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Effect of Extracellular adherence protein extracted from *Staphylococcus aureus* on mitotic index for some immunological organs in mice.

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Abstract

Staphylococcus aureus is one of the most common agents causing bacterial infections in human and animals, because it's ability to expresses a divers and array of virulence factors such as numerous protein adhesion ,Extracellular adherence protein (Eap) one of these protein ,have a major impact on the immune system by mediated adherence and colonization into host cells and effects on T and B cell proliferation. Hence, these studies show the role of Eap in proliferation of T and B cell in some immunological orgns .such as Bone marrow ,Spleen and Thymus , through out measurement of mitotic index (MI) for this organs.

Extracellular adherence protein (Eap) was extracted from the cell of *S.aureus* bacteria by using 1 M NaCL buffer (pH 5.5) ;Subsequently, the protein was purified by two steps including ion-exchange chromatography and gel filtration chromatography which give partial purified Eap with high agglutination activity.

After microscopically examination of slides prepared for each one of organs,the MI was determined and found it's value for bone marrow, thymus and spleen cells in treated mice with (2.5, 5, 10 µg/ml) of Eap induced a significant increase of mitotic index, while high dose of this protein (20, 30 µg/ml) showed significant decrease for all organ compared with control group.

الخلاصة

تعد جرثومة *S.aureus* واحدة من أكثر الجراثيم شيوعاً، فهي مسؤولة عن العديد من الإصابات الحادة في الإنسان والحيوان وذلك لامتلاكها العديد من عوامل الضراوة التي تزيد من امراضيتها مثل بروتينات الالتصاق السطحية الذي يعد بروتين الالتصاق الخارجي واحد من أهم هذه البروتينات لتوسطه عملية الالتصاق واجتياح الخلايا المضيفة ، فضلاً عن تأثيره على تكاثر الخلايا T وB المناعية.

تضمنت هذه الدراسة بيان تأثير بروتين الالتصاق الخارجي في تكاثر الخلايا المناعية في كل من نخاع العظم والطحال وغدة التوثة في الفئران من خلال حساب معامل الانقسام ألخيطي في هذه الأعضاء. حيث تم استخلاص هذا البروتين لأول مرة في القطر من جدار عزلة بكتريا *S.aureus* المشخصة محلياً باستخدام داريءالفوسفات الحاوي كلوريد الصوديوم بتركيز (1 مولا ر) واس هيدروجيني (5.5) آما تنقيته الجزئية فتمت باستخدام تقنية التبادل ألايوني والترشيح الهلامي ، آذ تم الحصول عليه بكمية مناسبة بلغت 200 مايكروغرام/ملليتر وبفعالية تلازنية عالية.

بعد الفحص ألمجهري للشرائح الزجاجية المحضرة لكل عضو مناعي وحساب معامل الانقسام الخيطي وجد ان هنالك زيادة معنوية لهذا المعامل في الأعضاء المناعية الثلاثة للفئران المُعاملة بالتراكيز (2.5 و5 و10 مايكروغرام/ ملليتر) من بروتين الالتصاق الخارجي مقارنة بالسيطرة في حين أظهرت الفئران المحقونة بالجرع العالية من هذا البروتين والبالغة (20 و30 مايكروغرام / ملليتر)انخفاضاً معنوياً في قيمة معامل الانقسام الخيطي للفئران في الأعضاء المناعية الثلاثة مقارنة بالسيطرة.

Introduction

S.aureus is a wide spread, persistent pathogen that causes a broad range of disease in human and animals from simple wound infection to more severe conditions such as septicemia,endocarditis and osteomylitis as in the case with many pathogens, initiation of *S.aureus* infection requires colonization of unique microenvironment within the host (Geisbrecht *et al.*,2005).

To initiate invasive infection, *S.aureus* must adhere to host extracellular matrix, by virtue of different surface proteins such as extracellular adherence protein

(Eap) that interaction with target structures on the eukaryotic cells, this regard a crucial step in it's pathogenesis (Flock and Ingmar, 2001).

The Eap is an extracellular protein with molecular weight (60 KD), caused agglutination of the bacteria cells, due to it's ability to rebind to the surface of *S.aureus* and because of the strong tendency of the Eap to form multimeric aggregates. Exogenously added protein significantly enhanced the adherence of *S. aureus* to fibroblasts and epithelial cells, based on the broad binding activity (Palma *et al.*,1999).

The Eap consisted principally of six repeated domain of (110) residues, each one of the repeats contain a sub-domain of (31) residues that had sequence homology with a segment in the peptide binding groove of the β. chain of the major histocomptibility complex (MHC) class 11 from eukaryotic cell (Haggar, 2005).

The Eap as an immunomodulating protein has high inducing activity for immune system as ability to produce antibody and delay type hypersensitivity reaction, thus the specific interaction with extracellular matrix proteins render Eap a potent anti-inflammatory factor, which may serve as a new therapeutic substance to block leukocyte extravasation in patient with hyperinflamation pathologies (Chavakis *et al.*,2002). This protein, affecting T-cells and antigen presenting cells and having an impact on antibody response (Flock and Ingmar, 2001). The Eap induce the B-lymphocyte cell to division and differentiation to Iq-secreting cell therefore have mitogenitic activity to immune cell specially this cell (Thuy *et al.*, 1978).

The objective of this study are to tried to establish a more convient partial purification protocol for Eap. and to study the immune effects of Eap on immunological cell.

Materials and Methods

A-Bacterial strain

S.aureus bacteria was isolated from the peripheral blood of patients with leukemia, then identified and characterized by biochemical depending on (Holt *et al.*,1994) ; (Macfaddin ., 2000)

B-Preparation of bacterial cell surface protein extract

S.aureus cell surface protein was prepared by pelleted bacteria from an overnight culture in 1 liter of Brain heart infusion broth ,then this pellet were resuspended in 100 ml of 1M NaCL, (pH 5.5) and the bacterial suspension was incubated at 37 ^{o}C for 2 h with gentle agitation. After centrifugation (5000 × g for 15 min at 4^{o}C) to precipitate bacterial cell and the supernatant was dialyzed against phosphate buffer saline (PBS) overnight .Then collected this supernatant that represented crude of extracellular adhesive protein suspension.

C-Isolation and partial purification of Eap .

According to the method of Flok & Ingmar(2001), the crude of Eap suspension was precipitated with 80 % saturation of ammonium sulfate (80% saturation), by added this salt gradually with gentle agitation in ice bath water at 4^{o}C for 30^{o}C and the protein precipitated by centrifugation (6000 g for 15 min). The precipitate proteins was dissolved into 0.02 M NaCL buffer , then dialyzed against PBS and used for further Purification by anion exchange chromatography (CM cellulose chromatography column with a diameter of 12 cm and a length of 2.8 cm) According to the procedure descended by Hussain *et al* (2001) with some modification using 1 M NaCL instead of LiCL to prepare a salt gradient (0.25-1 M) used for eluting the bound proteins after washing the column with several volumes, of PBS at flow rat (0.5 ml / min) to remove unbound proteins. the Eap eluted at 0.5 M NaCL ,fraction were collected (5 ml /fraction), the eluted protein was dialyzed against

PBS and chromatography again on Seharose.6B column (sigma) according to the procedure descended by Palma *et al* (1999), the column with distance 60×1.2 cm washed with 0.02 M PBS at a flow rate of 0.3 ml/ min, after added specimen, then fractions were collected (3 ml/ fraction) and assayed for protein content (absorbance at 280 nm) and agglutination activity (AA) as show in Palma *et al* (1999) the fractions that showed AA were pooled ,dialyzed first against PBS pH 7.2 for 2 hour with stirring at 4 ^{0}C ,these pooled fractions were concentrated by sucrose , assayed for protein concentration according to Lowry *et al* (1951) .this protein was used for mitogentic analysis .

D-Mitotic index assay

These assay occur according to Allen *et al*(1977) in low steps

Tissues collection (Bone marrow ,spleen and thymus)

Sex groups of /female-mice were injected intraperitoneally with 2.5-5-10-20-30 µg/ml of partial purified Eap and one group was injected with PBS as control. 2 hours prior to sacrifice ,0.25 ml of 500 µg of colchicine was injected intraperitoneally to arrest cells in the metaphase by inhibiting the operation of spindle mechanism .

The animals were killed ,Bone marrow cell ,spleen and thymus were homogenized in betridish contain equal volume of PBS. cells were collected and centrifuged at 4^{o}C , 2000 rpm for 10 min, and the pellet cell were then treated with 5 ml of hypotonic solution (0.075 M ,KCL) the tubes were incubation in water bath at 37 ^{0}C for 1 hour and then centrifuged at 2000 rpm for 10 min. ,freshly prepared fixation methanol was added drop–wise to give a total volume 5 ml ,three other washes with fixative solution made , 1 ml of the fixative was added to the cells after last wash .

Slide preparation and staining

The cells were dropped from a height of about 1 meters using Pasteur pipette on the cleaned microscopic slide that had been washed with methanol then distilled water. Slide was then dried on a 50^{0}C hot plate and stained with giemsa stain for 10 minutes ,washed with distilled water and examined mitotic index (MI) .

The MI was determined as a ratio of mitotic cell to interphase nucleic in 1000 cells.

$$MI = \frac{\text{No. of dividing cells}}{\text{Total No.of cells}} \times 1000 \ .$$

Results and discussion

A. Extraction Eap.

For extraction native form of Eap from the surface of *S.aureus*, bacterial pellet were resuspended with small volumes of phosphate buffers saline containing 1 M NaCL pH (5.5). Eap was efficiently and very selectively release from the bacterial surface upon incubation with buffers containing high NaCL concentration, the NaCL effects seems to be specific for amine functions and suggested that NaCL might complete with the free amino groups of Eap that could interacted with constituents of the cell wall (Palma *et al.*,1999).

B. Isolation and partial purification of Eap

When had trieded to isolation Eap from the bacterial cell NaCL extraction, extracts with biochemical methods, the rather unusual nature of the protein complicated the applicant of standard chromatography material. Ion exchange CM-cellulose was used according to Hussain *et al* (2001), the suspension 5 ml (200 µg/ml) was applied to column and eluted by NaCL graduate. The Eap was eluted with (o.5M) NaCL.

Two peaks of protein were observed (Fig 1). The first peak was represent by fractions (33-39) was dialyzed, concentrated and assayed for agglutination activity with bacteria cells which gave the high agglutination activity, while other peak was represent by fraction (44-47) was not gave agglutination activity. The concentration fraction caused agglutination activity was 600 µg/ml. By applying another procedure Haggar (2005), Found that the average yield of partial pure Eap was 850 µg/ml. As second step for purification, suspension 5 ml contain 500 µg/ml obtain from ion exchange chromatography applied to Sepharose 6B colum equilibrium with 20 mM NaCL (pH 7.6). The Eap was easily separated from other protein, which gave one peak was represented by fractions (24-38) (Fig 2). This fractions were dialyzed, concentrated and assayed for agglutination activity with bacteria cells. The concentration of fraction caused agglutination was (200 µg/ml).

Even though the use of sepharose matrix enabled the separation of Eap from other polypeptide , a compenation of many chromatography procedures was used to obtain high yield of purified Eap (Palma *et al.*,1999). Now immunoaffinty purification procedure yielded high ratio of partial pure Eap (Geisbrecht *et al.*,2005).

C. Mitotic index (MI %)

Organ with controls were varied largely are presented in table 1. The table show the mean value of MI in treated group with 2.5 -5 -10 µg/ml of Eap, which found to be significant higher ($p < or = 0.001$) than that for the control in all three organ. Also in same table showed the significant decrease ($p > or = 0.001$) in compared with control group when used the 20 -30 µg/ml from this protein .

The cell division machine, DNA and the genetic material are the most able parts in the cell affect by any parts of bacteria have mitogenitic activity, which effects of replication of DNA in cell for some immunological organ (spleen) as show in this study (Fig 3).

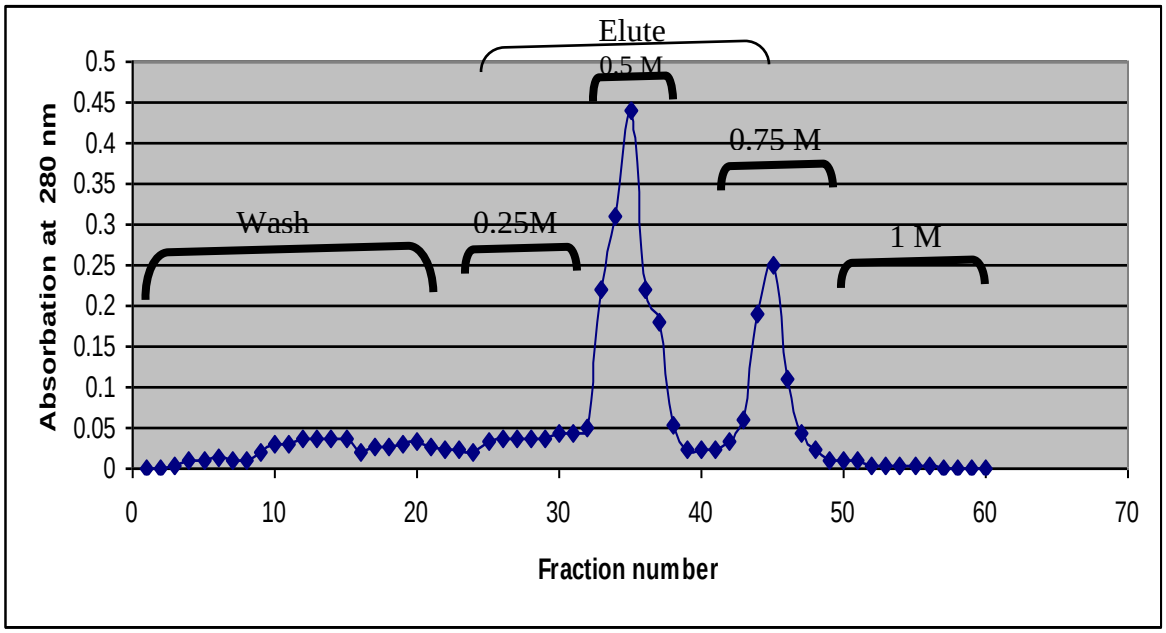
The result for this study gave idea that the Eap was found to have does dependent stimulatory effects on lymphoid cells division and prolefirations, because the effects of this protein on MI begin to low when increase it's concentration more than (10µg/ml).

This result approach with the study of Haggar (2005) which found that Eap have stimulatory effect of concentrations ranging from 3-9 µg/ml. While at higher concentration an inhibitory effect for immunological cell was seen.

In addition to ability of Eap to induce immune system this protein can prevented recruitment of inflammatory cells to the wound site with decrease in expression of tissue factor there for this protein serve as a lead compound for new anti-inflammatory and antiangiogenic the rapines in several pathologic (Athanasopoulos *et al.*, 2006).

Table (1): % Mitotic index in lymphoid organ

Concentration of Eap (µg/ml)	Number of mice	doses ml	% Mitotic index (average ±SD)		
			Bone marrow	Thymus	Spleen
0.00	5	0.5	18.18 ±0.612	7.68±0.155	12.8±0.435
2.5	5	0.5	19.53±0.466	10.12±0.286	20.94±0.92
5	5	0.5	21.72±1.55	18.54±0.793	23.28±1.5
10	5	0.5	24.29±0.23	14.34±1.025	25.42±0.77
20	5	0.5	16.22±0.795	6.24±0.304	11.84±0.32
30	5	0.5	11.54±1.33	6.06±0.415	11.64±0.70



Fig(1): Purification of Eap by using ion exchang chromatography(CM.cellulose 12×2.8 cm) with flow rat(0.5 ml/ min),(5 ml/fract) and salt gradient (0.25-1 M). After collection fraction for each peak , the fractions (33-39) gave high agglutination activity.

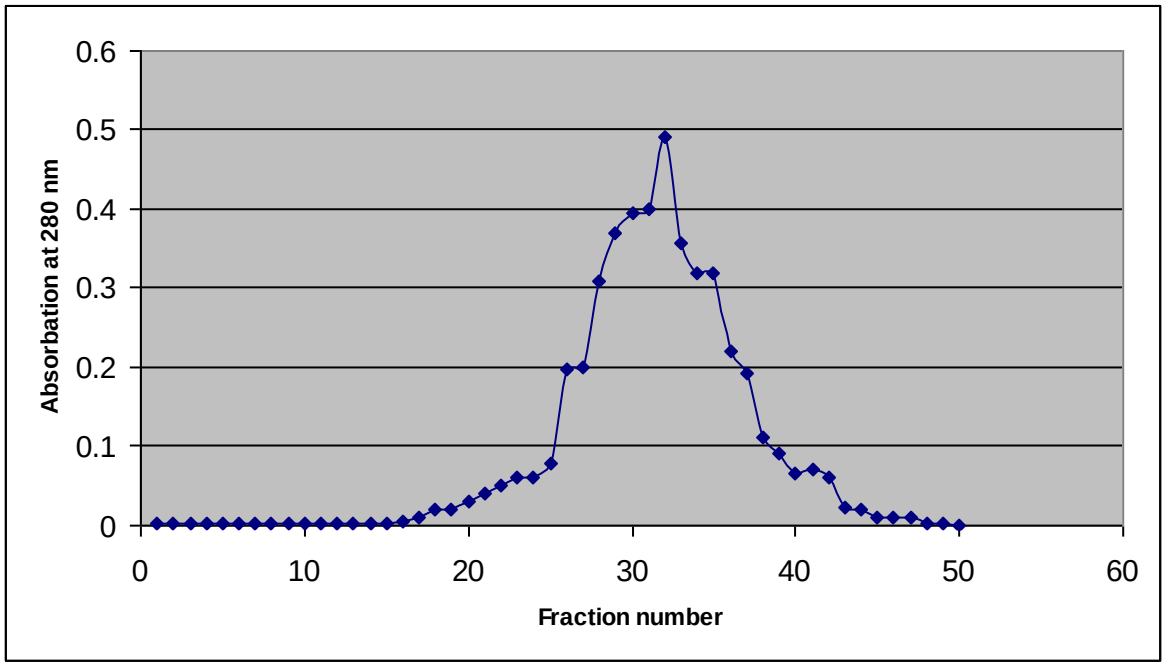


Fig (2) :Purification of Eap by using Gell filtration chromatography (Sepharose.6B 60 ×1.2 cm) with flow rate (0.3 ml/min) and (3ml/fract).fraction from (24-38) gave high agglutination activity after collection .

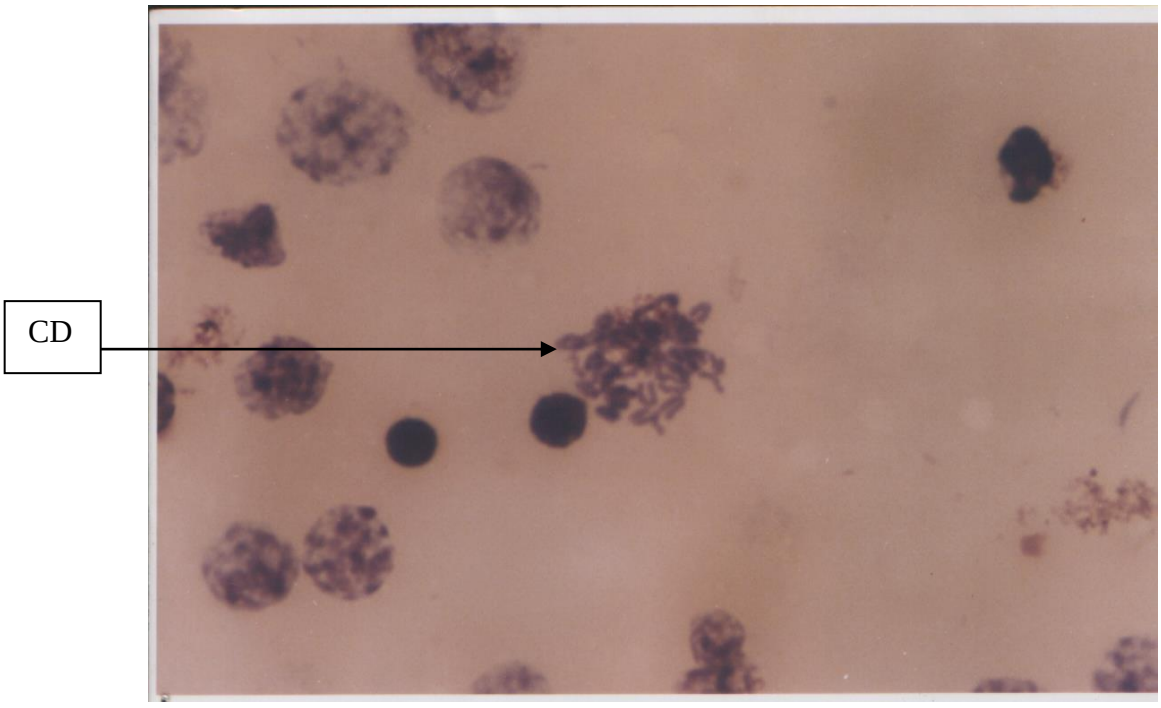


Fig (3) : Spleen cell division in Mice treated with(20 µg/ml) from partial purification extracellular adherence protein chromosomal division (CD), magnification power (1000 x)

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