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Studies on two methods for extraction of streptococcal T antigens

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SUMMARY

The extraction of group A streptococcal antigens by group C phage-associated lysin has been confirmed. In addition to the T antigen the extract contained M-protein, group-specific polysaccharide and mucopeptide antigen which was difficult to remove. This method of extraction of the T antigen was compared with the trypsin method. The latter method was found to be of advantage in giving a pure specific antigen.

INTRODUCTION

The serological classification of group A streptococci introduced by Griffith (1934) was established by means of slide-agglutination of intact cells. This reaction depends on the presence of T antigen and hence its use in T-typing of these streptococci.

However, precipitation reactions of T antigens were only practised on a small scale and the only available method of extraction of these antigens was by treatment with trypsin after heating the cells to a temperature of 70° C. (Lancefield & Dole, 1946; Pakula, 1951; McLean, 1953). Recently, Kantor & Cole (1960) reported the extraction of type 1 T antigen by means of group C streptococcal phage-associated lysin (Maxted, 1957; Krause, 1957).

In this communication results of studies aiming at confirming previous findings with the phage-associated lysin extraction method and comparing it with the already established method of extraction with trypsin are reported. T antigens of three T-types other than type 1 (types 2, 5 and 12) were extracted with this lysin.

MATERIALS AND METHODS

Strains of streptococci

The following four T types of group A streptococci were used in the investigation:

(1) N.C.T.C.	100079	type 1
(2) N.C.T.C.	8322	type 2
(3) N.C.T.C.	100080	type 5
(4) N.C.T.C.	100081	type 12

All cultures were grown in 2 litre amounts of Todd–Hewitt broth (Oxoid) for 3 days in the air at 30° C.

Preparation of the streptococcal cell walls

The cultures were killed by heating in a water-bath at 56° C. for ½ hr. The cells were then washed three times in sterile normal saline. Cell walls were prepared from the washed killed cells in a Mickle's disintegrator as described by Salton & Horne (1951).

Antisera

Group A streptococcal typing antisera (T and M specific antisera) and grouping antisera (group A specific and group A-variant specific antisera) were prepared as previously described (Erwa, Maxted & Brighton, 1969). The antisera used for typing by the agglutination method, T-typing, contain only the T-type antibody, while those used for M-typing contain both M and T antibodies.

Preparation of antigens

T antigen

This was prepared by two different methods as follows:

Trypsin method (Lancefield & Dole, 1946; Pakula, 1951). The streptococcal cells, killed at 70° C., were suspended in a suitable volume of phosphate buffer pH 7·8, and treated with 0·5 % crystalline trypsin at 37° C. for a few hours. The trypsinized suspension was then centrifuged and the supernatant fluid collected. This was acidified by N-HCl to pH 2·5 at which maximum precipitation of the T protein occurred. The precipitate was allowed to settle in the refrigerator overnight and then collected by centrifugation. It was then redissolved in a minimum amount of phosphate-buffered saline pH 7·2 and used in serological tests.

Extraction by group C phage-associated lysin. This muralytic enzyme was obtained by propagating the bacteriophage on group C streptococci (Maxted, 1957; Krause, 1957) grown in Todd–Hewitt broth. The lysin was reduced by the addition of a few drops of thioglycollic acid neutralized to pH 7·4 with N-NaOH. Twenty ml. amounts of the lysin were used to lyse cell walls from 2 litre cultures. The suspensions of cell walls in lysin were incubated at 37° C. for a few hours. The cell walls were then separated from the suspension by centrifugation at 10,000 rev./min. for 30 min. The supernatant fluid was taken as the crude extract. One half of the extract was treated with trypsin in order to destroy M-protein present. The other half was left untrypsinized.

For purification, each of the trypsinized and the untrypsinized parts of the crude extract was then divided into two lots. One lot was purified by precipitation of the protein at pH 2·5 by adding N-HCl dropwise. The precipitate was then redissolved in water and reprecipitated by acid and the addition of 2·5 volumes of absolute alcohol. The latter would remove any group-specific polysaccharide in the extract (Fuller, 1938). This procedure was repeated four or five times.

The second lot was purified by precipitation with saturated ammonium sulphate. Three different concentrations of ammonium sulphate were used, 50 %, 60 % and 70 %, and the extracts were contained in dialysis bags suspended in these solutions. This process continued overnight at refrigerator temperature (1–4° C.). The deposits were then collected by centrifugation, redissolved in water and dialysed in dis-

tilled water in the cold with several changes of water. The solution was then acidified to pH 2·5 and washed in absolute alcohol as before. The purified deposits were redissolved in a minimum amount of phosphate-buffered saline pH 7·2 and used for serological tests.

Group-specific polysaccharide

Group A and group A-variant specific polysaccharides were prepared from cell walls by the hot formamide method of Fuller (1938) and purified by several treatments with acid-alcohol and acetone as previously described (Erwa *et al.* 1969).

Mucopeptide

The formamide residue obtained after the extraction of the group-specific polysaccharide was treated with crystalline egg lysozyme solution (0·5 mg/ml) in citrate buffer pH 5·3 (Krause & McCarty, 1961). The pH was adjusted to 7·2 by N-NaOH and used for the serological tests.

Serological tests

The presence of T antigens was tested for by the capillary precipitation test of Swift, Wilson & Lancefield (1943). Group-specific precipitation reactions and mucopeptide reactions were detected by the ring precipitin technique of Lancefield (1933).

Absorption of group-specific antibodies and mucopeptide antibodies was carried out by adding equal volumes of extract and antiserum, allowing the absorption to continue at 37° C. for 1 hr. and then overnight at refrigerator temperature. The precipitate was separated by centrifugation and discarded.

RESULTS

Extraction of the T antigen by the trypsin method

Group A streptococci of T types 1, 2, 5 and 12 were extracted by trypsin treatment and purified as described above. They were then tested by the capillary precipitation method and ring precipitin technique. In the former method homologous and heterologous T and M antisera were used. In the latter, group A antiserum (grouping serum) was used in order to exclude any group reaction. The results are shown in Table 1. The extracts reacted specifically with their homologous T and M antisera and showed no cross-reactions with the control heterologous antisera. The reaction of the M antisera with T extracts of homologous types is explained by the fact that M antisera contain T antibodies as well since absorption is carried out to remove group-specific antibodies only. On the other hand there were no reactions with the grouping serum (group A antiserum) when tested with the extracts.

Extraction of the T antigen by the phage-associated lysin

Crude group C streptococcal phage-associated lysin was used to extract T antigens from cell walls of group A streptococci types 1, 2, 5 and 12 as described previously. The crude extract obtained from each type was divided into two parts.

Table 1. *Precipitation reactions of purified trypsin-extracted T antigens of types* 1, 2, 5, 12 *group A streptococci with homologous and heterologous T and M specific antisera and with group A specific antiserum*

T extract	Precipitation reactions with antisera of: Type 1 T1	Type 1 M1	Type 2 T2	Type 2 M2	Type 5 T5	Type 5 M5	Type 12 T12	Type 12 M12	Reactions with group A antiserum
Type 1	3+	4+	–	–	–	–	–	–	–
Type 2	–	–	2+	2+	–	–	–	–	–
Type 5	–	–	–	–	2+	4+	–	–	–
Type 12	–	–	–	–	–	–	2+	3+	–

– = no reaction.
± = trace, precipitation column < 0·25 cm.
1+ = weak, precipitation column 0·25–0·5 cm.
2+ = moderate, precipitation column 0·5–1·0 cm.
3+ = strong, precipitation column 1·0–1·5 cm.
4+ = very strong, precipitation column > 1·5 cm.

Table 2. *Results of precipitation reactions of phage lysin extracts of group A T types* 1, 2, 5 *and* 12 *streptococci with T and M type-specific antisera as well as group A specific antiserum*

T antigen		Reactions with type-specific antisera: T antisera T1	T2	T5	T12	M antisera M1	M2	M5	M12	Reactions with grouping antiserum
Type 1 extract	i	+	–	–	–	+	–	–	–	4+
	ii	±	–	–	–	+	–	–	–	4+
	iii	2+	–	–	–	2+	–	–	–	4+
	iv	2+	–	–	–	2+	–	–	–	4+
Type 2 extract	i	–	2+	–	–	–	±	–	–	4+
	ii	–	2+	–	–	–	±	–	–	4+
	iii	–	±	–	–	–	+	–	–	3+
	iv	–	+	–	–	–	2+	–	–	2+
Type 5 extract	i	–	–	±	–	–	–	±	–	4+
	ii	–	–	+	–	–	–	±	–	3+
	iii	–	–	+	–	–	–	+	–	4+
	iv	–	–	±	–	–	–	+	–	4+
Type 12 extract	i	–	–	–	+	–	–	–	+	4+
	ii	–	–	–	+	–	–	–	±	4+
	iii	–	–	–	+	–	–	–	±	4+
	iv	–	–	–	2+	–	–	–	+	4+

(i) Trypsinized crude extract.
(ii) Trypsinized acid-precipitated extract.
(iii) Untrypsinized crude extract.
(iv) Untrypsinized acid-precipitated extract.

For grades of the type-specific reactions see footnote for Table 1, and for group-specific reactions see footnote for Table 4.

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Table 3. *Precipitation reactions with T antigen extracted by means of phage lysin and subsequently digested with trypsin and precipitated with saturated ammonium sulphate*

T Antigen		Reactions with T antisera				Reactions with group A antisera
		T1	T2	T5	T12	
Type 1	X	+	−	−	−	3+
	Y	+	−	−	−	3+
	Z	+	−	−	−	2+
Type 2	X	−	+	−	−	3+
	Y	−	+	−	−	3+
	Z	−	+	−	−	2+
Type 5	X	−	−	+	−	3+
	Y	−	−	+	−	2+
	Z	−	−	+	−	2+
Type 12	X	−	−	−	+	3+
	Y	−	−	−	+	3+
	Z	−	−	−	+	3+

X = trypsinized extract precipitated with 50 % $(NH_4)_2SO_4$.
Y = trypsinized extract precipitated with 60 % $(NH_4)_2SO_4$.
Z = trypsinized extract precipitated with 70 % $(NH_4)_2SO_4$.

One part was treated with trypsin to destroy any M-protein present. The other part was left without treatment. A volume of each of the two parts was then acidified to pH 2·5 in order to precipitate the T antigen. Thus four lots of the extract resulted: (i) trypsinized crude extract, (ii) trypsinized acid-precipitated extract, (iii) untrypsinized crude extract and (iv) untrypsinized acid-precipitated extract. These four lots were tested serologically by the capillary precipitation test with homologous T and M antisera. They were also tested for group reaction with group A specific antiserum. The results are shown in table 2. All the four preparations reacted with the homologous T antisera without cross-reactions with the heterologous antisera. Reactions with M antisera of homologous types persisted even after removal of M-proteins by trypsin and this is due to the presence of T antibodies in the M antisera as explained above. Therefore, the four lots of type 1 extract reacted with T1 and M1 antisera, the four lots of type 2 with T2 and M2 antisera, etc.

However, all extracts reacted strongly with grouping sera, i.e. even after precipitation with acid and washing with alcohol (Table 2). In view of this, it was considered necessary to purify the extracts in such a way that the protein antigen would be completely separated from the carbohydrate antigen. The crude extracts were therefore first trypsinized and then treated with saturated ammonium sulphate solution to final concentrations of 50, 60 and 70 %. The T antigen preparations so obtained were labelled X, Y and Z respectively and tested as before. The results are set out in Table 3. As before there were no cross-reactions with T heterologous antisera but again the group reaction persisted and only slightly diminished in intensity (from 4+ to 2+ or 3+).

However, the reactions of the extracts with the grouping serum could be, to

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Table 4. *Results of precipitation reactions of T antigens obtained from trypsinized phage-lysin extracts by treatment with saturated ammonium sulphate and acid* (pH 2·5) *and tested against streptococcal group A and group A-var. antisera before and after absorption with their respective group-specific polysaccharides*

Antiserum		Precipitation reactions of T antigen extracts							
		Type 1		Type 2		Type 5		Type 12	
		a	*b*	*a*	*b*	*a*	*b*	*a*	*b*
Group A antiserum	Before absorption	3+	4+	3+	3+	4+	3+	3+	4+
	After absorption	3+	2+	2+	+	3+	2+	3+	3+
Group A-var. antiserum	Before absorption	3+	3+	3+	3+	3+	3+	3+	3+
	After absorption	3+	3+	3+	2+	3+	2+	2+	2+

a = T antigen precipitated by saturated $(NH_4)_2SO_4$.
b = T antigen precipitated by acid (pH 2·5).
– = negative reaction.
± = trace reaction after 2 hr.
\+ = weak reaction after 1–2 hr.
2+ = moderate reaction after ½–1 hr.
3+ = strong reaction after 5–30 min.
4+ = very strong reaction after < 5 min.

a large extent, due to the presence of mucopeptide in the extracts. Streptococcal mucopeptides are strongly antigenic (Abdulla & Schwab, 1965; Karakawa, Lackland & Krause, 1966), and antibodies to them are invariably found in sera prepared by hyperimmunization with streptococcal cells or cell walls of which they are a constituent. Therefore, precipitation tests of the extracts were repeated with group A antiserum and with the heterologous group A-var. antiserum as shown in Table 4 (the reactions before absorption with group-specific polysaccharides). Both antisera gave positive results, although the reactions with group A-var. were rather slow to appear, i.e. after 5–30 min. This finding suggests that the reaction is not due to group A polysaccharide. To confirm this, small quantities of group A and group A-var. antisera were absorbed with purified group A and group A-var. polysaccharides (Erwa *et al.* 1969) and retested as above. The results are also included in Table 4, and it is evident that the reactions with the group antisera persisted even after absorption with the respective group-specific polysaccharide. Generally the intensity of precipitation after absorption either remained unchanged or fell only slightly. Only with types 1 and 2 was there an appreciable fall.

Furthermore, a mucopeptide extract prepared as described in 'Materials and Methods' above was used for the absorption of homologous antibodies from group A and group A-var. antiserum. The absorbed serum showed effective loss of reaction with the T antigen preparations when tested by the precipitation technique as shown in Table 5. The reactions of the T antigens with the mucopeptide-absorbed sera were almost equal to those given by the mucopeptide and the same absorbed sera (1+ or ±).

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Table 5. *Precipitation reactions of T antigens obtained from trypsinized phage-lysin extracts by treatment with saturated ammonium sulphate and acid* (pH 2·5) *and tested against groups A and A-var. antisera before and after absorption with a streptococcal mucopeptide*

Antiserum		Precipitation reactions of T antigen extract								Reactions with the mucopeptide extract
		Type 1		Type 2		Type 5		Type 12		
		a	*b*	*a*	*b*	*a*	*b*	*a*	*b*	
Group A antiserum	Before absorption	3+	4+	3+	4+	4+	3+	3+	3+	3+
	After absorption	±	1+	±	1+	1+	±	1+	±	±
Group A-var. antiserum	Before absorption	3+	3+	3+	3+	3+	3+	3+	3+	3+
	After absorption	±	±	1+	±	±	±	1+	±	−

N.B. for explanation of signs see footnote for Table 4.

In summary the absorption tests carried out generally showed the failure of effective reduction of the intensity of reactions of the T extracts with the grouping sera when the latter were absorbed with purified polysaccharides. On the other hand, with the mucopeptide extract the reaction was almost abolished.

DISCUSSION

The results presented above demonstrate clearly the effectiveness of group C phage-associated lysin in releasing the T antigen from the streptococcal cell wall. The soluble antigen so obtained is readily detectable by precipitation reactions indicating a satisfactory serological reactivity.

However, extracts with phage-associated lysin present certain difficulties. The main difficulty encountered in our work was the release of other cellular antigens alongside the T antigen. The group-specific polysaccharide is one (Maxted, 1957; Krause, 1957), but its removal was easy as shown above. The M-protein is another such antigen (Kantor & Cole, 1960) and again we were able to get rid of it by trypsin treatment. However, the greatest challenge came with the attempts to remove the mucopeptide antigen which was found to be present in the extract. In this respect efforts to separate the mucopeptide from the T antigen were not fruitful and its serological reactivity persisted in all extracts.

Furthermore, the phage-associated lysin is rather unstable and has to be used in the reduced condition and like other enzymes it requires adjustment of the medium to optimal pH and temperature in order to give a satisfactory reaction.

On the other hand the T antigen extracted by the trypsin method (Lancefield & Dole, 1946; Pakula, 1951) gave highly specific reactions with homologous antisera and showed no cross-reactions of any sort connected with any of the four types of group A streptococci investigated here, thus indicating the lack of other contaminating cellular antigens. The trypsin destroys the M-protein. The group-

specific polysaccharide needs a more vigorous treatment for its release, such as hot formamide (Fuller, 1938), hot acid (Lancefield, 1933), muralytic enzymes (Maxted, 1948, 1957). Nevertheless trace amounts of polysaccharide which might be found in extracts made by trypsin digestion are easily removed after precipitation of the T antigen at pH 2·5 and washing with absolute alcohol. Moreover, since the cells remain intact after trypsin treatment, the mucopeptide antigen is not released in the extract. In general all of these facts endow the trypsin method extract with an advantage, since it is easy to prepare and purify as well as being highly specific in serological reactions. In our opinion this method of T antigen extraction is to be recommended for all practical purposes requiring serological purity and specificity of the antigen.

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