Purification and characterization of *Streptococcus pyogenes* superantigen (Spe-C)

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Received 12, May, 2009
Accepted 15, January, 2011

Abstract:

From 144 specimens of tonsillitis which were collected from patient, (children of 3 -12 year olds) there were 70 isolates beta hemolytic and 28 isolates were identified as *S. pyogenes.*

Sensitivity of *S. pyogenes* isolates to antibiotics was tested, all isolates were sensitive to amoxicillin and cephaloxia while higher resistant were to erythromycin.

One isolate whiche was 100 A had a stable characteristics and produce pyrogenic toxin was chosen for study and it was purified and characterized from the cell free supernatant of *S. pyrogenes* strain.

Key word: superantigen , pyrogenic toxin , Erythrogenic toxin , *Streptococcus* exotoxin.

Introduction:

Erythrogenic (pyrogenic) toxins of *Streptococcus pyogenes* and *Staphylococcus aureus* from a superantigen family based on genetic and shared biological properties which are defined by their ability to form trimolecular complexes with T-cell receptor (TCR) and major histocompatibility complex (MHC) class II of antigen presenting cell [1 , 2]. These exotoxins stimulate T-cell with particular B-chain (V_B) of TCR resulting in proliferation and induction of cytokines that cause hypertension, fever and shock [3, 4]. Their function in the pathogenesis of streptococcal infection is unknown. In addition to its role as a causative agent of symptoms associated with scarlet fever. *Streptococcus* pyrogenic toxin type-C may play a role is the early events of rheumatic fever. In particular toxin is the most common toxin found in recent clinical isolates and nearly all rheumatic fever [5].

First investigations on streptococcal pyrogenic toxin type C (SPC) was made by [6]. The Spe C amino acid) sequence appeared to be related to that of Spe A and less to these of *Staphylococcus* enterotoxins [6, 7].

The aim of this investigation is to study the role of *Streptococcus pyogenes* tonsolitis, isolate and characterization of superantigen type – C (Sep-C) and its effect in elevates of body temperature.

Materials and Methods:

Bacterial strains: local isolates from tonsillitis and scarlet fever patients (3 – 12 year old) from Child's Hospital Education central in Baghdad from March 2000 to June 2001.

Isolation and diagnosis of bacterial strains according [8].

Bacterial media : Brain – heart infusion broth , Brain heart infusion agar , nutrient broth (Oxoid) , blood

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group O from laboratory of the hospital.

Antibiotics : from (Oxoid). Antibiotics sensitivity test by the method of [9].

Chemical : CM-Sepharose was purchased from whatman , Sephadex G-100 was from pharmica , Molecular markers for electrophoretic analysis were obtained from pharmacia fine chemical.

Bacterial growth and the isolation of crude toxin.

This was done as described earlier [10]. In abbreviation : over night culture of *Streptococcus pyogenes* strain 100A were grown in the brain heart infusion with 0.2 % yeast extract and 1 % peptone , culture was incubated for 18 hr. in 37˚c. after the cultivation streptococci were killed with 0.15 H_2O_2 followed be separation of the biomass.

Purification of toxin: the cell free supernatant 1 liter was saturated by addition of ammonium sulphate gradually to a final concentration of 80 %. The pH was adjusted to pH 4.5 with 1M HCl. After 18 hr at 4˚c the precipitated protein were collected and dissolved in 0.01 M Tris buffer pH 8. This solution was dialysed against a 40 % ammonium sulphate solution at pH 4.5. After 24 hr. repeated exchanges of buffer solution the participitated immpuritiey were separated. Protein solution containing ETC was dialysed against 0.02 M acetate buffer pH 5.5. Further purification was achieved by CM-Sepharose column 12 x 1.8 cm equilibrated with 0.02 M sodium acetate buffer pH 5.5.

After washing the column with the same buffer and 0.05 M sodium acetate buffer pH 6.0 the ETC was eluted with 0.1 M sodium acetate buffer, pH 6.5. toxin fraction were collected and dialysed against 0.2 M PBS. each fraction was examined its ability to elevate rabbit temperature , it regard as pyrogenic toxin when 0.1 ml increase the temperature 0.5 c when it adminstated intravenously within 4 hr [11].

The biological active fraction (toxin) was collected and more purification was done by gel column Sephadex G100 (83 x 1.6) washed by phosphate buffer saline 0.01 M pH 6.5 molecular. Biological active fractions (pyrogen) was collected and concentrated by sucrose.

Protein concentration was determined by the method [12]. Molecular weight was determined by SDS-polyacrylamide electrophoresis , method was done according to [13] determination of isoelectric point (PI) by the method of [14].

Result and Discussion:

From 144 speciment of patient with tonsillitis and scarlet fever (3-12 years old) there were 70 isolates were beta hemolytic , it create 48.2 % from total speciment.

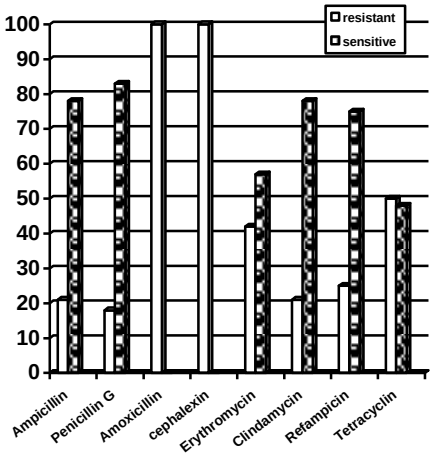

Fig. 1: Sensitivity of *S. pyogenes* strains to antibiotics

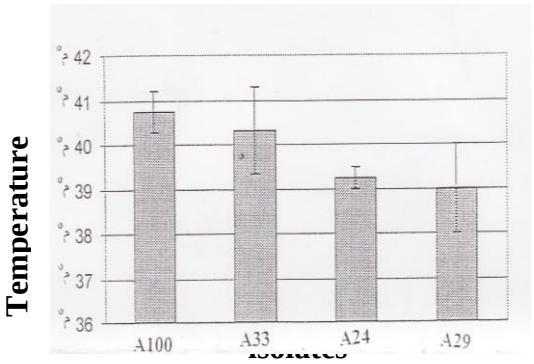

Fig 2: the effect of injected of 0.1 ml of free cell dialyzed supernatant of *S. pyogenes* , intravenous in rabbit of *S. pyogenes* , the column represents average and lines represent standard error

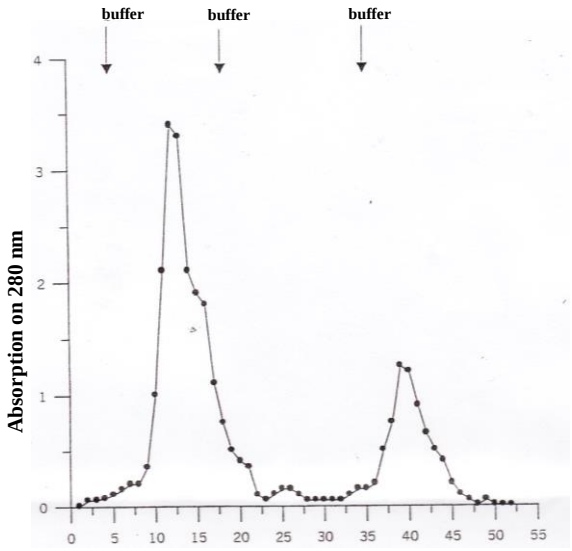

Fig. 3; Purification of crude ETC from *S. pyogenes* on CM-Sepharose. The major concentration of ETC appeared in peak 1 with 0.1 M Sodium acetate buffer pH 5.5 – 6.5.

Streptococcus pyogenes in 28 isolates it had 19.3 % from total speciment , diagnosis was depending on microscopic , biochemical test and confirmed with API 20 strep. All strains of *S. pyogenes* were sensitive to amoxicillin (AMX). Cefalexin (KF) while there were resistance for penicillin (PG) and Ampicillin (Amp) 16.8 %and 21 % respectively and Clindamycin (kln) 22.3 % , Refampicin (Re) 25.4 and high resistant for erythromycin (Ery) 42.7 % and tetracycline (Tet) 50.5 (Fig. 1).

From this study we found there were high resistance to common used antibiotics. Antibiotic susceptibility test must be done before using antibiotics randomly because the latter cause to produce high resistant strains for many antibiotics examination. The ability to produce pyrogenic toxin (spe c) was done according to [11]. The supernatant which contain the toxin was tested in young 3 month old white rabbits compared with control which the rabbits was injected intravenously with 0.1 dialyzable medium or PBS. four tested strain (100A , 33A , 24A , 29A) had the ability to produce pyrogenic exotoxin (superantigen) but it differ in its activity to elevate rabbit temperature within 4 hr. fig (2) this may be due to ability differences in activating T-cell and trigger they to reduce interleukin which used in elevate rabbit temperature [15].

Strain 100A was chosen of research because it have the highest effect during the purification of superantigen (Spc) their were many solution with different pH was used to collect highest quantity of toxin and lowest impurities, dialysis against 40 % ammonium sulphate 4.5 all these treatment not effect the biological activity of this toxin we conclude that this toxin was not effected by alkalinity and acidity.

In the purification of superantigen (pyrogenic toxin) type C by CM-Sepharose column it was found the main part was collected in the begging this indicate a weak attachment with CM-Sepharose (Fig. 3).

All fractions which have the ability to elevate rabbit temperature was collected and more partications was

done on sephadex G-100 column after and equilibration with saline buffer fraction was between 77 – 91 (fig. 4). Biological active fractions was collected and concentrated by sucrose the protein concentration was determined by [12] (Table-1). Purification with Sephadex G-100 was used by [16, 17, 18] they found their was no effect of this treatment on superantigen activity.

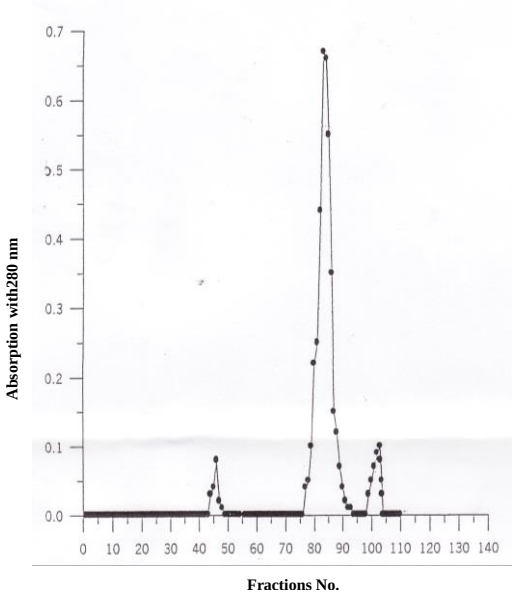

Fig.4; Purification of crude ETC on Sephadex G100 (1.5 × 83) equilibration with 0.01 M PBS pH 6.5, Flow rate 0.5 cm^3/min. (3 cm^3/Fraction).

Table 1: Purification steps of superantigen produced by *S. pyogenes*

Purification degree	Activity unit / mg	Protein concentration mg / cm^3	Volume cm^3	Purification steps
1	1.5	0.78	1000	Culture supernatant
10.15	11.2	1.12	10	Ammonium sulphate precipitation
12.91	13.56	0.226	8	Ion exchange column Cm-Sepharose
13.8	14.54	0.182	8	Gel column Sephadex G-100

Determination of molecular weight by SDS electrophoresis apparent that (Spe C) have 24.000 Da while [11] refer it have 21.000 Da by using high speed sedimention equilibrium menisens depletion method and [16] estimate it 24.324 Da depending on calculation of amino acid which formed it [18] refer that (SPe C) have 24 Da with SDS polyacryl amid electrophorysis (Fig. 5).

Isoelectric point (PI) was 6.8 by isoelectric focusing which the same value of [16].

Spe-C was protein affected by heating to 65˚c for 30 min or at 100˚c for 2 min this treatment lose its activity to arise rabbit temperature. Treatment with heat cause protein denaturation this lead to lose its ability to bind to T-cell receptor TCR and major histocompatibility class II (MHC II) to form trimolecular complex so there is no triggering to produce large quantities of interleukins [15].

Treatment (Spe-C) with proteases like trypsin and pepsin cause to lose its activity to elevate rabbit temperature this may be due to break down toxin molecule or may be cause to change the binding site and prevent the attachment with MH II and TCR [16]. Form this study we found that this toxin was not affected by *Streptococcus* proteases which produced by this bacteria in log phase while the pyrogenic toxin superantigen produced in stationary phase leaving the supernatant during toxin extraction in refrigerator not affect toxin activity (Fig. 6).

Fig. 5 : Poly acryl amid electrophoresis with SDS
Column 1 : Standard proteins
1.phosphorylase b
2.Albumin
3.Ovalbumin
4. Carbonic anhydrase
5. Trypsin inhibitor
6.Lact albumin
Column 2: Crude super antigen
Column 3: purified superantigen

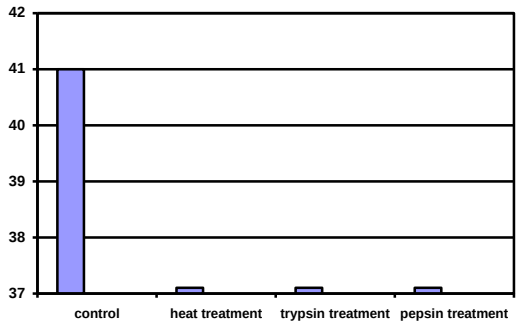

Fig. 6: The effect of temperature and proteinase (Trypsin and pepsin) on superantigen activity.

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تنقية وتوصيف المستضد الخارق نوع (Spe-C) المعزول من *Streptococcus pyogenes*

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الخلاصة :

تم جمع 144 عينة من مرضى التهاب اللوزتين بعمر (3-12) سنة. كان 70 عزلة منها محللة للدم نوع بيتا وقد تم تشخيص 28 عزلة على انها *Streptococcus pyrogenes*. اجري اختبار الحساسية للمضادات الحيوية وقد وجد ان جميع العزلات حساسة للاموكسسلين والسيفالكسين بينما تمتلك مقاومة عالية للارثرومايسين. تم اختيار عزلة واحدة وهي 100A ذات صفات ثابتة وتنتج السم الرافع للحرارة (pyrogenic toxin).

تم تنقية السم الرافع للحرارة نوع C من الرائق الخالي من الخلايا وتم دراسة وتشخيص السم. هذا السم وهو مستضد خارق له وزن جزيئي 24.000 دالتون ونقطة تعادل كهربائي (PI) 6.8 وهو يثبط بالحرارة ويهضم بالانزيمات مثل الببسين والتربسين.