EFFECT OF PENICILLIN RESISTANCE OF STAPHYLOCOCCI ON ANTIGENIC BEHAVIOR

by

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B. Sc. (Vet.) - Bombay University, 1954

A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Bacteriology

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1961

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INTRODUCTION

It has been observed that staphylococci which become resistant to penicillin show modified behavior as regards their morphology, metabolism and certain internal structures.

Staphylococci may acquire resistance *in vivo* or *in vitro* in the presence of low concentrations of the antibiotic. Penicillin resistance *in vivo* is attributed to the formation of the enzyme penicillinase (Spink and Ferris, 1947; Gilson and Parker, 1948), which destroys the antibiotic while that acquired *in vitro* is the effect of altered metabolism (Gale and Rodwell, 1949).

The morphological changes studied in the past have included the property of the cell's becoming Gram negative and general reduction in the size of the organism. With respect to metabolic changes, organisms synthesize certain amino-acids, and are independant of their presence in the medium (Gale and Tayler, 1947). It has also been observed that there is accumulation of certain polypeptides (Hotchkiss, 1950) and amino sugars in or around the cell wall material of penicillin-treated cells (Park and Strominger, 1957). Antibiotic resistant bacteria generally exhibit reduced virulence and are more susceptible to phagocytosis (MacFadden *et al*, 1960).

Under these altered conditions it is worthwhile to study the antigenic behavior of the organism. An attempt, therefore, has been made to review the literature on the subject and to study how the organism reacts in relation to the agglutination test, precipitation test and protein (tuberculin type) reaction.

REVIEW OF LITERATURE

Dufernoy and Pratt (1947) have shown that cells of Staphylococcus aureus under the influence of bacteriostatic concentrations of penicillin gradually lose their positive reaction to the Gram's Stain. They have also pointed out that the loss of Gram positivity was correlated with changes in the character and distribution of the vacuolar material in the cell and with the shift of -SH to S-S at the threshold of the boundaries of the zones of inhibition. Carpenter (1959) has mentioned that Gram positive bacteria induce relatively low antibody concentration.

Blair et al (1946) while studying the action of penicillin on staphylococci observed that during the development of resistance to penicillin, there was reduction in the ability to form pigment and retardation of hemolysis of blood agar and fermentation of mannitol. No alteration in the coagulase positivity was observed. However, morphologically there was considerable variation in the size of the cocci. Though some swollen forms were seen, the cocci appeared to be somewhat smaller than normal in size. Gram's stain showed irregular staining of the cocci. With restoration to sensitivity, normal morphology and biologic activity were observed. Further, they observed a more striking change, viz., the definite loss of virulence of the cultures and a measurable reduction in the toxicity of the strains.

Abraham et al (1941) while studying adaptation of S. aureus to penicillin, observed that increased resistance could occur to a concentration of 1:1000. They observed an important effect in the adaptation to penicillin through a reduction both in rate of growth in ordinary media and enzyme activity. Growth was observed to be slow both in broth and agar, and hemolysis on blood agar plates was much delayed. However, there was no

difference in coagulase activity. These workers have further shown that the adaptation of staphylococci to penicillin was not dependant upon the production of penicillin-destroying enzyme by the organisms. When treated with appropriate amounts of penicillin (1 ml extract and 1 mg of penicillin), the extract obtained after crushing the bacteria did not destroy the penicillin nor reduce its activity. This was also proved by growing Escherichia coli in certain concentrations of penicillin which remained unaltered even after rich growth of the organisms.

Clara and Houch (1943) have pointed out that cultures which become resistant to penicillin showed a marked decrease in virulence. The loss of virulence to mice was associated with increased resistance to penicillin.

Cummins and Harris (1956) while studying the cell wall structure of S. aureus recorded the presence of glucosamine in the cell wall and that hexoses and pentoses were not detected. Park and Strominger (1957) analysed cell wall structure of S. aureus, and the compounds present were glutamic acid, lysine, alanine, 3-O-carboxyethyl hexosamine. They observed that uridine pyrophosphate N-acetyl amino sugar peptide was a biosynthetic precurser of the bacterial cell wall and that accumulation of this compund in penicillin-treated S. aureus was the consequence of the interference by penicillin with the biosynthesis of the cell wall. Mitchell and Moyle (1954) have found that penicillin-treated Micrococcus pyogenes (S. aureus) showed decreased RNA synthesis compared to that of DNA, but initially the percentage of both RNA and DNA increased because of inhibition of synthesis of some unidentified component which contributes significantly to the cell wall dry-weight. Furthermore, in addition to the chemical changes, there were changes in the surface of the penicillin-treated cells as exhibited by their light scattering properties and suspension stability.

Raddison et al (1956) and MacFadden et al (1960) have observed increased susceptibility of antibiotic resistant organisms to phagocytosis.

Human A and B blood cells (Carpenter, 1959) have stable agglutinogens that are comprised of polysaccharide - amino-acid complexes containing galactose and an amino-sugar, N-acetyl glucosamine, and are closely related chemically.

Julianelle and Wieghard (1935a) in their experiments on the specificity of carbohydrates have observed that at least two immunological types existed among the staphylococci. The pathogenic strains belonged to group A and non-pathogenic to group B. It was also found that both virulent and avirulent strains were capable to elaborating type specific carbohydrates and the virulence, therefore, was not associated with the soluble specific substance but rather with a particular type of polysaccharide. They have also observed that the supernatant fluid of centrifuged broth cultures and acid-extracts of sedimented bacteria showed precipitation in homologous immune sera. The evidence indicated that the carbohydrates were apparently contained within the cell in striking contrast to ectoplasmic distribution of type specific polysaccharides of encapsulated bacteria. They also reported that the type specific antibodies in the sera of immunized rabbits varied in individual animals but the agglutinin titers were high in all the antisera. They studied the effect of different methods of immunization on precipitin formation and found that type specific precipitins were stimulated only following intravenous injections of bacteria into the experimental animal.

The same investigators further (Julianelle and Wieghard, 1935b) observed that the soluble specific substance which determined the type specificity of the staphylococcus, was a poor antigen in the form in which it existed in the cell. It was unable to stimulate specific antibody response

in rabbits. On purification it lost even the weak antigenic properties it possessed. Acetylation or adsorption of the polysaccharide on collodian particles did not affect measurably the lack of antigenicity. It could be precipitated only in antibacterial sera. The carbohydrate on injection in the skin of patients infected with staphylococcus elicited an immediate wheal and erythematous reaction which was specific to type.

The proteins separated from the bacteria were species specific and were common to both virulent and avirulent groups of staphylococci. The proteins were antigenic and induced the formation of precipitins which reacted with protein solutions obtained from either type. The proteins reacted both in anti-protein and anti-bacterial sera. In addition, they observed that the proteins exhibited skin reactive properties which produced a delayed, inflammatory reaction specific of the species rather than the type.

MATERIALS AND METHODS

Strains of Staphylococci

The strains of staphylococci used were isolated from milk samples from Kansas State University dairy cows which had staphylococcal mastitis. The details of the strains are shown in Table 1.

Table 1. Source and bacteriophage characteristics of staphylococci used in the experiments.

Strain No.	Cow No.	Phage type	Remarks
I	30-B-1	42 D (CTD)*	Organism isolated from the milk of cow No. 30 B; from right fore quarter.
II	30-B-2	29, 52, 52A, 80, 3B 3C, 71, 6, 7, 40E, 47, 54, 81. (Conc.)**	Strain isolated from milk of cow No. 30-B; from right rear quarter.
III	100-C-1	75, 42D. (Conc.)	From cow No. 100-C, right fore quarter.
IV	184-C-3	75, 42D. (CTD)	From cow No. 184-C, left fore quarter.
V	277-2	6, 7, 42E, 47, 54, 81 (CTD)	From cow No. 277, right rear quarter.
VI	280-3	54, 75, 42D. (Conc.)	From cow No. 280, left fore quarter.
VII	342-1	75 (CTD)	Cow No. 342, right fore quarter.
VIII	465-3	75 (CTD)	Cow No. 465, left fore quarter.

*CTD - By critical test dilution method.

**Conc. - By concentration method.

The eight strains used were all hemolytic and coagulase positive. They were all sensitive to penicillin.

Media Used

The parent strains were maintained on nutrient agar slants. The cultures were renewed every month. For inducing penicillin resistance, glucose salt broth was used.

The composition of the glucose salt broth was:

Potassium citrate.	1.0 g
Ammonium phosphate, $(NH_4)_2H\ PO_4$.	0.5 g
Potassium bicarbonate, KHCO3.	0.5 g
Potassium phosphate K_2HPO_4.	1.5 g
Peptone.	20.0 g
Dextrose.	0.5 g
Distilled water	1000.0 ml

Dissolve over flame. Adjust to pH 7.0. Sterilize for 30 minutes at 20 lbs. pressure.

The penicillin used was crystalline potassium Penicillin G, Pfizer, Lot No. 02435 with an expiration date of July, 1963.

Inducing Development of Penicillin Resistance

For each strain, twelve tubes, each containing 10 ml of the medium were used. Initially the amount of penicillin incorporated in the medium was at a very low but increasing concentration.

The inoculum for the first time consisted of 24-hr agar slant growth suspended in sterile saline. Two to three drops of this saline suspension were added to each tube. Tubes No. 11 and 12 served as controls. The tubes were incubated at 37°C for 36 to 48 hours and were kept at room temperature for 24 hours more. The growth was observed every 24 hours.

As there was turbidity produced in all the tubes except in tube No. 12 of each series (tube No. 11 produced heavy turbidity) showing some evidence of growth in the rest of the tubes, material from tube No. 10 of each series was inoculated onto nutrient agar slants and incubated 24 hours to detect living resistant staphylococci.

A second series of tubes with increasing concentrations of penicillin was inoculated with the broth culture of tube No. 10 of the previous series for each strain. This time, however, no turbidity was noticed after 72 hours. A separate set of tubes, as in the second series was again prepared with increasing concentrations of penicillin and the inoculum used was the agar suspension of the staphylococci inoculated from broth tube No. 10 for each strain of the previous series. With the usual incubation period, growth was obtained in some tubes of each strain. The concentration of penicillin in broth was thus increased gradually and the organisms from tubes showing growth and containing the highest concentration of penicillin were used as inocula. Eight series of inoculations were required to enhance the resistance to 50 units of penicillin per ml. Every time tube No. 11 produced heavy turbidity, and tube No. 12 showed no growth. Table 2 shows the protocol of concentrations of penicillin used for the first series for each strain.

Table 2. Protocol of concentrations of penicillin in the medium for the first series.

Strain No.	Tube Numbers											
	1	2	3	4	5	6	7	8	9	10	11	12
	Penicillin concentration - Units per ml.											
	0.01	0.02	0.03	0.04	0.05	0.06	0.07	0.08	0.09	0.10	Nil	100

Occasionally the resistance acquired was tested to see if the organisms were still susceptible to the concentration of penicillin by disc method

(5 unit penicillin per disc) even though they had acquired resistance to a slight extent in the tube test.

When strain No. I showed a consistant increase in penicillin resistance it was selected for further study. The strain was designated as I-r (resistance strain No. I) and the parent strain as I-s (sensitive strain No. I).

Production of Antisera

Preparation of Bacterial Suspension for Inoculation Into Rabbits. Oeding's method (1957) modified to a certain extent was used for the production of antisera against the two strains. Glucose salt agar was used in Blake bottles for growing the bacteria in large amounts. The surface of the medium was dried by keeping the bottles in the incubator for a few hours and penicillin solution to the concentration of 50 units per ml was added to the medium. The bottles were inoculated from a broth culture containing 50 units of penicillin per ml and incubated 48 hours at 37°C. The growth was harvested with 20 ml of sterile saline for each bottle. The suspensions were then filtered through sterile cheesecloth and centrifuged. The sedimented cells were adjusted to Nephelometer tube No. 7 with sterile saline. The cell suspension was killed by adding 0.3 percent formaldehyde and incubated 48 hours. The sterility of the suspension was tested by inoculating a drop on to nutrient agar and into nutrient broth. A suspension was prepared in the same way from the parent strain I-s except penicillin was omitted from the medium.

Inoculation of Rabbits. Four rabbits were taken for each strain (I-s and I-r). Eight intravenous injections were given to every rabbit, in three series, the dose being increased in each series with increasing interval for later injections. Four to five ml of blood was removed from

each rabbit before inoculation and the serum obtained was preserved for use as control serum.

Table 3 shows the schedule of rabbit inoculations.

Table 3. Schedule of inoculations of rabbits with bacterins.

Date	Strain I-r Rabbit No. and dose				Strain I-s Rabbit No. and dose			
	1	2	3	4	5	6	7	8
9-5-61	1 ml	1 ml	1 ml	1 ml	1 ml	1 ml	1 ml	1 ml
11-5-61	1 ml	1 ml	1 ml	1 ml	1 ml	1 ml	1 ml	1 ml
13-5-61	1 ml	1 ml	1 ml	1 ml	1 ml	1 ml	1 ml	1 ml
16-5-61	2 ml	2 ml	2 ml	2 ml	2 ml	2 ml	2 ml	2 ml
20-5-61	2 ml	2 ml	2 ml	2 ml	2 ml	2 ml	2 ml	2 ml
25-5-61	2 ml	2 ml	2 ml	2 ml	2 ml	2 ml	2 ml	Died
30-5-61	4 ml	4 ml	4 ml	4 ml	4 ml	4 ml	4 ml	
4-6-61	4 ml	4 ml	4 ml	4 ml	4 ml	4 ml	4 ml	
8-6-51						Died	Died	

One week after the last injections, the rabbits were bled from the heart and 20 to 50 ml of blood were collected, depending upon the size of the rabbit. The serum obtained was stored in a home freezer until used. These rabbits were subsequently used for the skin test to be described later.

Tests Carried Out

The following tests were carried out with I-s and I-r strains as antigens and/or their antisera:

1. Agglutination Test.
2. Agglutination Test with Human Anti-A and Anti-B Sera against I-s and I-r Antigens.

3. Agglutination Test with Normal Prior and Immune Sera against Human A and B Group Blood Cells.

4. Precipitation Test.

5. Protein (Tuberculin type) Reaction in the Rabbit.

Agglutination Test. Preparation of antigen. Blake Bottles containing glucose salt agar with 50 units of penicillin per ml were inoculated with the resistant (I-r) broth culture. The bottles were incubated 48 hours at 37°C and the growth harvested with 0.3 percent phenolized saline. The growth was filtered through cheesecloth to removed fragments of medium and centrifuged. The sediment was suspended in phenolized saline to give a thick suspension and then stored. From this thick suspension, antigen for agglutination tests was prepared. The opacity of the antigen was adjusted to nephelometer tube No. 1. Antigen was prepared in a similar manner from the I-s strain using the plain glucose salt agar.

Procedure. The agglutination test was carried out with serum samples from the rabbits collected before and after immunization. Two-fold geometric dilutions of the serum were used, starting from 1:20 to 1:5120. A control tube with antigen alone was maintained for every sample. For each sample, two sets were used, one for I-s antigen and the other for I-r antigen. The tubes were incubated at 37°C for 48 hours and read.

Agglutination Test with Human Anti-A and Anti-B Sera with I-s and I-r Antigens. Two-fold geometric dilutions of the serum were used, starting from 1:20 to 1:5120. The antigens used were I-s and I-r for each serum. The tubes were incubated at 37°C for 48 hours and read.

Agglutination Test with Normal Prior and Immune Sera with Human A and B Group Blood Cells. Preparation of cell suspension. Blood from individuals belonging to group A and group B was collected separately in one percent

citrate saline solution. The blood cells were then washed three times with saline solution. After the last centrifugation, the volume of the packed cells was measured and a one percent cell suspension was prepared from each group of cells.

For the agglutination test the serum was diluted in two-fold geometric dilution, starting from 1:10 to 1:320. A control consisting of cell suspension alone was maintained for every serum sample. The tubes were incubated at room temperature and the results were read after 24 and 48 hours.

Precipitation Test. The antigenic extract was prepared by the autocalve method (Rantz and Randall, 1955). The bacterial suspension of each type I-s and I-r was adjusted to nephelometer tube No. 10, using ordinary salone as diluent. Five ml of suspension of each type was autocalved at 15 lbs. pressure for 15 minutes. On cooling, the material was centrifuged and the clear supernatant constituted the antigen for the precipitation test. The test was carried out with "the constant serum and diluted antigen" method. All the serum samples - prior and immune - were diluted to 1:20. The test was carried out in small capillary tubes using the ring method. The diluted serum (0.1 ml) was first placed in the tube and then the antigen (0.1 ml) was added over the serum layer. Ring formation at the interface was regarded as a positive reaction and was recorded after an interval of one hour and three hours, respectively.

It had been separately observed that a saline extract of the organisms also gave a positive preceipitation reaction with the immune and prior sera. Such an extract, prepared by keeping the saline suspension in the refrigerator overnight and then centrifuging, was used only for qualitative testing.

Protein (Tuberculin type) Reaction. A bacterial suspension was adjust-

ed in saline to 10X tube No. 10 nephelometer. Five ml of the suspension was heated in a steam sterilizer at 100°C for two hours. The suspension was then cooled and centrifuged. The supernatant constituted the reagent for the test. The reagent was prepared for each of the (I-s and I-r) strains.

The rabbits which were inoculated with I-r and I-s strains for antiserum preparations were injected intradermally with 0.1 ml of the reagents. One apparently healthy rabbit was taken as a control (Rabbit No. 6). The hair on the back of each rabbit was closely clipped and the area sterilized with 95 percent alcohol. The reagent from the I-s strain was injected on the left side and that from the I-r strain on the right side. Inoculation in each case produced a distinct bead at the spot. The animals were observed for erythema at the site of injection, induration, necrosis or sloughing. The results of the reaction were recorded after 24 and 72 hours.

RESULTS

Agglutination Tests

The agglutination tests carried out with prior serum of rabbit No. 1 against I-s and I-r antigens gave positive reactions (2+ and/or greater) in 1:160 and 1:320, respectively. Serum from the same rabbit after immunization with resistant strain I-r, gave agglutination reactions up to 1:1280 with either antigen.

Prior serum of rabbit No. 2 did not show a positive reaction even in the 1:20 dilution of the serum with I-s antigen; however, a positive reaction with the same serum up to 1:80 dilution was seen with I-r antigen.

Prior serum of rabbit No. 3 gave agglutination up to 1:160 with I-s antigen and up to 1:320 with I-r antigen. The immune serum gave a reaction up to 1:2560 with either antigen.

Prior serum from rabbit No. 4 showed agglutination up to 1:320 with either antigen. The immune serum reacted up to 1:2560 with I-s antigen and up to 1:1280 with I-r antigen.

Prior serum of rabbit No. 5 agglutinated the I-s antigen up to 1:80 dilution and I-r antigen up to 1:160 dilution of the serum. The immune serum produced in this case by injecting the sensitive strain (I-s) reacted up to 1:2560 with I-s (homologous) antigen and up to 1:5120 with I-r antigen. These results are shown in Table 4.

Table 4. Agglutination test results with prior and immune sera of rabbits against I-s and I-r antigens.

Rabbit No.	Type of serum	Antigen used	Serum dilution and tube Nos. 1/20 (1)	1/40 (2)	1/80 (3)	1/160 (4)	1/320 (5)	1/640 (6)	1/1280 (7)	1/2560 (8)	1/5120 (9)	Nil (Control) (10)
Rabbits immunized with I-r (resistant) strain -												
1	Prior	I-s	4+*	4+	4+	4+	1+	-	-	-	-	-
	Prior	I-r	4+	4+	4+	4+	2+	-	-	-	-	-
	Immune	I-s	4+	4+	4+	4+	4+	4+	2+			
	Immune	I-r	4+	4+	4+	4+	4+	4+	2+	1+	-	-
2	Prior	I-s	1+	Trace	Trace	-	-	-	-	-	-	-
	Prior	I-r	3+	3+	2+	1+	-	-	-	-	-	-
	Immune	I-s	4+	4+	4+	4+	4+	4+	4+	2+	1+	-
	Immune	I-r	4+	4+	4+	4+	4+	4+	4+	2+	1+	-
3	Prior	I-s	4+	4+	4+	2+	1+	-	-	-	-	-
	Prior	I-r	4+	4+	4+	4+	3+	1+	-	-	-	-
	Immune	I-s	4+	4+	4+	4+	4+	4+	4+	2+	1+	-
	Immune	I-r	4+	4+	4+	4+	4+	4+	4+	2+	1+	-
4	Prior	I-s	4+	4+	4+	4+	2+	-	-	-	-	-
	Prior	I-r	3+	4+	4+	4+	3+	1+	-	-	-	-
	Immune	I-s	4+	4+	4+	4+	4+	4+	4+	2+	1+	-
	Immune	I-r	4+	4+	4+	4+	4+	4+	2+	1+	1+	-
Rabbit immunized with I-s (sensitive) strain.												
5	Prior	I-s	3+	2+	2+	1+	-	-	-	-	-	-
	Prior	I-r	3+	3+	3+	2+	1+	-	-	-	-	-
	Immune	I-s	4+	4+	4+	4+	4+	4+	4+	3+	-	-
	Immune	I-r	4+	4+	4+	4+	4+	4+	4+	4+	3+	-

*Number indicates relative completeness of the agglutination reaction.

Agglutination Test With Human Anti-A and Anti-B Sera With I-s and I-r Antigens

Human anti-A serum showed agglutination of I-s and I-r antigens up to 1:80 dilution of the serum. Human anti-B serum reacted with I-s and I-r antigens up to 1:40 dilution of the serum. These results are shown in Table 5.

Table 5. Agglutination test results with human anti-A and anti-B sera against I-s and I-r antigens.

Serum	Antigen	Serum dilution									
		1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	Nil Control
Anti-A (Human)	I-s	4+*	4+	3+	1+	-	-	-	-	-	-
Anti-A (Human)	I-r	4+	4+	3+	1+	-	-	-	-	-	-
Anti-B (Human)	I-s	4-	3-	1-	-	-	-	-	-	-	-
Anti-B (Human)	I-r	4+	3+	1+	-	-	-	-	-	-	-

*Number indicates relative completeness of the agglutination reaction.

Agglutination Test With Prior and Immune Sera From Rabbits With Human A and B Group Blood Cells

Prior serum from all the rabbits showed slight agglutination with human A and B group blood cells. Immune serum from rabbit No. 1 and 2 agglutinated human A and B group cells up to 1:40 dilution of the serum. Immune serum of the rabbit No. 3 showed agglutination of the cells up to 1:20 dilution. Immune serum from rabbit No. 4 agglutinated group A cells up to 1:40 dilution and group B cells up to 1:20 dilution. Immune serum from rabbit No. 5 (immunized with sensitive strain) agglutinated A and B cells up to 1:10 dilution of the serum. These results are shown in Table 6.

Table 6. Results of agglutination tests using prior and immune sera with human A and B cells (1.0 percent cell suspension).

Rabbit No.	Serum	Antigen (Type of cells)	Dilution of serum 1/10	1/20	1/40	1/80	1/160	1/320	Nil Control
1	Prior	A	Sl*						
	Prior	B	Sl	Sl					
	Immune	A	+	+	+	Sl			
	Immune	B	+	+	+	Sl			
2	Prior	A	Sl						
	Prior	B	Sl	Sl					
	Immune	A	+	+	+	Sl			
	Immune	B	+	+	+	Sl			
3	Prior	A	Sl						
	Prior	B	Sl	Sl					
	Immune	A	+	+	Sl				
	Immune	B	+	+	Sl				
4	Prior	A	Sl						
	Prior	B	Sl						
	Immune	A	+	+	+	Sl	Sl		
	Immune	B	+	+	+	Sl			
5	Prior	A	Sl	Sl					
	Prior	B	Sl	Sl					
	Immune	A	+	Sl	Sl				
	Immune	B	+	Sl	Sl				

*Sl - slight agglutination.

Precipitation Reaction

Prior sera (diluted 1:20) from rabbits No. 1, 2, 3, and 5 reacted only up to 1:10 dilution of the I-r antigen. The remainder of the reaction were negative with either antigen. The immune sera were also diluted 1:20 and used against diluted antigens. Immune serum from rabbit No. 1 showed a precipitation reaction up to 1:160 with the I-s antigen and up to 1:320 with the I-r antigen. Immune serum from rabbit No. 2 reacted up to 1:80 with I-s antigen and up to 1:640 with I-r antigen. Immune serum from rabbit No. 3 reacted up to 1:320 with I-s antigen and up to 1:640 with I-r antigen. Immune serum from rabbit No. 4 gave precipitation reaction up to 1:40 dilution of I-s antigen and up to 1:80 of I-r antigen. Immune serum from rabbit No. 5 (immunized with the sensitive strain) showed a precipitation reaction up to 1:160 with I-s antigen and up to 1:640 with I-r antigen. These results are presented in Table 7.

Table 7. Precipitation test results with prior and immune sera (1:20 dilution) against I-s and I-r antigens.

Rabbit No.	Type of Serum (1:20 dilution)	Antigen	1 hour Antigen dilution							3 hours Antigen dilution						
			1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/10	1/20	1/40	1/80	1/160	1/320	1/640
1	Prior	I-s	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Prior	I-r	-	-	-	-	-	-	-	+	-	-	-	-	-	-
	Immune	I-s	+	+	-	-	-	-	-	+	+	+	+	+	-	-
	Immune	I-r	+	+	+	-	-	-	-	+	+	+	+	+	+	-
2	Prior	I-s	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Prior	I-r	-	-	-	-	-	-	-	+	-	-	-	-	-	-
	Immune	I-s	+	+	+	-	-	-	-	+	+	+	+	-	-	-
	Immune	I-r	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3	Prior	I-s	-	-	-	-	-	-	+	-	-	-	-	-	-	-
	Prior	I-r	-	-	-	-	-	-	+	+	-	-	-	-	-	-
	Immune	I-s	+	+	+	+	+	-	+	+	+	+	+	+	+	-
	Immune	I-r	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4	Prior	I-s	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Prior	I-r	-	-	-	-	-	-	+	-	-	-	-	-	-	-
	Immune	I-s	+	+	+	-	-	-	-	+	+	+	-	-	-	-
	Immune	I-r	+	+	+	+	-	-	-	+	+	+	+	-	-	-
5	Prior	I-s	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Prior	I-r	-	-	-	-	-	-	+	+	-	-	-	-	-	-
	Immune	I-s	+	+	+	+	-	-	+	+	+	+	+	+	-	-
	Immune	I-r	+	+	+	+	+	+	+	+	+	+	+	+	+	+

+ = positive (ring formation)

- = Negative (no ring formation)

The Protein (Tuberculin type) Reaction

The tuberculin-type reaction produced in all rabbits with the reagent from the resistant strain was much more severe than that produced by the reagent from the sensitive strain. The reaction produced by the reagent from the sensitive (I-s) strain was in the form of an erythematous zone, varying from 1 to 2 cm in diameter at the site of inoculation with slight induration in one case and no induration in others. Seven days after the injection there was no mark left behind. The reaction produced by the reagent from the resistant strain (I-r) showed a dark hemorrhagic spot at the site of inoculation with induration and a wide erythematous zone of 1.75 to 2.5 cm in diameter. Seven days after the injection, the reaction in two rabbits terminated in sloughing of the skin from the dark necrotic spot with a persistant narrow erythematous zone. The reaction seen in the other animals was in the form of slight reddening only at the site of inoculation. The reaction produced by I-r reagent in the control was cleared up to a great extent by 72 hours, and by seven days no signs remained on either side. The results of the reaction are shown in Tables 8 and 9.

Table 8. Results of intradermal inoculation of protein extract in rabbits after 24 hours.

Rabbit No.	Left Side Injected with reagent from I-s Strain.	Right Side Injected with reagent from I-r Strain.
1	Erythematous zone, no induration. 2.0 X 2.0 cm.*	Central portion dark hemorrhagic, indurated with erythematous zone surrounding. 2.2 X 2.5 cm.
2	Slight erythematous zone only. 1.0 X 1.0 cm.	Central portion slightly dark, indurated with erythematous zone surrounding. 2.5 X 2.5 cm.
3	Erythematous zone, no induration. 1.5 X 1.5 cm.	Central portion slightly dark, indurated and erythematous zone surrounding. 1.75 X 2.0 cm.
4	Erythematous zone, no induration. 1.0 X 1.5 cm.	Central portion dark, hemorrhagic, indurated with erythematous zone surrounding. 2.0 X 2.5 cm.
5	Erythematous zone, part indurated. 1.5 X 1.5 cm.	Central portion dark, hemorrhagic, indurated and surrounded by erythematous zone. 2.0 X 2.5 cm.
6**	Erythematous zone, no duration. 1.0 X 1.0 cm.	Central portion slightly dark, indurated and erythematous zone surrounding. 1.5 X 1.5 cm.

* Size of the erythematous zone.

**Control rabbit.

Table 9. Results of the intradermal inoculation of protein extract in rabbits after 72 hours.

Rabbit No.	Left Side Injected with reagent from I-s Strain.	Right Side Injected with reagent from I-r Strain.
1	Subsiding, slight reddish zone seen.	Induration persistant, central necrotic area formed.
2	Slight erythema.	Small dark necrotic, center, induration and erythematous zone present.
3	Slight reddening.	Small, dark, necrotic center, erythematous zone present.
4	Slight erythema.	Dark necrotic spot, induration present.
5	Slight erythema.	Dark red spot, induration present.
6*	Slight reddening.	Slightly reddish zone.

*Control rabbit.

DISCUSSION

In order to prevent any adverse effect of the nutrient broth on penicillin it was thought advisable to use glucose salt broth for inducing resistance in staphylococci to penicillin. The organisms produced normal growth in the medium and proved to be quite satisfactory for the purpose.

While inducing resistance to penicillin most of the strains acquired the ability to grow easily in the presence of the antibiotic. Strain II, however, did not show increased resistance beyond 1.5 units per ml. Strain III showed granular sediment during growth. Strains IV and V exhibited slow development. Strains VI and VII were more adaptable than IV and V. Strains I and VIII showed continuous and rapid development of resistance to penicillin.

Strain No. I was, therefore, used for the tests in the experiments. During the development of resistance, the organism showed a decreased tendency towards pigment production and an increased tendency towards viscidity of growth. The growth throughout was slow and moderate. This confirms the findings of Abraham et al (1941).

Morphologically the resistant organisms showed some swollen forms. In general, they were smaller than normal and were easily decolorized. The death of the three rabbits used for immunization with the sensitive strain may be explained on the ground that the material from the sensitive strain proved more toxic than that of the resistant strain. Antibody production as judged by the agglutination reaction was practically similar with the resistant and the sensitive strain except in one rabbit it was slightly less which may be due to individual rabbit reactivity towards the antigen. This may show that there is no great difference in the antigenicity

of sensitive and resistant strains in agglutinin stimulation.

Normal serum of the rabbits used also contained natural agglutinins against staphylococci and these agglutinins showed a slightly increased tendency towards agglutination of the resistant strain. The immune serum, either produced by injecting the resistant or the sensitive strain, showed practically the same range of agglutination with resistant and sensitive antigens.

The altered metabolism and staining characteristics are apparently not associated with the agglutinin stimulating property. However, with normal serum or serum prepared with the sensitive strain, a slightly increased tendency was seen in agglutination of the resistant strain.

Human anti-A serum agglutinated the sensitive and resistant strains up to 1:80 dilution and human anti-B serum up to 1:40 dilution of the serum. This shows that the human A and B cells have some common antigenic substance similar to that in the sensitive or the resistant strains. It may be the amino sugar which is common in all these cells which give the positive reactions. Similarly the immune serum produced by the injection of the resistant strain agglutinated human A and B cells up to 1:40 (in one case up to 1:20) while the serum produced by injecting the sensitive strain agglutinated the cells only up to 1:10 dilution. This might have been due to the accumulation of the amino-sugars in the resistant strain which in turn might have increased specificity for the human blood cells.

Natural precipitins against staphylococci have been detected in the normal serum of the rabbits used by a qualitative test. The immune serum, however, showed an increased tendency to produce precipitation in higher dilutions of the resistant antigen. This may similarly be attributed to the increased accumulation of certain carbohydrates or polysaccherides in the cell structure of the resistant strain.

The protein (tuberculin type) reaction showed a distinct difference in the behavior of the two reagents. The severe type of reaction produced by the material from the resistant strain indicated that it had in addition to the polysaccharides some toxic substances that gave rise to the marked reaction. This may be interpreted on the grounds that while the organisms develop penicillin resistance, they assume a Gram negative character. Simultaneously they may acquire the property to produce more endotoxins as they become more resistant. Production of exotoxins may or may not be altered but the cells apparently produce decidedly more endotoxin which can be obtained by the heat treatment method used for the preparation of the protein extract. Gram negativity and endotoxin production may be closely correlated. The antigenic material (protein extract) derived from the Gram positive, parent strain did not produce the severe reaction even in the rabbit which was immunized with the same strain. The reaction occurring in the apparently healthly rabbit (control) may be ascribed to the naturally occurring antibodies in the animal or antibodies which might have been produced by some unknown lesions suffered prior to inoculation.

SUMMARY

1. Strains of Staphylococcus aureus which become penicillin resistant show variation as regards to morphology, metabolism and virulence.

2. Strains of S. aureus, isolated from mastitis milk samples from cows, showed variation in the development of resistance to penicillin.

3. The resistant strain selected for the study showed slow growth and a reduced tendency to form pigment and had an increased tendency to lose the Gram's stain.

4. Immune sera were prepared against the sensitive and the resistant strains in rabbits.

5. Certain serological tests such as the agglutination tests, and precipitation tests were carried with the sera and the antigens. The protein (tuberculin type) reaction was carried out on the immunized rabbits and one control.

6. The resistant strain did not show any altered capacity as regards the stimulation of agglutinins in the rabbit. However, the resistant strain showed a slightly increased tendency to agglutinate in the presence of normal rabbit serum and serum produced against the sensitive strain.

Human anti-A and anti-B blood grouping sera agglutinated both of the antigens to the same extent. Antisera against the resistant strain agglutinated the human A and B group blood cells to a higher degree than that produced against the sensitive strain.

7. Antigen prepared from the resistant strain was precipitated in a higher dilution with serum produced against either type. The "serum constant and antigen dilution" method was used. Serum was diluted 1:20 in all cases.

8. The protein (tuberculin type) test was carried out in the immunized rabbits with protein extract obtained from both strains by the heat treatment method. The reagent from the resistant strain produced a severe reaction compared to the reagent from the sensitive strain. The results were recorded after 24 hours and 72 hours. In some cases the reaction produced by meterial from the resistant strain persisted even after 7 days.

Certain serological tests and reactions with serum against penicillin-sensitive and resistant strains of S aureus have been carried out and their results discussed.

ACKNOWLEDGMENT

The author wishes to express his great indebtedness and sincere appreciation to his major instructor, Prof. V. D. Foltz, Professor of Bacteriology, for his guidance and helpful suggestions during the course of the research work. His appreciation also goes to Dr. T. H. Lord, Professor of Bacteriology, for his valuable suggestions in the preparation of this thesis.

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Penicillin-resistant Staphylococci: Mechanisms involved in the development of resistance. J. Clini Investig. 26:379-393, 1947.

Staphylococci show some altered characteristics when they become resistant to penicillin. Staphylococcus aureus contains certain polysaccharides and proteins which are type specific and species specific, respectively. Attempts have been made to study some antigenic characters of the resistant staphylococci.

Resistance to penicillin has been induced into a strain of S. aureus isolated from a mastitis milk sample from a cow. The resistance was enhanced to 50 units of the antibiotic per ml of the medium by serial transfers of the strain to tubes containing increasing concentrations of penicillin.

Immune sera were produced in rabbits against the parent (sensitive) strain and its resistant derivative. Certain sorological tests were carried out with these antisera. Agglutination tests were carried out with the prior sera (serum samples collected before immunization) and immune sera. It was observed that the agglutinin stimulating capacity of the resistant strain was not altered. The sensitive and the resistant strains were agglutinated practically to the same extent by the immune sera against the resistant strain. However, the resistant strain showed a slightly increased tendency towards agglutination by the agglutinins in the normal sera and those produced by injecting the sensitive strain. Human anti-A and anti-B blood group sera agglutinated the sensitive and the resistant strains to the same extent. Antiserum against the resistant strain agglutinated human A and B group blood cells to a higher titer than the serum produced against the sensitive strain. As regards the precipitation reaction the antigen prepared from the resistant strain showed a precipitation reaction at a higher dilution than the antigen from the sensitive strain with serum produced against either strain.

The protein (tuberculin type) reaction was severe in the rabbits with protein extract obtained from the resistant strain as compared to that produced by the material obtained from the sensitive strain.