ANTIGENIC AND ENZYME SYSTEM CHANGES IN BETA HEMOLYTIC STREPTOCOCCI RESISTANT TO PENICILLIN, STREPTOMYCIN, BACITRACIN AND AUREOMYCIN [1, 2, 3, 4]

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Previous reports from this laboratory (1, 2) have presented preliminary data suggesting that there are antigenic and enzyme system changes in beta hemolytic streptococci resistant to penicillin and streptomycin. These data were all qualitative and were unconfirmed by quantitative methods.

The present study is a continuation of this work. The same strains of group A and C streptococci were made resistant separately to bacitracin and aureomycin. Representative members of each group, resistant to all four antibiotics, were studied for alterations in: *1*) streptolysin S; *2*) streptokinase; *3*) proteinase; *4*) ribonuclease activity.

That such variations might be expected was first suggested by the observation that reversible changes in the hemolytic behavior of streptococci from beta to alpha or gamma type of hemolysis occurred when the organisms were growing on maximal concentrations of either penicillin or streptomycin.

MATERIALS AND METHODS

Strains of Streptococci

Seven group A strains and three group C strains isolated in 1946–1947 from throats of patients with acute upper respiratory infections were employed throughout the study. The strain designations for the group A organisms were: S_1, S_2, S_3, S_4, S_5, S_6, and U_5; and, for the group C strains, U_1, U_2, and U_3.

Development of Resistance

The method employed for inducing penicillin and streptomycin resistance has been described (1, 2). A similar technique was used for inducing bacitracin and aureomycin resistance. Briefly, this consisted of daily transfer of organisms from a blood agar plate containing the maximal quantity of antibiotic that would permit growth to a new series of plates containing graded amounts of antibiotic. For the streptomycin, bacitracin, and aureomycin series a total of 40 such transfers were made, and for the penicillin series 60 transfers.

Streptolysin S Production

The method described by Todd (3) was employed for all tests of streptolysin S production. Thirty ml Todd-Hewitt medium containing 20% normal horse serum inactivated at 56° C. for 30 minutes were inoculated with 1 ml of an 18 hour streptococcal broth culture in Todd-Hewitt medium prepared with 2% Pfanstiehl peptone. After incubation at 37° C. for 12 hours, the cultures were thoroughly chilled in an ice water bath, centrifuged at 3,000 rpm for 20 minutes and the supernate filtered through Selas candles (No. 02 porosity with a maximum pore size of 0.85 micron). Only cultures of resistant strains with densities that matched that of the control strain, *i.e.*, the parent organism transferred 40 times on plain blood agar medium, were employed. The densities were determined in a Coleman Junior Spectrophotometer. Filtrate dilutions ranging from 1:4 to 1:200 were prepared in cold physiological saline so that, after the addition of 0.5 ml of a 5% suspension of fresh triple-washed rabbit RBC's, the final volume was 2.0 ml. The hemolytic end point was considered to be the dilution which showed approximately 50% hemolysis after incubation for 30 minutes in a 37° C. water bath. The lysin then was titrated against anti-streptolysin S rabbit serum (4).

Streptokinase Activity

The method employed for determining the streptokinase activity of the supernate of the broth culture was essentially that described by Kaplan (5). The fibrinogen [5]

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[3] Aureomycin was supplied by the Lederle Laboratories Division, American Cyanamid Co., through the Antibiotics Study Section of the National Institutes of Health.

[4] Bacitracin was supplied by the Commercial Solvents Corporation, through the Antibiotics Study Section of the National Institutes of Health, and by Dr. Frank L. Meleney of Columbia University.

[5] The fibrinogen employed was Fraction I of the plasma proteins prepared by the Department of Physical Chemistry, Harvard Medical School, Boston, Mass., from blood collected by the American Red Cross, and generously supplied by Dr. Edwin J. Cohn and Dr. John T. Edsall.

was used in a concentration of 600 mg dissolved in 100 ml phosphate-buffered physiological saline at pH 7.4. Purified human thrombin was prepared so that each ml of buffered saline contained approximately 3 units. The fibrinolytic titer was expressed as the reciprocal of the dilution in which there was complete dissolution of the clot after incubation in a water bath for one hour at 37° C.

Proteinase Activity

The streptococcal proteinase activity in broth cultures was measured by the method described by Elliott and Dole (6). Briefly, this consisted of observing the rapidity and degree of coagulation of a skimmed milk-thioglycollate substrate by sterile broth culture filtrates. Twenty-four hour cultures of streptococci in Todd-Hewitt broth containing 2% Pfanstiehl peptone were filtered through Selas candles (No. 02 porosity). Maintaining sterility, two-fold dilutions of filtrate were made in phosphate buffer pH 7.0. The milk-thioglycollate substrate was added with an automatic pipette, and the mixture was incubated at 37° in a water bath. The test was read at one, three, five, and nine hours and the activity expressed as the reciprocal of the dilution showing coagulation at nine hours.

Ribonuclease Activity

The ribonuclease activity of culture supernates was determined by the turbidimetric method described by McCarty (7). The substrate, yeast ribonucleic acid, was purified by the method of Kunitz (8). The optical densities were determined on a Coleman Junior Spectrophotometer at a wave length of 425 mμ, at five minute intervals over a 30 minute period.

RESULTS

The group A strains, in general, developed little resistance to penicillin and aureomycin, and considerable resistance to streptomycin and bacitracin. The group C strains showed similar group behavior to each antibiotic. The results are given in Table I.

TABLE I

Antibiotic resistance acquired by beta hemolytic streptococci

Group	Strain	Fold change after 40 transfers on			
		Penicillin medium*	Streptomycin medium	Bacitracin medium	Aureomycin medium
A	S_1	4	140	100	2
A	S_2	2	70	20	6
A	S_3	17	140	40	6
A	S_4	0	140	100	6
A	S_5	4.5	240	40	5
A	S_6	4	180	13	6
A	U_5	6	70	8	8
C	U_1	16	3,000	20	10
C	U_2	14	600	300	60
C	U_3	4	200	300	20

* After 60 transfers

The rate at which a given strain of beta hemolytic streptococcus acquired resistance to an antibiotic was not uniform in the case of penicillin, streptomycin, bacitracin, and aureomycin. For example, strain S_4 after 60 serial transfers on penicillin medium showed no change in sensitivity, after 40 serial transfers on aureomycin medium only slight resistance, but considerable resistance to streptomycin and bacitracin after 40 subcultivations on media containing these antibiotics.

Streptolysin S production was reduced for each group A organism after acquiring either penicillin or streptomycin resistance. The same strains after acquiring bacitracin or aureomycin resistance maintained the same level of hemolysin production as the control strain. The group C organisms behaved somewhat differently in their production of streptolysin S. The three penicillin-resistant and three bacitracin-resistant organisms all produced less hemolysis than the control strains. The streptomycin-resistant and aureomycin-resistant strains showed little if any change. The results are given in Table II.

TABLE II

Streptolysin-S production of groups A and C beta hemolytic streptococci

Group	Strain	Hemolytic units per ml.				
		Control*	Pen.† res.	Strep.‡ res.	Bac.‡ res.	Aureo.‡ res.
A	S_1	75	35	—	20	75
A	S_2	35	10	20	35	35
A	S_3	75	10	13	75	50
A	S_4	75	—	35	75	100
A	S_5	20	4	—	10	—
A	U_5	120	30	—	—	120
C	U_1	75	13	75	20	35
C	U_2	120	13	—	45	120
C	U_3	75	20	75	50	75

* Control organism is the parent strain after 40 transfers on plain blood agar.

† Penicillin-resistant organism is one that has been transferred 60 times on medium containing increasing concentrations of penicillin.

‡ Streptomycin-, bacitracin-, and aureomycin-resistant organisms are those that have been transferred 40 times on media containing increasing concentrations of each antibiotic.

TABLE III

*Streptokinase activity of groups A and C beta hemolytic streptococci**

Group	Strain	Control	Pen. res.	Strep. res.	Bac. res.	Aureo. res.
A	S_2	320	320	320	160	160
A	S_3	80	20	40	160	80
A	S_4	320	—	160	160	40
C	U_1	<5	<5	<5	10	<5
C	U_2	40	<5	<5	5	20
C	U_3	20	<5	5	10	<5

* Streptokinase activity expressed as the reciprocal of the broth culture dilution showing complete lysis of a fibrin clot in one hour.

The streptokinase activity of group A streptococci was reduced in one of two strains after acquiring penicillin resistance, and one of three strains after acquiring aureomycin resistance. The streptomycin- and bacitracin-resistant organisms had essentially the same fibrinolytic activity as that of the control organism. The results are given in Table III.

Two of the three group C strains studied had demonstrable fibrinolytic activity. All of the antibiotic-resistant variants of these two had less activity than the control strain.

Only two of the seven group A strains, and none of the group C strains employed in this study, had proteinase activity. These results are given in Table IV. In the S_2 strain the penicillin-, streptomycin-, and bacitracin-resistant variants had less proteinase activity than did the control strain. The aureomycin-resistant variant had the same enzymatic activity as both the parent and the control. In the S_4 strain, only the streptomycin-resistant organism had less proteinase production than its control.

Ribonuclease activity was demonstrated in all seven group A strains studied, but was absent in the three group C streptococci. In four of the

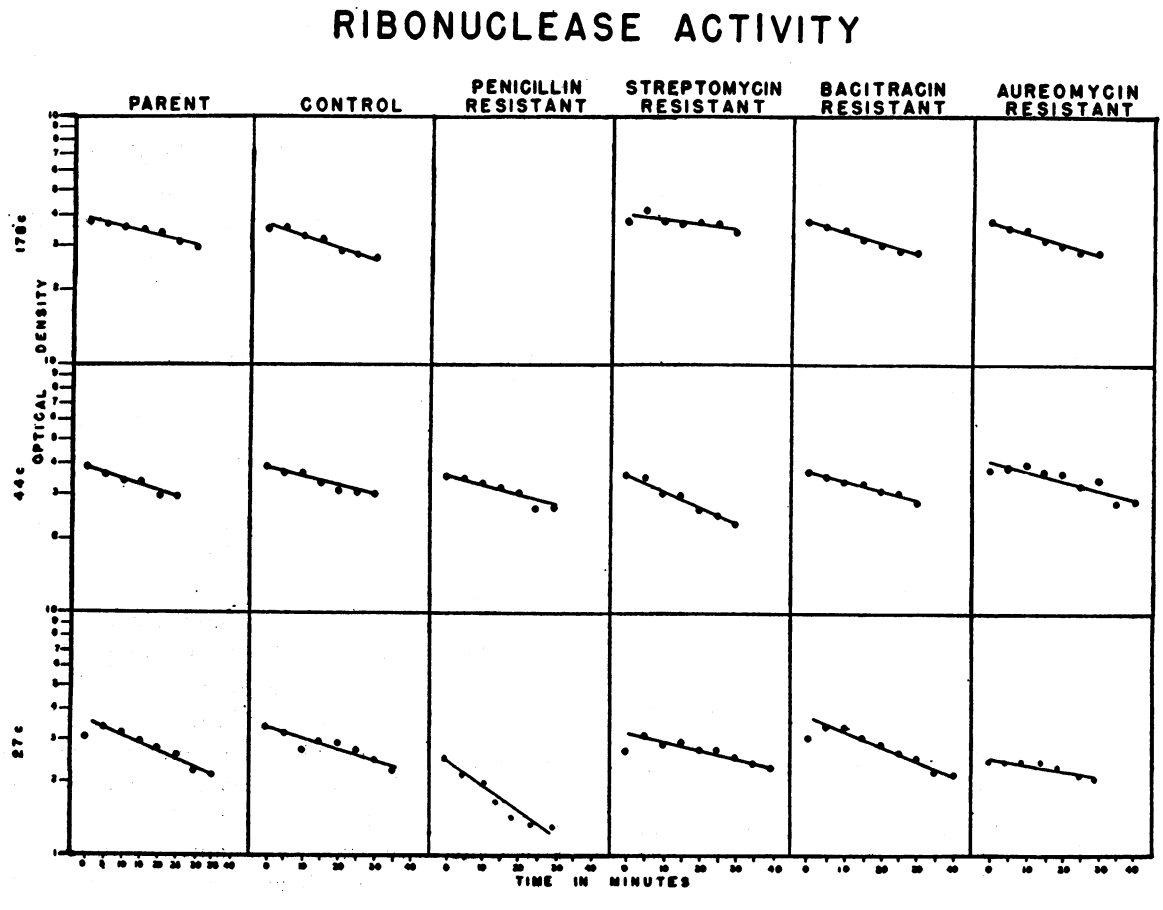

FIG. 1

TABLE IV

*Proteinase activity of groups A and C beta hemolytic streptococci**

Group	Strain	Control	Pen. res.	Strept. res.	Bac. res.	Aureo. res.
A	S_2	128	<4	16	<4	128
A	S_4	128	—	32	128	256
A	S_5	<4	<4	—	<4	<4
A	S_6	<4	<4	—	<4	<4
C	U_1	<4	<4	<4	<4	<4
C	U_2	<4	<4	<4	<4	<4

* Proteinase activity expressed as the reciprocal of the dilution of filtrate showing coagulation of milk at nine hours.

seven group A organisms, the antibiotic-resistant variants had essentially the same level of activity as that of the control. In the S_2, S_3, and S_4 organisms, two of the three streptomycin-resistant, and two of three aureomycin-resistant organisms had less ribonuclease activity than the parent or control strains. None of the penicillin- or bacitracin-resistant variants had a reduction of activity. The results are given in Figure 1.

DISCUSSION

Tables V and VI correlate changes in the bacterial enzyme systems of organisms with induced antibiotic resistance. In these tables, ++++ refers to activity comparable to that of the control strain, while +++ is within the limits of experimental error. In such quantitative determinations, + and ++ probably indicate a definite decrease or total loss in enzymatic activity. No strain resistant to each of the four antibiotics had a decrease in activity of all four enzyme systems (or antigenicity). Only one strain—the streptomycin-resistant variant of S_2—had a decrease in three of the four enzyme system activities. The penicillin-resistant variants of S_2 and S_3, and the streptomycin-resistant variant of S_4, had a loss in two of the four types of activity. Five of the 11 variants described in Table V had a loss in one of the four types of activity, and only two organisms, both resistant to bacitracin, had no change in behavior from that of the corresponding control strain. It may be concluded that some group A streptococci, after acquiring either penicillin or streptomycin resistance, may demonstrate reduction in enzymatic capacity or lose some antigenicity, while after acquiring aureomycin or bacitracin resistance, most of the streptococci change little in activity from that of the parent strain.

TABLE V

Correlation of enzyme system changes with antibiotic resistance

Group	Strain	Antibiotic	Streptolysin S	Streptokinase	Proteinase	Ribonuclease
A	S_2	Pen.	+	++++	+	++++
		Strept.	++	++++	++	++
		Bac.	++++	+++	+	++++
		Aureo.	++++	+++	++++	+
A	S_3	Pen.	+	++		++++
		Strept.	+	+++		++++
		Bac.	++++	++++		++++
		Aureo.	+++	++++		+
A	S_4	Strept.	+++	+++	++	+
		Bac.	++++	+++	++++	++++
		Aureo.	++++	+	++++	++++

++++ equal to activity of the parent and control strains.
+ eight-fold or greater decrease from that of parent and control strains.

TABLE VI

Correlation of enzyme system changes with antibiotic resistance

Group	Strain	Antibiotic	Streptolysin S	Streptokinase
C	U_1	Pen.	+	++++
		Strept.	++++	++++
		Bac.	++	++++
		Aureo.	+++	++++
C	U_2	Pen.	+	+
		Strept.		+
		Bac.	++	+
		Aureo.	++++	+++
C	U_3	Pen.	++	+
		Strept.	++++	++
		Bac.	+++	+++
		Aureo.	++++	+

++++ equal to activity of the parent and control strains.
+ eight-fold or greater decrease from that of parent and control strains.

The parent group C strains studied produced neither proteinase nor ribonuclease. In all three strains, the penicillin- and streptomycin-resistant variants tended to show a significant decrease in activity from that of the control strains in both streptolysin S and streptokinase production. The bacitracin- and aureomycin-resistant organisms maintained these activities at the same level as the parent strains.

It has not been determined what the reduced activity of the enzyme systems contributes to the un-

derstanding of the mechanism of action of acquired antibiotic resistance. Further, it is not known why penicillin and streptomycin resistance appears to alter the behavior of streptococci to a much greater extent than does either bacitracin or aureomycin resistance. The degree of resistance for streptomycin and bacitracin and that for penicillin and aureomycin are comparable.

The variables of medium, time, temperature and amount of growth so far have been eliminated as the probable explanation for the reduced enzymatic production by the resistant variants. Each series of organisms was grown for the same period of time, at the same temperature, in a single lot of medium and on the same day. In every instance, the density of the resistant variants was matched turbidimetrically with that of the parent and control organisms.

The possibility exists, of course, that during the numerous serial transfers another strain of streptococcus was introduced through air-borne contamination or through a change in serial order of several strains on a single plate. This is partially answered by the fact that all of the resistant variants for all four antibiotics were of the same group as the parent organism. Strains that were ungroupable after acquired penicillin resistance have not been included in this study. Final proof will come from typing the parent and resistant strains. This is now in progress.

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