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A Modified Method for Preparation of *Staphylococcus aureus* Peptidoglycan Towards Making a Sub – Unit Vaccine

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ABSTRACT

The immounological responses to *S. aureus* cell wall antigens, namely crude or whole cell, teichoic acid (TA) peptidoglycan prepared in conventional way (CPG), cell wall (CW), lipoteichoic acid (LTA) were investigated by number of immunological methods e.g. RIA, ELISA, immunodiffusion and western blotting techniques.Also a method for preparation of peptidoglycan (MPG) was modified and used as vaccine for our protection studies. In general the responses in rabbits (serum titre) to fractions of *S. aureus*, varied, crued antigen showed the highest end-point titer while lipoteichoic acid showed the lowest end-point. The highest end-point titre was always seen between homologous sera and antigens. Modified pepdidoglycan and cell wall (PG + TA) showed the similar titre against *S. epidermidis* hyperimmueserum. The cross – reactivty was also seen between fractions by *S. aureus* Bate . *S. aureus* Wood (lack protein A) *S. epidermidis* and streptococci against various immune sera tested by Elisa and gel diffiusion. The results demonstrated that protective antibody can be obtained between different strain or species of bacteria due to the presence of common antigen or antigenes especially PG in gram - positive bacteria.

INTRODUCTION

Infection due to *S. aureus* have been and continue to be an important cause of community and hospital acquired infection. *S. aureus* infection is the second most common cause of wound infection to *E. coli* as an overall cause of infection and the most common cause of wound infection.

The widespread use of antibiotics has led to the re- emergance of multiple – resistant strains and methicillin resistant strains have caused problems in many countries including USA, Australia, and recently in the UK (1,2).

Numerous studies have reviewed the clinical features of staphylococcal infections. Bacteraemia in association with other infection, especially pneumonia, osteomyelitis have been investigated (3,8). *S. aureus* is also causative organism of many superficial infections like boils ulcer skin excema. Many of the recent studies of *staphylococcal* infection have been involved with the disease called toxic shock syndrome(TSS) which is similar to entertoxin F. Many questions are remained about the nature of *staphylococcal* infection, how organism gain access to the blood stream or the reason for such a high proportion of positive culture and the role of complement; the nature of their contribution is not clearly nuderstood. The other problem with *staphylococcal* infection may be the absence of underlying disease in experimental animals and especially in human volunteers.

An effective *staphylococcal* vaccine has become an important requirement for the following reasons: 1) increasing antibiotic resistant. 2) to protect high rick patients including those who are immunosuppressed. 3) patient with recurrent sepsis and animals with mastitis. Attempts to prepare an effective vaccine from extracellular *S. aureus* produts or cell-associated commponents have been in progress for more than 50 years. A vaccine consisting of killed organism (heat- inactiveted or formalized) or staphylococcal toxoid have not proved effective. However, it was possibble to show some immunological responses(5).

In this study, a vaccine was developed which would offer protection against a wide range of *S. aureus* strains and possibly other gram – positive bacteria.

The use of peptidoglycan was studied, because it is the structure which has been shown to play a major part in the opsonic recognition of *staphylococcal* and also has a great capacity to activate both the classical and alternative pathways of human complements.

MATERIALS AND METHODS

Bacterial Strains

The strains used in this study were *S. aureus* wood strain 46.NCTC, *S. aureus* Bate(boold isolate), and clinical isolates from teaching hospital.

Culture of Bacteria

Strain of bacteria were originally obtained as freeze dried ampoule (from NCTC) or from cultures on boold agar plates. All cultures were subcultured on 10% boold agar plates and checked for bacterial purity.

Growth Condition

For routine culture preparation of staphylococci two or three colonies of bacteria were transferred from blood agar plates to 20 ml of brain heart infusion (BHI) in universal bottles and incubated at 37°C with gentle shaking, if large volume of

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culture was needed, organism grown in universal bottles were transferred after appoximately 6 hrs to 2 lit of BHI closed cotton wool.

Viable Counts of Organisms

In all cases of antigen prepartion, organisms were harvested and washed twice in PBS. The viable count was then estimated by serial ten fold dilution in normal sterile saline.(ranging from 10^{-1} to 10^{-7}). For each dilution 25µl was spotted on a blood agar plates and plates spread evenly over the plate. Plates were incubated for 18 hr at 37°C and colonies between 50 –100 were counted .

Crude Preparation of *S. aureus* Antigen

S. aureus strain were grown in two liters of BHI and harvested after 18 hrs and washed twice in pbs, the suspension of 10^{11} cfu/ml was prepared and sonicated with glass bead for 30 min, temperature were kept between 35-37°C using thermocouple. After sonication the titration of bacteria on blood agr plates showed reduction to 10^{10}cfu/ml. The glass bead were removed and the supernatant after centrifugation used as crude antigen and the pellet was used for preparaion of cell wall fractions.

Fraction Prepartion

The following fractions, cell wall (CW), peptidoglycan (CPG) teichoic acid (TA) were prepared by the method of Peterson et al (2) and lipoteichoic acid was prepared by the method of Coley et al (3).

Preparation of Modified Peptidoglycan (MPG)

The MPG was prepared by modified method of Christensson et al (8). *S.aureus* was used for PG preparration which was used as vaccine, bacteria were grown, harvested, washed and resuspended in 5 ml of 10^{11} cfu/ml saline. Then the bacteria was treated with 10% and 5% trichloroacetic acid (TCA) at 4°C and 90°C, respectively.

RESULTS

Different fractions of *S. aureus* strain Bate were analyzed for amino acid and amino sugar profile by method of Hare. Table 1 shows the results of amino acid and amino sugar measurement of the fractions (4).

Table 1.**The quantitative measuremenet of amino acid and amino sugar from fractions of *S. aureus* (nmol/ml)**

Aminoacid & Amino sugar	LTA Lipoteichoic Acid	TA Teichoic Acid	CPG Conventional Preparation of Peptidoglycan	MPG Modified Peptidoglycan
Asp	22	370	2400	1300
Thre	6.5	32	1200	640
Ser	23	85	2400	640
Glu	19	300	5800	1500
Pro	-	100	640	360
Gly	110	970	16000	920
Ala	10	5400	9000	1500
Cyst	-	-	-	8.4
Val	4.9	38	1300	780
Met	-	6	490	210
Ileu	3.3	29	1200	660
Leu	4.1	23	1600	880
Tyr	-	-	-	330
Phe	-	-	-	510
His	-	-	610	260
Lys	4.9	400	5000	890
Arg	7.5	32	900	440
Glucosammine	-	25000	10000	4500
Muramic acid	-	-	8900	3800

(tetra peptide component) e.g. alanine, glutamic acid and lysine as well as interpeptide beidge which is mainly made of glycin in *S. aureus* were shown to be present in very high amount. MPG

The results of the analysis of the fractions showed that PG prepared in conventional way and MPG were relativity pure, in addition, the main amino acids are involved in peptide subunit

Antigenic Activity of MPG

MPG was prepared from both strains (Bate and Wood)showed some antigenic activity against all human and patient's sera. The patient's serum on average showed a higher end - point dilution than the nomal sera against MPG from both strains, this activity was higher against Bate MPG than Wood MPG when it was measured against the patient's serum and slightly higher against normal human sera. The end - point dilution of 15 ormal sera and a patient's serum are shown in table 2a .MPG also cross - reacted with hyperimmune sera raised against *S. aureus* and *S. epidermidis* 2, however, this activity was much higher against homologous serum. The end- point dilution of hyperimmune sera against MPG (prepared from both strains)are shown in Table 2b.

showed the presence or traces of amino acid including those which have not been seen in PG. The reason for relative purity of TA from PG contamination was shown by the excessive ratio of glucosamine to muramic acid in the cell wall, further evidence of PG separation from TA was obtained by gel diffusion and by measuring anitbody against these fractions.

Polypeptide Analyses of Cell Wall Fraction

The analyses of cell wall fraction, by SDS - PAGE showed approximately 50 polypeptide band for crude antigen. MPG showed 5 clear and about 3- 4 faint bands ranging from 25 k to 85 k, teichoic acid showed two clear bands with 25 k and 47k, which were different from sharp bands of MPG. No bands were detected from LTA and PA showed a diffuse band and a thin band below it with MW of 43 kd (Fig.1).

Table 2a. **Cross – antigenic reactivity of MPG prepared from *S. arueus* Bate and Wood against hyperimmune sera to crude *S. arueus* Bate, Wood, *S. epidermidis* 2 and homologous serum**

Hypreimmune sera	Beta "MPG"	Wood "MPG"
***S. arueus* Bate MPG**	25119	891
***S. arueus* Bate Crude antigen**	15849	1413
***S. arueus* Wood Crude antigen**	1122	10.000
***S. epidermidis* Crude antigen**	794	891

Table 2b. **Comparison between the end - point sera dilution of normal human sera and patient's serum against MPG prepared from two strains of *S. arueus***

Human sera	Bate "MPG"	Wood "MPG"
(1)	708	749
(2)	398	355
(3)	354	282
(4)	126	251
(5)	1259	981
(6)	562	251
(7)	355	200
(8)	355	178
(9)	178	178
(10)	282	316
(11)	251	251
(12)	224	200
(13)	141	158
(14)	126	112
(15)	112	112
Patient's serum	1000	631

these fractions varied substantially against human and hyperimmune sera. The crude antigen showed a higher activity against human sera than other fractions and these activities were high against human sera (no 1,5). The antigen activity of the

Antigenic activity of *S. aureus* fraction was investigated on some human sera and against fractions of Wood strain. The results indicated that the antigAntigenice activity of *S. aureus* fractions was investigated some human sera and agaenecity of

cell wall and MPG were also high against these two sera. TA showed less activity than MPG and CW but higher than LTA. The crude antigen showed highest activity against homologous serum and cross - reacted with hyperimmune sera raised against *S. aureus* Wood and *S. epidermidis* antigen. MPG showed the highest activity against homologous serum and cross- reacted with other hyperimmune sera (Table 3).

Comparison between cross- antigenic activity of *E. coli* and *S. faecalis* to hyperimmune sera raised to fraction of *S. aureus* Bate, *S. epidermidis* crude antigen and homologous sera to both antigens indicated that *S. faecalis* has stronger antigen reactivity and higher end - point titre than *E. coli* in all instances. This is probably due to the similarity in the structure of streptococci and hence the cross- reactivity between antigen and antibody *E. coli* cross- reacted in a different way to *S. feacalis* with the above sera (Tables 4 and 5).

Table 3. **The end - point dilution of normal human sera against of S. arueus Bate fractions and their cross- reactivities against hyperimmune sera raised to *S. epidermidis*, *S. arueus* Wood and homologous sera**

Human sera	**Crude**	**MPG**	**TA**	**CW**	**LTA**
Serum(1)	1000	-	293	794	158
Serum(2)	708	-	79	446	112
Serum(3)	708	-	281	316	100
Serum(4)	158	-	126	126	89
Serum(5)	1259	-	398	1122	251
S. arueus Bate crude	22358	15849	1122	17783	251
S. arueus wood crude	4464	1122	251	3162	158
S. epidermidis	3162	794	63	794	80
Homologous sera	-	25119	5012	79437	631

Table 4. **The end - point sera dilutions of various hyperimmune sera against *S. faecalis* and *E. coli***

Hyperimmune sera	***S. faecalis* antigen**	***E. coli* antigen**
***E. coli*(crude)**	178	25119
***S. faecalis* (crude)**	31623	100
***S. aureus Wood* (crude)**	2518	316
***S. aureus Bate*(crude)**	3162	447
***S. epidermidies* (crude)**	1585	316
***S. aureus Bate* (CW)**	1413	282
***S. aureus Bate* (MPG)**	1000	282

Table 5. **The end - point sera dilutions of various hyperimmune sera against *S. faecalis* antigen**

Hyperimmune sera	*S. epidermidis* antigen
S. epidermidis	19953
***S. aureus* Bate**	8912
***S. aureus* Wood**	5623
***S. aureus* Bate MPG**	1778

Antiobody Cross–Reactivity

To examine whether the antibody raised against fractions of *S. aureus* in rabbit cross- react with other cell wall antigens, hyperimmune sera to various fractions were used against homologous cell wall antigens. By using RIA the end-point sera dilution of these were measured (Table 6). These experiment showed that cross – reactivity between the cell wall hyperimmune and wall and antigens were varied.

Table 6. **End point dilution of hyperimmune sera to fractions of *S. arueus* Bate and crude Wood against fractions of *S. arueus* Bate**

Hyperimmune sera to Bate fractions and Wood crude antigen	CW	CPG	MPG	TA	LTA
Crude	44668	28184	25119	5623	708
MPG	22387	4467	25119	158	126
CW	28183	15849	12589	17783	126
CPG	10000	22387	2517	89	112
TA	1000	447	178	7943	200
LTA	100	224	12	178	562
Wood crude	5623	891	1000	708	158

The hyperimmune sera to MPG and crude antigen showed the same end - point titre against MPG and immune sera to cell wall (mixture of PG and TA) showed the highest end - point titer. The activity of homologous sera in general was higher than the cross-reactivty against other cell-wall antigens. Hypermimune serum to MPG showed the third highest end-point titer against cell wall antigen.

Immunodiffusion

Homologous serum raised against crude antigen of *S. aureus* Bate showed at least 6 different immunoprecipitin lines (Fig. 2). MPG and CPG seemed to share a line of identity with one of the lines obtained with the crude antigen (wells 2 and 4) Fig. 2.

Hyperimmune serum raised against *S. aureus*_Bate showed a precipitin line against homologous TA (well 6) which was different from the MPG and CPG precipitin line (wells 1 and 5). Commercially bought PA (well 3) was placed adjacent to crude *S. aureus* Bate antigen (well 2 and 4) and hyperimmune serum raised to crude *S. aureus* Bate in the center well. A distinct line which obtained in crude antigen was similar to that PA and another line which is the non- specific reaction between PA and IgG present in hyperimmune serum was also seen.

Western Blot

The autoradiograph of the blot which was treated with hyperimmune serum to MPG showed at least 9 to 10 polypeptide bands bands against homologous antigen (Fig. 3). The bands which were detected showed MWs ranging from 71K to 83K.Polypeptide with MW of approximately 42K was present in a large amount and another band with MW of 74 KD was also quite clear. However, the latter was present in a large amount against hyperimmune serum to crude antigen, as is shown in Fig.3.

DISCUSSION

Characterization of Cell Wall Antigens

The amino acid analysis of modified peptidoglycan (MPG) showed that glutamic acid, glycine, alanine and lysine were the major amino acids, consistent with author the work (6), in particular *S. aureus* showed the same amino acids, the major constituants of PG in *S. aureus*.

The investigation of both peptidoglycan (MPG and CPG)on immunodiffusion gel tested against hyperimmune serum to crude whole cell antigen showed different single precipitin lines. In summery, although the prepartion and analysis of purified cell wall fractions is time- consuming and contamination of the fractions can easily occur, it is not difficult to accomplish and, if necessary, may be compared with synthetic standards. Lipoteichoic acid showed very low profile of amino acids and no precipitine line was detected against homologeus serum.

Comparison Between Polypeptide Patterns of *Staphylococcal* Strains

In general the polypeptide pattern of *S. aureus* strains (all clinical isolates) showed some similarities. However, the density of some bands and the number of a few bands especially those with high molecular weights, differed from strain to strain; most of the bands in staphylococci had molecular weights ranging from 158K and 26K.

The conclusion is that quantitative measurment of cell wall antigens prepared from *S. aureus* (strain Bate) as measured by amino acid analyser, correlated with the ploypeptide pattern of these antigens on SDS-PAGE. These were also shown by the number of immunoprecepitine lines on immunodiffusion gel.

Comparison between various preparation of antigens showed that a high concentration of viable bacteria and long sonication with controlled temperature during sonication, exposes most of bacterial antigens. In this study it was shown that patients with low incidence of peritionitis showed high titre of lgA in their sera and dialysate against MPG where as high levels of lgG were seen in patients with high and low rate of infection. This study indicated that passive immumization of a CAP patient with anti- MPG may be an important step forward for treatment.

Fig. 1. **Comparison between different fraction of *S. aureus* Bate conventionally prepared PG, lysostaphin, crude antigen treated with lysostaphin, non- treated crude with lysostaphin, modified PG, TA, LTA and PA are show in tracks a to h. Lysostaphin and crude antigen after three minutes sonication are shown in tracks 1 and j. The molecular weights of some polypeptide bands are shown by the number ($\times 10^3$) on the vertical axis. Electrophoresis of the samples were done in 10% polyacrylamide gels.**

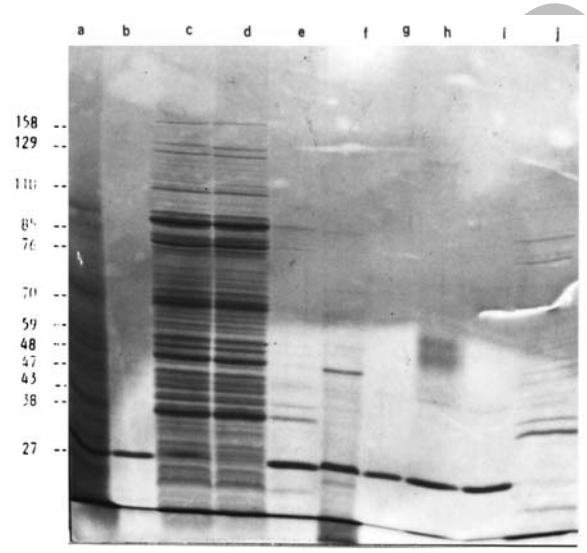


Fig 2. **Immunodiffusion gel (print and diagram) showing the reactions between *S. aureus* Bate immune serum (centre well) with the modified peptidoglycan MPG (well 1), crude antigen (well 2), protein A (PA) (well 3), crude antigen (well 4), conventionally prepared PG (CPG) (well 5) and TA (well 6) prepared from the same strain.**

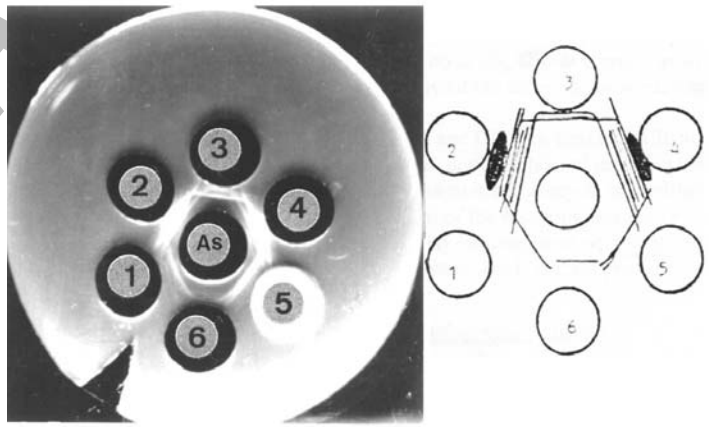
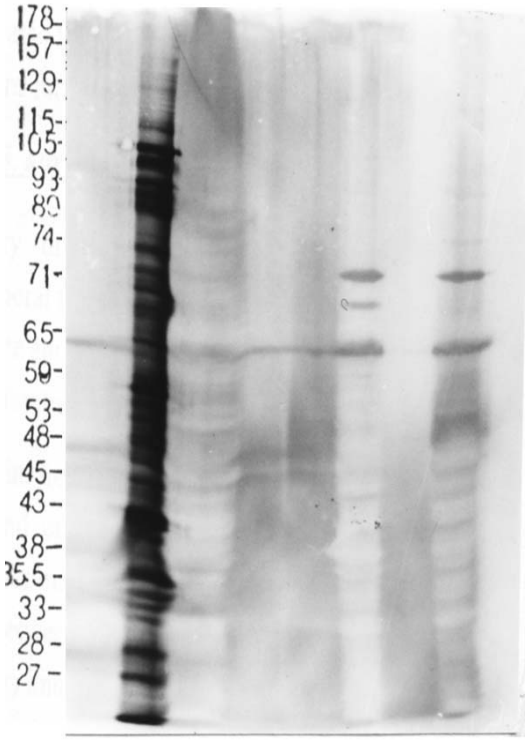


Fig. 3. **The Western Blotting of MPG**



The Immunogenecity of *S. aureus* Cell Wall Antigens

The immunogenecity of *S. aureus* Bate and Wood cell wall fractions was investigated in rabbits using RIA. In general the highest end – point titres was seen between homologous sera and antigens. *S. aureus* Bate and Wood cell wall antigens cross – reacted with byperimmune sera raised to *S. epidermidis* of fractions of *S. aureus* Bate.

The highest end-point titre between cell wall antigens was seen between crude and other hyperimmunesera. MPG and cell wall (prepared from *S. aureus* Bate) showed a similar titre against *S. epidermidis* hyperimmune serum, although cell wall showed higher titre to immune sera' raised also seen between fractions of *S. aureus* Bate, Wood, *S. epidermidis* and streptococci, against various immune sera on gel diffiusion gels. The above results demonstrate that protective antibody can be obtained between different strains or species of bacteria due to the presence of common antigen or antigens, especially PG in gram - positive bacteria.

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