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ANTIGENIC CHARACTERS OF CAMP-FACTORS PRODUCED BY DIFFERENT STRAINS OF STREPTOCOCCUS AGALACTIAE

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Abstract

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Antigenic relations of CAMP-factors produced by different strains of *Streptococcus agalactiae* were studied within the framework of this species. Prepurified forms of CAMP-factors produced by the serotypes Ia, Ib, Ic, II, III X, and R were used to prepare antisera by repeated intravenous injections into rabbits. Two techniques of precipitation were used for serological tests, namely the agar immunodiffusion with prepurified CAMP-factors as antigens and the modified Elek-Ouchterlony test employing the native form of this exosubstance. CAMP-Factors gave positive precipitation with homologous and heterologous rabbit antisera. Three precipitation lines were formed in the cases of homogeneity, one of them beeing very conspicuos, and two lines only originated when CAMP-factor and antiserum were heterologus. As a rule these two lines were faint. Our results proved existence of both specific and common antigenic fractions in CAMP-factors. The Elek-Ouchterlony test was carried out for investigation of 300 *S. agalactiae* strains, based on serotyping of their CAMP-factors. All strains were isolated by us from clinical materials, and they were tested with rabbit antisera to CAMP-factors of the serotype strains. Among 150 strains from humans, CAMP-factors reacted mostly in the same way as those of strains Ic and III, among 150 strains from bovine udders, in the same way as CAMP-factors of strains X and R.

native and prepurified CAMP-factors, serotypes of Streptococcus agalactiae, CAMP-factor antisera, agar immunodiffusion, Elek-Ouchterlony test.

The exosubstance of *S. agalactiae*, called CAMP-factor (P u l s f o r d 1954) is characterized by its synergistic hemolytic reaction with staphylococcal beta toxin (C h r i s t i e et al. 1944), or with corynebacterial phospholipase D (F r a s e r 1964; S k a l k a et al. 1979b) on sheep or bovine erythrocytes. This synergistic action was explained either by lipase activity of CAMP-factor (K a r C h o u d r y 1978) or by its binding to ceramide liberated by the prior action of staphylococcal sphingomyelinase on sheep erythrocytes (B e r n h e i m e r et al. 1979). Evidently, CAMP-factor is produced by all *S. agalactiae* strains, even during their growth in the L-phase (S k a l k a et al. 1979b; P h i l l i p s et al. 1980; F l o r e s and F e r r i e r i 1983). Antigenicity of CAMP-factor was described (B r o w n et al. 1974; S k a l k a et al. 1980; F l o r e s and F e r r i e r i 1983), as well as its toxic and even lethal effect for rabbit and mouse (S k a l k a and S m o l a 1981). The CAMP-factor producing streptococci belong to Lancefield group B according to the antigen present in their cells (L a n c e f i e l d 1933), nevertheless, a serological subdivision to types is possible within this group (L a n c e f i e l d 1934; P a t t i s o n et al. 1955; W i l k i n s o n and M o o d y 1969; J e l í n k o v á 1983 a,b; H e n r i c h s e n et al. 1984). The investigation of antigenicity of CAMP-factors produced by different *S. agalactiae* strains, and their diagnostical employment was the topic of the present study.

Materials and Methods

Media

Brain heart infusion CM225 and Brain heart infusion agar CM375 (Oxoid Ltd.) were used. For blood agar preparation, the solid base was supplemented by 5 % washed sheep erythrocytes and 2.5 mg $ml.^{-1}MgSO_4$.

Bacterial strains

S. agalactiae (Str B) from the Czechoslovak National Collection of Type Cultures (CNCTC) were used, namely 8/70 for type Ia, 9/70 for type Ib, 5/70 for type Ic, 3/70 for type II, 4/70 for type III, 24/60 for type X, 25/60 for type R, and 8/66 for the strain posessing exclusively the group antigen (Šourek 1981) and referred further as B.

Additionally employed streptococcal strains were *S. pyogenes* Str A 3/55 and 2/56, *S. zooepidemicus* Str C 4/49, *S. equisimilis* Str C 7/49, *S. dysgalactiae* Str C 12/77, *S. equi* Str C 3/78, and further Str E 42/59, Str F 4/65, Str P 19/55, Str P 21/55, Str U 1/70, Str V 1/75, all from CNCTC.

S. aureus CCM 6188 served both for routine CAMP-test and for preparing prepurified form of its beta toxin. The strain of Corynebacterium pseudotuberculosis CNCTC Cor 39/70 was used for hemolytic synergism test.

Moreover, 300 *S. agalactiae* strains were examined, 150 of them isolated from humans and 150 from bovine udders.

Prepurified bacterial exosubstance

CAMP-factors and staphylococcal beta toxin were prepared by aceton precipitation, examined fro their activity which is expressed in activity units (AU), and stored as described earlier (Skalka et al. 1979a). The term prepurified is used in order to distinguish this from the native and purified froms.

Assay for hemolytic synergism

All strains of streptococci were examined fro their hemolytic synergism with staphylococcal beta toxin and corynebacterial phospholipase D by a modified CAMP--test as described earlier (Skalka et al. 1980). Type strains of *S. agalactiae* and their CAMP-factors were also verified on blood agar with a staphylococcal beta toxin content of 7.5 AU ml^{-1}.

CAMP-factor antisera

CAMP-factor antisera were prepared by repeated intravenous injection of prepurified exosubstance into rabbits according a schema described earlier Skalka et al. 1980). CAMP-factors of *S. agalactiae* type culture were used.

The antisera gained were inactivated at 56°C for 30 min and absorbed with heat--killed pellets of homologous and heterologous *S. agalactiae* type strains. Then the antisera were centrifuged and their supernatants were collected and used.

A commercial antiserum for serological identification of group B streptococci, delivered by ÚSOL (Prague), was used for checking.

Serological assays for CAMP-factors

Serological assays were performed both with prepurified CAMP-factors and with their native forms, produced on agar media by the growth of *S. agalactiae* strains. Both forms of CAMP-factors were examined with homologous and heterologous sera.

In order to examine prepurified CAMP-factors, double agar immunodiffusion was performed. Wells of 1 cm diameter were cut in the medium at a distance of 1 cm each from the other. The wells were filled with the antisera of prepurified CAMP--factors under study. The dish was kept for 48 h at 37°C, and after this time the result was read.

A modification (Skalka and Švastová 1985) of the technique described by Elek (1948) and Ouchterlony (1948) was performed for native CAMP-factors. It was convenient both for one strain tested with all antisera, and for four strains tested simultaneously against one serum. In the first case, the streptococcal strain was streaked on agar medium in the middle of the Petri dish and the dish was incubated at 37°C for 24 h. Wells of 1 cm diameter were cut in the medium at a distance of 1 cm from the culture line. The wells were filled with the CAMP-factor antisera. The dish was kept for an

additional 48 h at 37°C, and after this time the result was read. When testing strains against one antiserum, four strains were streaked around one well. The rest of the proceeding remained unchanged.

Results

The production of CAMP-factor by different *S. agalactiae* serotype strains was not of equal intensity. Differences were to be observed both with the modified CAMP--test using *S. aureus* and *C. pseudotuberculosis* and with streptococcal strains growing on blood agar with staphylococcal beta toxin (Fig. 1). A similarly different

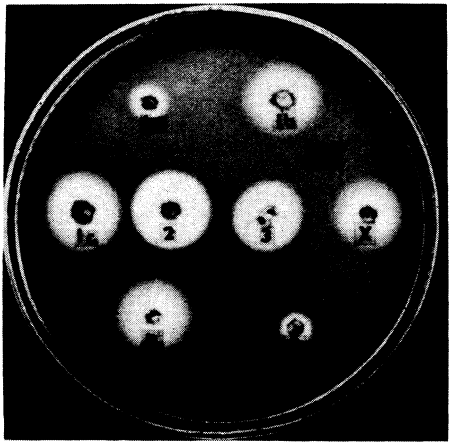

Fig. 1
Synergistic hemolytic effects of *S. agalactiae* serotype strains on nutrient medium (BT) containing 5 % washed sheep erythrocytes and staphylococcal beta toxin 7.5 AU ml^{-1}. The culture spots are marked according to the serotypes, with exception of II (=2) and III (=3).

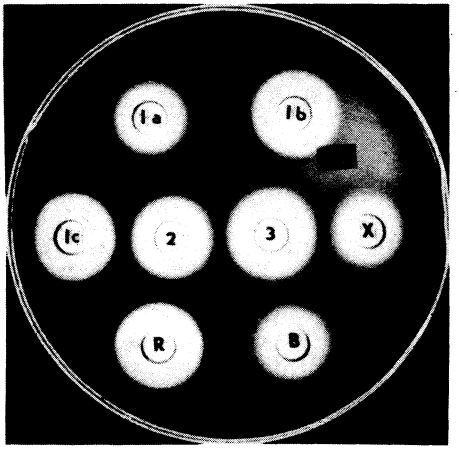

Fig. 2
Prepurified CAMP-factors of *S. agalactiae* serotype strains are placed in the wells which were cut into the same medium as on Fig. 1.

intensity of CAMP-factor production was also detected in *S. agalactiae* strains from clinical material. However, a prepurified CAMP-factor was to be obtained of every serotype strain, and the differences occuring in its native form could be reduced by convenient adjustment (Fig. 2).

As could be checked by collecting blood samples and examining sera, a sufficient amount of precipitation antibodies was present in sera from hyperimmunized rabbits following the fifteenth dose of antigen. As soon as serum and corresponding antigen reacted satisfactorily, the rabbits were exsanquinated by cardiac puncture. In the case of an insufficient reaction, the animals received further doses of antigen and the checking was repeated.

The reaction between antiserum and homologous antigen resulted in formation of three precipitation lines. This was achieved both with prepurified CAMP-factor in agar immunodiffusion and with its native form in Elek-Ouchterlony (EO) test. The first line was closest to the antigen, thus to the well containing prepurified homologous CAMP-factor or to the streak of the homologous strain, and it was very conspicuous. The second line was located in the middle between antiserum and antigen beeing less marked. The third precipitation line, occasionally missing, was similarly distinct as the second one, and adjoined the well with antiserum. A splitting or confluence of described precipitation lines could be observed in some cases. The second and third precipitation lines originated also in the cases of heterogeneity of antigens and antisera.

The first precipitation line always was intensive between antisera and corresponding CAMP-factors of the *S. agalactiae* strains Ib (Fig. 3), Ic (Fig. 4), X (Fig. 5), and R (Fig. 6), while less marked reactions were observed in homologous

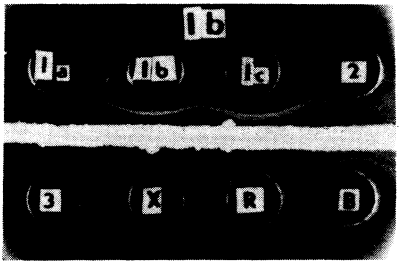

Fig. 3
On nutrient agar is a streak (Ib) of *S. agalactiae* serotype Ib. In the wells are CAMP-factor antisera signed according serotype of the producer strain.

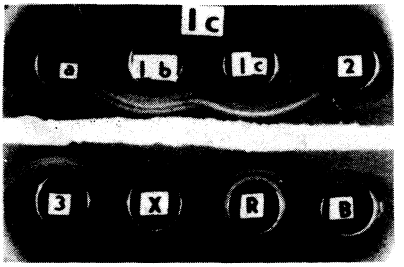

Fig. 4.
The same reaction as in Fig. 3, but with the strain *S. agalactiae* serotype Ic.

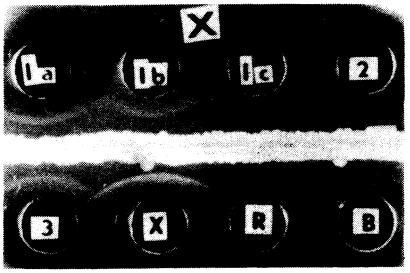

Fig. 5.
The same reaction as before, but with the strain *S. agalactiae* serotype X.

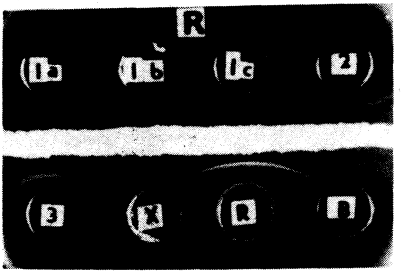

Fig. 6.
The same reaction as before, but with the strain *S. agalactiae* serotype R.

cases of the rest of CAMP-factors produced by the serotype strains and their corresponding antisera (Fig. 7). As for the first line, the homologous and cross reactions of CAMP-factors produced by the strains Ib and Ic, and their corresponding antisera, were of particular interest. An intensive precipitation line arose both homologous and heterologous reactions, beeing compact in homologous case and splitted in two branches in the heterologous one (Fig. 3 and Fig. 4).

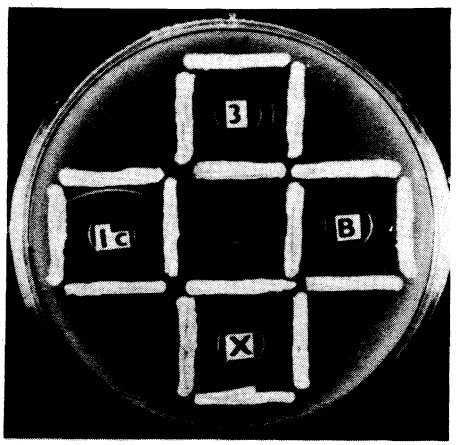

Fig. 7
CAMP-factor antisera marked according to the producer strains are in the wells. Streaks of four *S. agalactiae* strains are around each well. One of the strains is homologous and three are heterologous to the CAMP-factor antiserum.

The second precipitation line appeared in any heterologous case both when testing prepared antisera against one strain and when testing one antiserum against four antigens, either native of prepurified. It always joined analogous neighbouring lines assuming so the character of a common line. Similar features though less marked, displayed the third precipitation line.

CAMP-factors of the 150 *S. agalactiae* strains isolated from humans reacted with the antisera prepared as follows: 9 (6 %) gave identical result as CAMP-factor of the Ia strain, 7 (4.6 %) as Ib, and 3 (2 %) strains in the same way with the antisera of CAMP-factors Ib and Ic. Identically with CAMP-factor of the strain Ic reacted 28 (18.6 %) strains, with CAMP-factor II 15 (10 %) strains, and with CAMP-factor of the strain III 29 (19.3 %) strains. Fourteen (9.3 %) reacted simultaneously with antisera of the CAMP-factors produced by the strain III and R. CAMP-factors of three (2 %) strains were antigenic identical with that of the strain X, and two (1.3 %) strains displayed a reaction of their exosubstances with antisera of CAMP-factors of the strains X and R. The same reaction, as gave the strain R, was observed in 17 (11.3 %) strains, and as the strain B in 4 (2.6 %) strains. The rest of 14 (9.3 %) strains, as a rule, formed the second and mostly also the third precipitation line while in no case the first one. Consequently these strains could be placed to no described category (Table 1).

The typing of 150 *agalactiae* strains isolated from bovine udders gave following results: CAMP-factors of 2 strains (1.3 %) reacted like CAMP-factor of the strain Ia, CAMP-factors of 5 (3.3 %) strains reacted like that of the strain Ib, and the simultaneous positivity with CAMP-factor antisera Ib and Ic was observed in 2 (1.3 %) strains. The same result as CAMP-factor Ic was observed in 9 (6 %) strains, and exosubstances of 11 (7.3 %) strains reacted like CAMP-factor of the strain III. Three (2 %) strains reacted simultaneously with CAMP-factor antisera III and X, and 7 (4.6 %) with antisera III and R. The same result as with the strain X was gained with 29 (19.3 %) strains, and as with the strain R with 36 (24 %) strains. Thirteen

Table 1

Serotyping of S. agalactiae strains by means of the EO-test

Antisera against CAMP-factors of S. agalactiae serotype strains	Number of positively reacting S. agalactiae strains from	
Ia	9	2
Ib	7	5
Ib + Ic	3	2
Ic	28	9
II	15	4
III	29	11
III + X	0	3
III + R	14	7
X	3	29
X + R	2	13
R	17	36
B	4	2
not alloted	14	27
total	150	150

(8.6 %) strains precipitated both with CAMP-factor antisera X and R. Two (1.3 %) strains only reacted with CAMP-factor antiserum B. No allotment was possible for 27 (18 %) strains displaying reactions with all CAMP-factors antisera but forming only the second and third precipitation lines (Table 1).

Streptococcal strains of other serological groups than the group B which were used for checking gave results with all CAMP-factor antisera.

The commercial antiserum for identification of group B streptococci, used for controlling purposes in this study, gave no positive precipitation with both prepurified and native CAMP-factors.

Discussion

The availability of CAMP-factor for identifying of *S. agalactiae* is undeniable. Its production can be determined by means of the original technique for hemolytic synergism with staphylococcal beta toxin (C h r i s t i e et al. 1944) or with the corynebacterial phospholipase D (S k a l k a et al. 1979). The employment of this phenomenon caused studies on the nature of synergistic hemolytic interaction (K a r C h o u d r y 1978; B e r n h e i m e r et al. 1979).

The up to date scarce studies concerning antigenic properties of the CAMP--factor claimed the presence of neutralizing antibodies in blood sera - though as a rule in low titres - following experimental and spontaneous infections by *S. agalactiae* (B r o w n et al. 1974; F l o r e s and F e r r i e r i 1983). Our experience failed to confirm such type of CAMP-factor antibodies, even in the rabbit antisera evidently containing precipitins, but we noted the neutralizing activity in normal sera of rabbits, cattle and humans, up to the dilution 1/60 (S k a l k a et al. 1980; S k a l k a and S m o l a 1981).

Little attention has been payed to the problem whether CAMP-factors produced by different strains of group B streptococci are of identical different antigenicity. Antigenic identity of CAMP-factors was described by F l o r e s and F e r r i e r i (1983), nevertheless, the authors investigated only the strains belonging to the serotype III.

Our results confirmed and employfied this observation. They rendered evidence that CAMP-factors produced by different strains of *S. agalactiae* contained identical or common antigenic fraction(s) arising precipitation lines referred to as second and

third, as well as an antigenic fraction giving the precipitation line referred to as first which can be considered as specific for some clumps of *S. agalactiae* strains. The antigenic relation between CAMP-factor and the serotyp pertinence of producer strain is utmost remarkable.

Admittedly, the prepurified CAMP-factor could contain some traces of soluble cellular antigenic substances, neither can be excluded the contamination of native CAMP-factor with such ones, but the negative precipitation of both prepurified and native CAMP-factors with the commercial antiserum makes the importance of such eventuality insignificant.

Antigenní diferenciace CAMP-faktorů produkovaných různými kmeny Streptococcus agalactiae

V rámci druhu *Streptococcus agalactiae* byl zjišťován antigenní vztah mezi CAMP-faktory produkovanými reprezentativními kmeny jednotlivých sérotypů, a to Ia, Ib, Ic, II, III, X a R, a kmeny čerstvě izolovanými z biologických materiálů. Separované formy CAMP-faktorů typových kmenů byly použity k intravenózním aplikacím králíkům, ze kterých se získala antiséra. Pro antigenní určování byla použita dvojitá agarová imunodifuze s prepurifikovanými CAMP-faktory jako antigeny a modifikovaný Elek-Ouchterlonyho test, využívající nativní formu této exosubstance jako antigen. V reakcích korespondujícího antigenu a jeho antiséra vznikaly tři linie precipitátu. První z nich se pozorovala pouze v homologních případech, druhé dvě vznikaly v testech homologních i heterologních. To prokázalo existenci společné a specifické antigenní frakce v CAMP-faktorech. Pro vyšetření 300 kmenů *S. agalactiae* byl použit jen modifikovaný Elek-Ouchterlonyho test, nevyžadující speciální přípravu antigenu. Kmeny byly hodnoceny na podkladě podobnosti reakce jejich CAMP-faktorů s reakcemi CAMP-faktorů typových kmenů. Ze 150 kmenů humánní provenience byla tato podobnost nejčastěji s reakcemi CAMP-faktorů typů Ic a III, ze 150 kmenů z mléčné žlázy skotu dával největší počet kmenů reakce podobné CAMP-faktorům typů X a R.

Антигенная дифференциация CAMP факторов, продуцированных разными штаммами Streptococcus agalactiae

В рамках вида *Streptococcus agalactiae* определялись антигенные отношения между CAMP-факторами, продуцированными представительными штаммами отдельных серотипов, а именно Ia, Ib, Ic, II, III, X и R , и штаммами, изолированными из биологических материалов. Сепарированные формы CAMP-факторов типовых штаммов были внутривенно использованы у кроликов, от которых были получены антисывопотки. Для антигенного определения была использована двойная агаровая иммунодиффузия с заранее очищенными САМП-факторами в качестве антигенов и модифицированный тест по Элек-Уштерлони, использующий естественную форму данной экзосубстанции в качестве антигена. В реакциях цоответцтвующего антигена и его антисыворотки возникали три линии преципитата. Первая из них встречалась лишь в гомологичных случаях, следующие две линии возникали в гомологичных и инадекватных случаях. Это стало доказательством существования совместной и специфической антигенной фракции в САМП-факторах. Для исследования 300 штаммов *S. agalactiae* был использован только тест по Элек-Уштерлони, не требующий специальной подготовки антигена. Оценка штаммов проводилась а основе подобия реакции их САМП-факторов с реакциями САМП-факторов типовьх штаммов. Из 150 штаммов человека данное подобие чаще вцего встречали с реакциями САМП-факторов типов Ic и III, из 150 штаммов молочной железы крупного рогатого скота самое большое число штаммов по своей пеакции были похожи на САМП-факторы типов X и R.

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