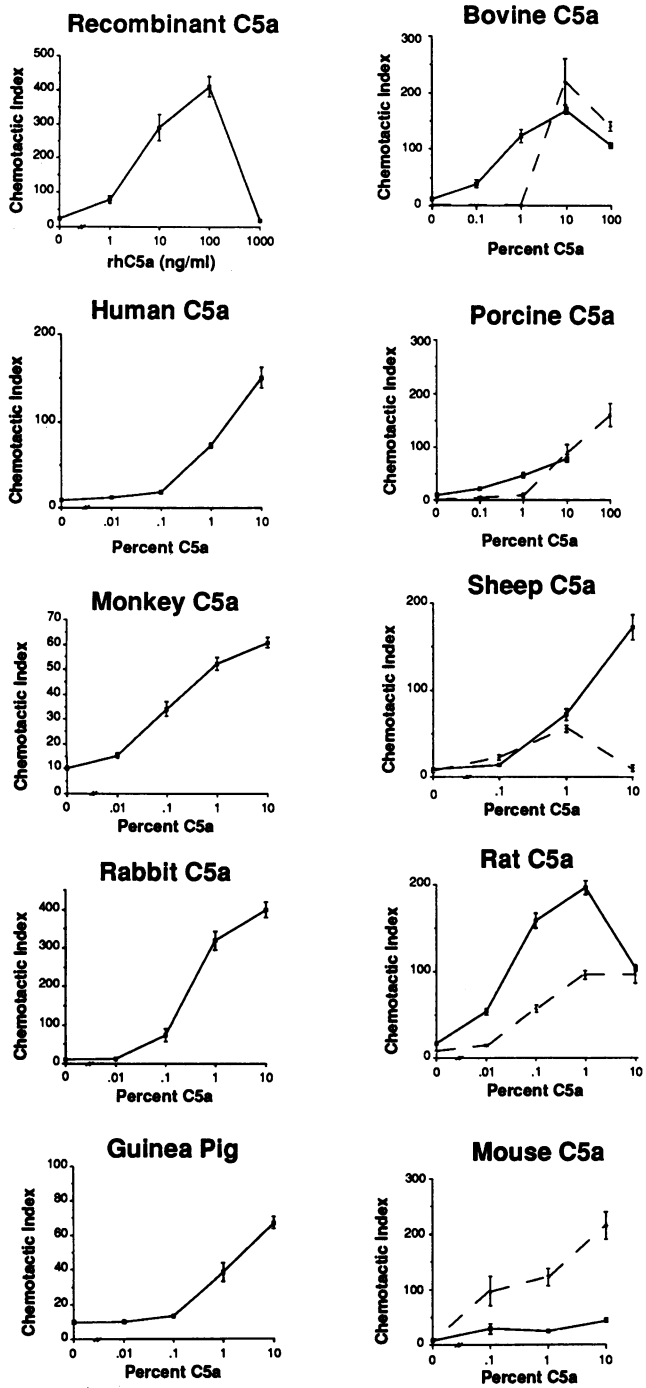

FIG. 2. Concentration response of human (——) or animal (– – –) PMN chemotaxis to increasing concentrations of rhC5a or partially purified human or animal C5a. Animal PMNs were isolated from the same species as the C5a. The data shown are the means ± the standard errors of the means of the following minimum numbers of determinations: rhC5a, $n = 9$; human C5a, $n = 8$; monkey C5a, $n = 12$; rabbit C5a, $n = 4$; guinea pig C5a, $n = 12$; bovine C5a with human PMNs, $n = 11$, except 0.01 and 100%, where $n = 4$; bovine C5a with bovine PMNs, $n = 16$; pig C5a with human PMNs, $n = 30$; pig C5a with pig PMNs, $n = 24$; mouse C5a with human PMNs, $n = 16$; mouse C5a with mouse PMNs, $n = 13$; rat C5a with human PMNs, $n = 12$; rat C5a with rat PMNs, $n = 7$; sheep C5a with human PMNs, $n = 8$; sheep C5a with sheep PMNs, $n = 4$. The numbers on the abscissa refer to the concentrations of the C5a preparations present in the lower chamber of the chemotactic apparatus.

TABLE 2. Ability of GBS C5a-ase to inactivate C5a with human PMN chemotaxis as an indicator

C5a species (concn; n)	Mean chemotactic index[a] ± SEM			
	Buffer	C5a	C5a + C5a-ase-positive GBS	C5a + C5a-ase-negative GBS
Recombinant human (100 ng/ml; 12)	24 ± 2	412 ± 20	54 ± 10[b]	351 ± 32[c]
Human (1%; 8)	9 ± 3	70 ± 2	12 ± 2[b]	61 ± 1[c]
Monkey (10%; 12)	10 ± 1	61 ± 2	28 ± 2[b]	53 ± 3[c]
Cow (1%; 7)	13 ± 1	123 ± 12	63 ± 9[b]	110 ± 11[c]
Sheep (1%; 8)	11 ± 2	72 ± 10	76 ± 10[c]	64 ± 10[c]
Pig (10%; 30)	9 ± 1	79 ± 7	74 ± 10[c]	69 ± 12[c]
Guinea pig (10%; 12)	10 ± 1	67 ± 3	60 ± 3[c]	51 ± 2[c]
Rabbit (1%; 4)	11 ± 1	319 ± 24	357 ± 39[c]	347 ± 33[c]
Rat (0.1%; 12)	17 ± 1	159 ± 8	168 ± 11[c]	174 ± 9[c]
Mouse (10%; 11)	7 ± 1	44 ± 4	45 ± 5[c]	53 ± 5[c]

[a] C5a preparations were incubated with a strain of GBS that expresses C5a-ase (7360 or COH-1) or a strain of GBS that does not express C5a-ase (GW or Z223) and tested for the ability to stimulate human PMN chemotaxis. The concentrations of rhC5a or the C5a preparations in the lower wells of the chemotaxis chambers are shown in parentheses.
[b] $P < 0.01$ compared with untreated C5a.
[c] $P > 0.1$ compared with untreated C5a.

distinct from the detectable decrease in relative molecular mass seen on SDS-PAGE following cleavage of human C5a with C5a-ase (3, 5, 15). This suggests that rat C5a is not cleaved by GBS C5a-ase, despite the presence of a His-Lys bond near its carboxy terminus.

DISCUSSION

The studies reported here show that GBS C5a-ase inactivates C5a preparations from humans, monkeys, and cows but not C5a preparations from six other animal species. We previously showed that GBS C5a-ase cleaves the His-Lys bond present near the carboxy terminus of human C5a. This His-Lys bond is conserved in the C5a proteins of all five species whose amino acid sequences have been deduced (Fig. 3). Thus, it seems likely that the monkey and bovine C5a proteins are also inactivated by enzymatic cleavage at a His-Lys bond. There are several possible explanations for the resistance of the C5a proteins of the other six species to

TABLE 3. Ability of GBS C5a-ase to inactivate animal C5a with PMNs from the same species as an indicator[a]

C5a species (concn; n)	Mean chemotactic index ± SEM			
	Buffer	C5a	C5a + C5a-ase-positive GBS	C5a + C5a-ase-negative GBS
Cow (10%; 16)	1 ± 1	188 ± 36	10 ± 6[b]	277 ± 38[c]
Pig (10%; 17)	1 ± 1	90 ± 15	75 ± 11[c]	85 ± 14[c]
Sheep (1%; 4)	7 ± 1	56 ± 4	68 ± 5[c]	70 ± 2[c]
Rat (0.1%; 8)	10 ± 1	55 ± 8	62 ± 14[c]	70 ± 13[c]
Mouse (1%; 14)	6 ± 1	123 ± 14	156 ± 14[c]	159 ± 11[c]

[a] C5a preparations were incubated with a strain of GBS that expresses C5a-ase (7360 or COH-1) or a strain of GBS that does not express C5a-ase (GW or Z223) and tested for the ability to stimulate chemotaxis in PMNs prepared from the same animal. The concentrations of the C5a preparations in the lower wells of the chemotaxis chambers are shown in parentheses.
[b] $P < 0.01$ compared with untreated C5a.
[c] $P > 0.1$ compared with untreated C5a.

	65					70					
Human	N	I	S	H	K	D	M	Q	L	G	R
Cow	D	E	H	H	K	N	M	Q	L	G	R
Pig	E	E	S	H	K	N	I	Q	L	G	R
Mouse	E	S	P	H	K	P	V	Q	L	G	R
Rat	N	Q	S	H	K	G	M	L	L	G	R

FIG. 3. Carboxy-terminal amino acid sequence of human (10), cow (11), pig (12), mouse (28), and rat (17) C5a proteins. The numbers refer to the amino acid positions in human C5a. The boxed portion indicates the C5a-ase cleavage point.

inactivation by C5a-ase. The first is that some of these C5a molecules lack the His-Lys bond, although this is not the case for the pig, mouse, and rat proteins. Western blot analysis of C5a-ase-treated rat C5a (Fig. 4) supports the hypothesis that the His-Lys bond in rat C5a is resistant to cleavage by C5a-ase because of the conformation of the rat C5a molecule. A second possibility is that C5a-ase does cleave the C5a of some of the species but the residual C5a molecules retain full agonist activity. This implies that the carboxy terminus of the C5a molecule in some species contributes relatively less to the agonist potency of the molecule. This situation would be quite different from the well-studied structure-function relationship between human C5a and its agonist properties, in which truncation of the carboxy terminus, by either enzymatic degradation or site-directed mutagenesis, drastically reduces the agonist activity of C5a (18, 21). Finally, differences in posttranslational modification of the various C5a molecules might alter the susceptibility of the C5a proteins of different species to cleavage by GBS C5a-ase. The only known posttranslational modification of C5a occurs in human C5a, which has an oligosaccharide side chain covalently attached to amino acid 64. This oligosaccharide chain is unlikely to account for the species specificity of GBS C5a-ase, since rhC5a and the bovine and porcine C5a proteins lack this modification (11, 12). Testing these various hypotheses requires further experimentation.

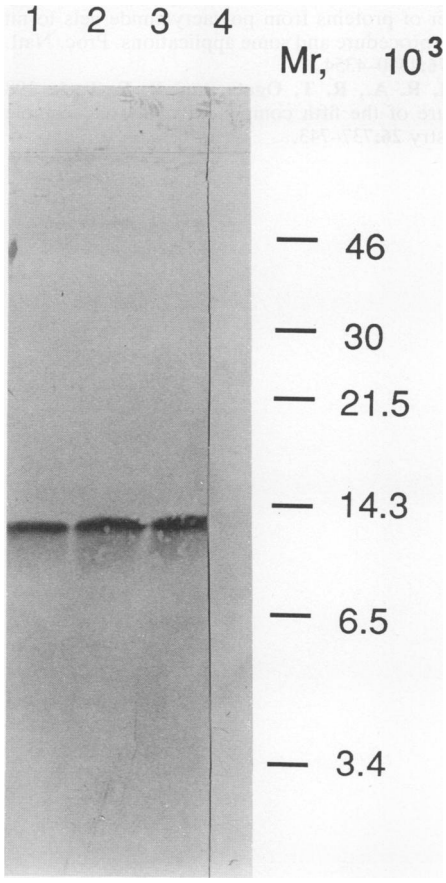

FIG. 4. Immunoblot of rat C5a treated with GBS strains that either express or lack C5a-ase. Lanes: 1 and 4, rat C5a; 2, rat C5a treated with a GBS that expresses C5a-ase; 3, rat C5a treated with a GBS that does not express C5a-ase; 1 to 3, rabbit anti-rat C5a; 4, preimmune rabbit serum.

Animals which have been used as models of human GBS infection include monkeys (24), pigs (13), sheep (23), rats (26), rabbits (25), and mice (8). Except for monkeys, the ability of GBS C5a-ase to cleave C5a cannot contribute to the pathophysiology of experimental GBS infections in these animal models. This limitation should be considered when extrapolating results obtained with these animal models to human infections. GBS is also an important pathogen in cows, as an agent of bovine mastitis (20), and we speculate that the ability of C5a-ase to inactivate bovine C5a contributes to the pathogenesis of that disease.

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