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Molecular Species of R-Protein Antigens Produced by Clinical Isolates of Group B Streptococci

AUREA E. FLORES AND PATRICIA FERRIERI*

Department of Laboratory Medicine and Pathology and Department of Pediatrics, University of Minnesota Medical School, Minneapolis, Minnesota 55455

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Clinical isolates of group B streptococci from body fluids and mucosal surfaces were examined for production of a trypsin-resistant antigen known as R protein. R protein was extracted with 1% trypsin from cells grown in a semidefined medium. The extracts were tested by immunodiffusion in agarose with a panel of antisera for detection and precise identification of the four species of R protein described by Wilkinson. R antigen was present in 49 of 131 (37%) of the strains tested. Analysis by serotype revealed that 0 of 2 type Ia, 0 of 11 Ib, 1 of 16 (6%) Ia/c, 12 of 15 (80%) II, 0 of 20 II/c, 35 of 49 (71%) III, 0 of 6 IV, and 1 of 12 (8%) nontypeable strains produced R antigen. Production of the R protein and the trypsin-resistant or α component of the c protein appeared to be mutually exclusive. R antigen was more prevalent in isolates from blood (50%) than in those from mucosal sites (27%) for type II strains; no difference was seen for type III strains from these sites. Concordant results were obtained with five paired body fluid-mucosal surface isolates from individual patients and with isolates from 17 mother-baby pairs. The most frequent species of R antigen was R4 (45 of 49), followed by R1 (4 of 49). These two species of R protein were biochemically (trypsin resistant and pepsin sensitive) and immunologically identical to the R-protein antigens produced by prototype strains of groups A, B, and C streptococci.

The R-protein antigen was described first in group A type 28 streptococci by Lancefield and Perlmann (13). This protein was named R for its trypsin-resistant nature, which contrasts with that of M protein. R antigen has been found also in some strains of group A streptococci of other types, as well as in groups B, C, F, G, and L (11, 13, 17–19, 22, 25).

R proteins of various immunological specificities have been described (12, 25). Wilkinson has classified the R antigens of some strains of streptococci from groups A, B, and C into four species (1, 2, 3, and 4) according to their immunoprecipitin reactions in agarose (25).

Since R protein is not produced by all strains within any streptococcal group or type, it is of interest to study the prevalence of this marker, especially among group B streptococci (GBS). Data from Jelinkova indicate that 11% of human GBS typed from 1960 to 1975 in the Prague reference laboratory produced R antigen and that the antigen was found most often in type II strains (7). Jensen reported that in urogenital patients, type III/R was the most common serotype, accounting for 36% of the isolates (8). Recently, Linden et al. found that among strains from mothers and babies, R protein was present in 51% of type II and 87% of type III strains (16).

We have studied 131 strains of GBS representing various serotypes obtained from body fluids (blood or cerebrospinal fluids [CSF]) or mucosal surfaces to determine the prevalence of R protein among these strains, as well as to identify the specific species of R antigen produced by them. An additional 22 isolates were studied to investigate the concordance of results in body fluid-mucosal surface isolates from the same patient and from mother-baby paired specimens.

MATERIALS AND METHODS

Strains. Reference R-protein-positive streptococcal strains were obtained from R. Lancefield and J. Jelinkova (Prague). These included group A strains of type 3 (D58X), 28 (B960), 33 (C107/24/8), 43 (C126/59/6), and 48 (C510); group B prototype III (D136C) and nontypeable (NT) (Compton 25/60) strains; and a group C (B337) strain.

A total of 131 clinical strains of GBS representing serotypes Ia, Ib, Ia/c, II, III, IV, and NT were tested for the production of R antigen. These strains had been isolated from blood and CSF or from mucosal surfaces (vagina, cervix, ear, nares, umbilicus) of mothers and infants or both. Isolates from more than one body fluid or site of an individual patient and isolates from mother-infant pairs were excluded to avoid skewing of results. Twenty-two additional isolates were studied to form body fluid-mucosal surface or mother-baby pairs. Isolates had been stored lyophilized or as frozen log-phase cultures in Todd-Hewitt broth at −70°C.

Antisera. A panel of rabbit antisera was available in our laboratory for the detection of the various species of R-protein antigen. Antisera were prepared with Formalin-killed cells of group B prototype III strain 71-735 for detection of R1 and with L-phase cells of wild-type III strain 76-043 for detection of R4. R2 was detected with group A M-33 serum from Lancefield, which contained antibodies to the M protein and this species of R protein. Serum with antibodies to R3 and R4 was provided by Jelinkova. Specific antisera were available also for grouping, typing, and detection of the individual components of the c protein, formerly known as the Ibc protein (6, 9).

Growth conditions and extraction procedures. For identification of R protein, cultures were grown overnight at 35°C in Carey chemically defined medium supplemented with acid-hydrolyzed casein (1). Cells were harvested by centrifugation, washed once with sterile saline, and extracted for 2 h at 37°C with 1% trypsin (Sigma Chemical Co., St. Louis, Mo.) in Sorensen buffer (0.067 N, pH 8.2).

For grouping, typing, and detection of the components of the c protein, the strains were grown overnight at 35°C in

* Corresponding author.

Todd-Hewitt broth. The cells were harvested and extracted with hot HCl (10).

Immunological methods. To detect the R antigen, trypsin extracts were tested against the panel of R antisera by immunodiffusion in 0.8% agarose plus 1% polyethylene glycol (5). Extracts of the R prototype strains were included to identify precipitin reactions. HCl extracts of the group B isolates were tested by immunodiffusion in agarose for type-specific carbohydrate (23) and for the trypsin-sensitive (TS), or β, and trypsin-resistant (TR), or α, components of the c protein (9). After overnight incubation at room temperature, slides were pressed, washed, and stained with Coomassie brilliant blue. Grouping was done by the capillary precipitin method with the HCl extracts (24). Statistical analysis of the results was done with the chi-square test.

Concentrated culture supernatants. The R antigen secreted into the culture supernatant was studied in a limited number of isolates. The supernatants from overnight cultures in Carey medium were saved after the cells were removed by centrifugation. They were filtered through 0.45-μm filters and either processed immediately or stored at −20°C. The supernatants were dialyzed extensively against Tris buffer (0.05 M, pH 8.0) (Sigma) and then concentrated 25- to 100-fold with Carbowax (polyethylene glycol compound 20; Union Carbide, Institute, W. Va.). After brief dialysis, the concentrated supernatants were tested by immunodiffusion.

Enzyme studies. The R protein antigens in 1% trypsin extracts or concentrated culture supernatants were treated either with 5% trypsin in Sorensen buffer (pH 8.2) or with 0.5% pepsin (Sigma) in acidified distilled water (pH 2.0) (4, 26). A drop of enzyme solution was added to 0.2-ml volumes of the 1% trypsin cell extract or of the concentrated supernatant. These volumes had been adjusted to pH 8.2 (with 5 N NaOH) or pH 2.0 (6 N HCl). Diluents were added to the control tubes. After incubation for 2 h at 37°C, the pHs of all volumes were adjusted to neutrality. Treated, untreated, and control samples were tested by immunodiffusion to observe the effects of these two enzymes or of the pH on the R antigen.

RESULTS

Extraction of cells of GBS strains grown in the semidefined medium with 1% trypsin gave excellent yields of R-protein antigen. This method produced extracts that gave stronger precipitin reactions, particularly for R2 and R3, than extracts made with hot HCl. With our panel of antisera, the four species of the R antigen could be detected easily in the trypsin cell extracts or in concentrated culture supernatants. The immunoprecipitin reactions in agarose of the species of R protein in our extracts were identical to those of species from prototype strains. In addition, the species of R antigens of the clinical isolates, like those of the R-protein prototype strains, were resistant to treatment with 5% trypsin (some species of R more than others) but sensitive to digestion with 0.5% pepsin (data not shown).

The R-protein antigen was detected in the cell extracts of 49 (37%) of the 131 clinical isolates of GBS examined (Table 1). The R1 species was detected in 4 isolates, and the R4 species was detected in 45 isolates. Neither R2 nor R3 could be detected in any of the 131 isolates. R antigen was detected most frequently in the cell extracts of type II strains without the c antigen and of type III strains (80 and 71%, respectively). Of particular interest was the observation that none of the type II/c isolates possessed the R antigen (0 of 20), while 12 of 15 (80%) of type II/non-c isolates did have R protein ($P = 0.0001$). R antigen was not found in any of the type Ia, Ib, or IV isolates.

TABLE 1. Species of R-protein antigen detected in cell extracts from clinical isolates[a] of group B streptococci

Serotype	No. tested (% positive)	No. of isolates with R-protein species[b]:	
		1	4
Ia	2		
Ib	11		
Ia/c	16 (6)	1	
II	15 (80)		12
II/c[c]	20		
III	49 (71)	2	33
IV	6		
NT	12 (8)	1	
Total	131 (37)	4	45

[a] From body fluids (blood, CSF) and mucosal surfaces.
[b] Detected in 1% trypsin cell extracts of strains by immunoprecipitin reaction in agarose. Classification of R proteins was done by the method of Wilkinson (25). No isolates had R-protein species 2 or 3.
[c] Type II polysaccharide and one or both components of the c-protein antigen.

When the presence of R protein was examined in relation to the source of isolation (blood, CSF, mucosal surfaces, or unknown), it was found that in the type III isolates, the R antigen was detected in almost the same percentage of blood and CSF isolates (17 of 26 [65%]) and of mucosal surface isolates (17 of 21 [80%]) (Table 2). With type II isolates, however, R antigen was found almost twice as often in isolates from blood and CSF (6 of 12 [50%]) as in isolates from mucosal surfaces (6 of 22 [27%], $P = 0.01$). R antigen was detected in approximately the same percentage of the total isolates from blood (38%), CSF (35%), mucosal surfaces (40%), and all sources (37%).

The relationship of the R-protein antigen to the TR and TS components of the c-protein antigen in clinical isolates was also examined (Table 3). The results are summarized for all serotypes. The R antigen was not detected in any of 13 isolates containing the TR and TS components of the c protein or in any of 41 isolates with the TR component only. The presence of R protein correlated highly with the absence of both components of the c protein (47 of 65 versus 2 of 66, $P = 0.0001$) and in particular with the absence of the TR component (49 of 77 versus 0 of 54, $P = 0.0001$). However, R protein was present in 2 of 12 isolates which contained the

TABLE 2. Distribution by clinical source of isolates with R-protein antigen in serotypes of group B streptococci

Serotype	R-antigen detection in strains isolated from[a]:				% Detection
	Blood	CSF	Mucosal surfaces	Unknown	
Ia	0/0	0/2	0/0	0/0	
Ib	0/5	0/2	0/4	0/0	
Ia/c	0/12	0/3	1/1	0/0	6
II	6/12	0/0	6/22	0/1	34
III	9/11	8/15	17/21	1/2	71
IV	0/0	0/1	0/4	0/1	
NT	0/0	0/0	1/11	0/1	8
Total (%)	15/40 (38)	8/23 (35)	25/63 (40)	1/5 (20)	

[a] Detected in 1% trypsin cell extracts by immunoprecipitin reaction in agarose. Values are number positive/number tested.

TABLE 3. Relationship of R-protein antigen to TR and TS components of c-protein antigen in clinical isolates of group B streptococci[a]

c-Protein component[b]	No. of isolates with R antigen[c]/no. tested
TR + TS	0/13
TR only	0/41
TS only	2/12
Neither	47/65

[a] Results summarized for all serotypes.
[b] Detected in Lancefield hot-HCl cell extracts by immunoprecipitin reaction in agarose (23).
[c] Detected in 1% trypsin cell extracts by immunoprecipitin reaction in agarose. R antigen was present in 37% of the isolates tested.

TS component only (one type Ia/c isolate and one nontypeable isolate).

Since the majority (47 of 49) of the isolates possessing R antigen were either type II or type III, the relationship of the c and R proteins was examined in these two serotypes (Fig. 1). Type II isolates were more likely to have the c protein (57%) than the R antigen (34%), while the majority of type III strains had R antigen (71%) and only a few (2%) had the c protein. Neither the c nor the R antigen was detected in 3 (9%) of the type II isolates and 13 (27%) of the type III isolates.

When the distribution of trypsin-resistant proteins on the 131 clinical isolates of GBS was examined, it was observed that the R4 species of the R antigen and the TR component of the c protein were detected most frequently (34 and 41%, respectively) (Fig. 2). R1 was detected in 3% of the isolates. Thus, a trypsin-resistant protein was found in 79% of all clinical GBS isolates.

Paired body fluid-mucosal surface specimens from five individual patients were examined for the presence of the R antigen (Table 4). Concordant results were obtained with four pairs which produced R antigen and one pair that did not. Likewise, 100% concordance was obtained with 17

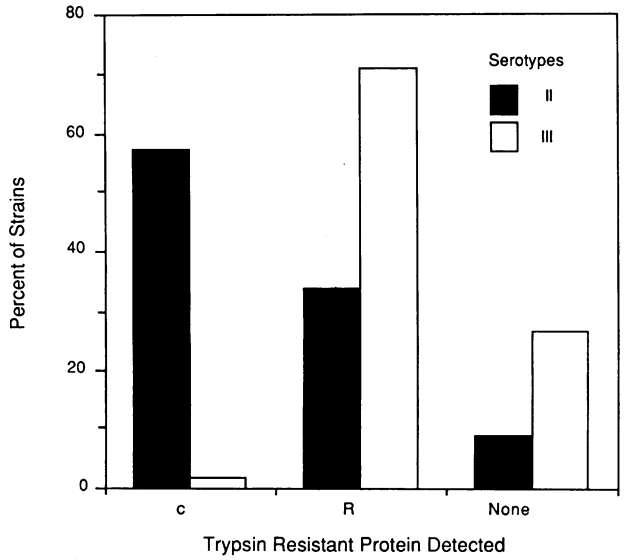

FIG. 1. Distribution of c and R proteins in clinical isolates of serotypes II and III group B streptococci. Cell extracts of 35 type II and 49 type III group B streptococci were examined by immunodiffusion in agarose with a panel of rabbit antisera for the four species of R antigen and the two components of c antigen.

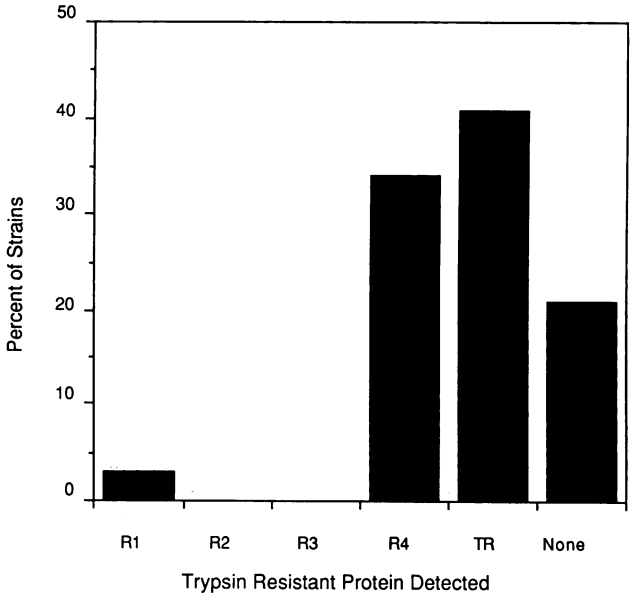

FIG. 2. Presence of trypsin-resistant proteins in cell extracts from 131 clinical isolates of group B streptococci. Extracts were tested by immunodiffusion in agarose for the presence of trypsin-resistant proteins. Each extract was tested against a panel of rabbit antisera to detect the R1, R2, R3, and R4 species of R antigen and the TR component of c antigen.

mother-baby paired isolates (Table 5). In 14 pairs, the R antigen was detected in the isolates of both the mother and her baby (4 pairs colonized with type II and 10 pairs colonized with type III). In two type III and one type IV pairs, the R antigen was not found in the isolates of either the mother or her baby. Congruence was also observed, since the same species of R antigen (R4) was produced by isolates of paired specimens, whether from body fluid-mucosal surfaces or from mother-baby pairs.

DISCUSSION

The cell extracts obtained by our method of using 1% trypsin, a modification of that of Lancefield and Perlmann (13), gave better results than did hot HCl extracts. The biochemical and immunological reactions of the species of R antigen detected in our clinical isolates were characteristic of R protein (13) and identical to those of R protein prototype strains of group A, B, and C streptococci (25). Only the R1 and R4 species of R were detected among our GBS clinical isolates, with R4 being the predominant species of R found.

TABLE 4. Presence of R protein in strains of group B streptococci isolated from paired body fluid-mucosal surface specimens

Serotype	No. of pairs of body fluid[a]-mucosal surface isolates		
	R protein present[b]	R protein absent	Concordant results[c]
II	2	0	2
III	2	1	3

[a] Blood or CSF.
[b] Detected in 1% trypsin cell extracts of isolates by immunoprecipitin reaction in agarose.
[c] Concordant results in both isolates.

TABLE 5. Presence of R protein in strains of group B streptococci isolated from mother-baby pairs

Serotype	No. of pairs of mother-baby isolates		
	R protein present[a]	R protein absent	Concordant results[b]
II	4	0	4
III	10	2	12
IV	0	1	1

[a] Detected in 1% trypsin cell extracts of isolates by immunoprecipitin reaction in agarose.
[b] Concordant results in both isolates.

In our isolates, R1 and R4 were detected singly, and none of the isolates appeared to possess both species. This is in contrast to most of the R-protein prototype strains from group A and C streptococci examined by Wilkinson (25), which had more than one species.

R antigen was detected in 37% of the 131 clinical GBS isolates that we examined. This is slightly lower than the prevalence of R antigen (46%) in GBS strains reported by Linden et al. (16) or the prevalence (44%) found by Jensen (8). Our finding, however, is higher than that of Jelinkova (7), who reported that 11% of GBS strains possessed R antigen. The differences may reflect variation in the antigenic composition of strains from various times and geographical locations, or they may be due to differences in sampling or methodology or both.

Among the GBS clinical isolates examined, R protein was found almost exclusively in serotypes II and III. It was found rarely in Ia/c and NT strains but was not found in any of the 19 isolates of serotypes Ia, Ib, and IV examined. Similar findings have been obtained by other investigators (8, 16).

Of interest was the fact that there may be some relationship, although not yet understood, between the surface-localized R- and c-protein antigens of GBS. This was exemplified by the detection of R antigen in 80% of type II isolates without the c antigen but in none of 20 type II/c protein isolates. There was a highly significant difference in the prevalence of isolates with R antigen among strains without c protein (47 of 65 [72%]) compared with strains with TR and/or TS components of the c protein (2 of 66 [3%]). This negative correlation between the c and R proteins appeared to be associated with the TR component in particular, since R protein was not detected in any of 54 isolates with TR, while R protein was found in 49 of 77 (64%) of isolates without the TR component of the c protein.

Because of the apparent relationship between R and c proteins, and because 91% of type II and 73% of type III isolates possessed either R or c protein, the occurrence of each was examined in the isolates of these two serotypes. The occurrence of the two proteins showed quite different patterns in the two serotypes. Type III isolates were more likely to have R protein, while type II isolates were more likely to have c protein. Jensen (8) reported similar findings; i.e., a predominance of R protein in type III isolates but of c protein in the type II strains.

When the presence of the TR component of c protein was plotted along with the various molecular species of R as individual categories of trypsin-resistant proteins, it was evident that the R4 species and the TR component were detected most frequently among the 131 isolates studied. Overall, 79% of the isolates possessed a trypsin-resistant protein, while 21% did not. Therefore, trypsin-resistant proteins appeared to be significant in the surface-localized antigenic composition of GBS. The role of these proteins and of trypsin-sensitive proteins in the pathogenesis of infection and immunity to GBS infections is under study (2).

The high percentage of our isolates which possessed either R antigen or c protein (the TR component in particular) is of importance in view of the suggestion by some investigators that protein antigens may play a role in opsonophagocytosis of GBS, particularly of serotype II. Payne and colleagues (20, 21) suggested that the presence of the complete c protein may account for the resistance to opsonophagocytosis of some type II strains. Linden (14) observed that injection of rabbit anti-R-protein antibodies into mice protected the animals from subsequent challenge with type II but not with type III GBS. In addition, Linden et al. (15) found that adequate levels of immunoglobulin G antibody to R protein were critical in preventing serious infection during the neonatal period with type II or type III GBS which carried the R antigen. Although we did not examine sera for anti-R antibodies, we did find concordance with respect to the presence or absence of R, as well as to the species of R-protein antigen detected in 17 pairs of mother-baby isolates and in body fluid-mucosal surface paired isolates from five patients. These and similar observations (16) indicate that the R protein is a stable antigen on these strains and is expressed consistently.

Although the data presented were based on examination of 1% trypsin cell extracts, we also found the R protein in the culture supernatants. We had previously reported that the L phase of growth of a group B type III streptococcus released R protein, molecular species 4, into culture supernatants even after 254 serial subcultures in the cell wall-defective state (4). Thus, R antigen appears to be released readily into the growth medium like the TS and TR components of c protein, as described previously in reports from our laboratory (3, 9).

In summary, our findings are unique not only because we present data on the prevalence of the various molecular species of R antigen among clinical isolates of GBS, but also because we examined the same isolates for the occurrence of R protein and c protein and particularly its TR component. These findings may contribute to our understanding of the basic biology of GBS and, in particular, of the cell surface proteins of these bacteria and their interactions with the host.

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