EXPERIMENTAL AND CLINICAL STUDIES ON GRAMICIDIN

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Research Article

EXPERIMENTAL AND CLINICAL STUDIES ON GRAMICIDIN[1]

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A bactericidal substance isolated by Dubos (1) from a soil bacillus has a marked bactericidal action against gram-positive bacteria. This substance is toxic for laboratory animals when administered by the intravenous route (2). We have recently shown that one of the toxic effects of this substance is its hemolytic activity. The crude substance (tyrothricin) consists of two fractions, tyrocidine and gramicidin, as reported by Hotchkiss and Dubos (3). The observations of Dubos were based on experiments in which bacterial cultures, as well as studies on animals, were employed. The present study deals with (*1*) observations on the effect of the bactericidal substance and its fractions on the growth of a number of gram-positive organisms in tissue culture media, (*2*) observations on the hemolytic property of gramicidin, and (*3*) observations on the clinical use of gramicidin. The tissue culture method offers two chief advantages: first, one may observe the effect of the substance on pathogenic bacteria growing in the presence of tissue fragment, serum and tissue extract; second, one may observe the effect of the substance on the growth or maintenance of various types of cells and on erythrocytes suspended in the tissue culture clot. It seems likely that the conditions under which this study was made more closely approach those which obtain *in vivo* than do conditions usually employed in bacteriologic studies.

The tissue culture preparation used in the bactericidal studies was similar to the modified Maximow technic described by King, Henschel and Green (4). The culture was planted on a 22 mm. round coverslip and consisted of a drop of heparinized rabbit's plasma and three drops of tissue extract made by extracting seven-day chick embryos with rabbit's serum. To the preparation was added an explant from a mesenteric lymph node of a rabbit; each explant measured approximately 1.5 mm. across. The total volume of each culture was approximately 0.2 cc. Bacterial cultures used in this study were grown in dextrose brain broth. One cubic centimeter of rabbit's serum was added to brain broth cultures of pneumococci and hemolytic streptococci. Dilutions of young dextrose brain broth cultures were made in plain broth and added to the tissue extract in the proportion of one part of a suspension of bacteria in plain broth to forty parts of tissue extract. The final concentration of the original bacterial culture in the tissue culture clot was one to ten million. This innoculum resulted in the appearance of twenty or more bacterial colonies in each tissue culture preparation with the exception of certain strains of hemolytic streptococci which grew only in the vicinity of the tissue fragment. It was necessary to use a dilution of one to one million cultures of these strains.

Three fractions of the substance elaborated by the soil bacillus were used: the crude bactericidal substance, tyrothricin, and its two fractions, gramicidin and tyrocidine. The lots of tyrothricin used in this study were furnished respectively by Dr. René J. Dubos and by Sharp and Dohme. They were found to be similar in bactericidal activity. Dr. Dubos kindly furnished us with purified gramicidin and purified tyrocidine. Also used in this study were samples of purified gramicidin and tyrocidine prepared from tyrothricin by Osterberg according to the method of Hotchkiss and Dubos. Since these products of the soil bacillus are insoluble in saline solutions, suitable suspensions of the material dissolved in 95 per cent alcohol were made in Tyrode's solution. Such suspensions containing varying amounts of bactericidal substance were added in the ratio of 1 to 10 to plasma and tissue extract used in preparing the tissue culture clot. The greatest amount of bactericidal substance used was 300 micrograms per cubic centimeter of medium. Similar dilutions of 95 per cent alcohol in Tyrode's solution were used in control cultures. Four cultures were prepared for each experimental condition. Cultures were incubated at 37° C. in a specially constructed circulation type incubator (5), and final readings were made after the culture had been incubated for forty-eight hours. The cultures were examined by using a magnification of seven diameters. The least amount of bactericidal substance which would completely prevent the appearance of bacterial colonies in all four cultures after incubation for forty-eight hours was determined for each bacterial strain tested.

Tissue cultures to which bactericidal substances and bacteria had been added, but which showed no evidence of bacterial growth in forty-eight hours, were put into tubes of dextrose brain broth to which 1 cc. of horse serum or rabbit's serum had been added. These cultures were incubated at 37° C. for five days in order to determine whether or not any viable bacteria were present.

[1] Read before the meeting of the American Society for Clinical Investigation, Atlantic City, New Jersey, May 5, 1941.

The following bacterial species have been studied: (*1*) four strains of smooth, encapsulated Diplococcus pneumoniae, that is, one of type I, two of type III, and one of type XIX; (*2*) five strains of group A Lancefield hemolytic streptococcus;[2] (*3*) two strains of the viridans group of streptococci (Streptococcus salivarius) isolated from the blood of patients with endocarditis; (*4*) four strains of Streptococcus faecalis; and (*5*) six strains of Staphylococcus aureus.

The results of bactericidal tests are shown in Table I. There was a considerable degree of species resemblance in the reaction of the various strains to gramicidin. Dubos also observed this in his *in vitro* experiments. The amount of gramicidin necessary to inhibit the growth of all of the representatives of each species was as follows: Diplococcus pneumoniae, 0.5 to 2.5 micrograms per cubic centimeter; hemolytic streptococcus, 5 to 20 micrograms per cubic centimeter; Streptococcus faecalis, 20 to 60 micrograms per cubic centimeter; Streptococcus salivarius, 10 to 60 micrograms per cubic centimeter; and Staphylococcus aureus, 100 to 300 micrograms per cubic centimeter. Three strains of Staphylococcus aureus were not inhibited by 300 micrograms of gramicidin per cubic centimeter. For the most part, a slightly greater amount of tyrothricin had to be used to cause inhibition of bacterial growth as compared to gramicidin. In general, it was necessary to use a much greater concentration of tyrocidine than of gramicidin to obtain the same degree of inhibition (Figures 1 and 2).

TABLE I

Amounts of bactericidal substance causing inhibition

Organism	Number	Tyrothricin	Gramicidin	Tyrocidine
		μg. per cc.	μg. per cc.	μg. per cc.
Diplococcus pneumoniae				
Type I		2.5	1.0	100
Type III	1	1.0	0.5	20
Type III	2	1.0	1.0	20
Type XIX			2.5	40
Hemolytic streptococcus (Group A Lancefield)	1	10	5.0	80
	2		5.0	100
	3		10.0	80
	4		5.0	100
	5		20.0	120
Streptococcus viridans group	1		10	60
	2		60	120
Streptococcus faecalis	1	20	40	300
	2		60	——*
	3		20	260
	4		60	300
Staphylococcus aureus	1		100	140
	2		——*	——*
	3		300	——*
	4		——*	——*
	5		——*	——*
	6		300	300

* Not inhibited by 300 μg. of bactericidal substance.

Although no bacterial growth occurred in cultures containing a sufficient amount of bactericidal substance, not all of the bacteria were killed. When such cultures were placed in brain broth, serum added, and incubated at 37° C. for several days, an occasional tube would show growth of the organism originally introduced into the tissue culture. This occurred when any of the bactericidal fractions were used and for all species except the Staphylococcus aureus. In experiments with this species, however, there were few negative clots available for study. Little is known concerning the mode of action of gramicidin. In the tissue culture preparations the presence of 1 to 10 mgm. of para-aminobenzoic acid per cubic centimeter did not inhibit the action of gramicidin on pneumococci.

Studies on hemolysis

The hemolytic effect of gramicidin has recently been reported from our laboratories (6). This observation was made concerning hemolysis that occurred when the crude substance, tyrothricin, was used. Further experiments have been carried out in which gramicidin and tyrocidine were used. When 0.5 microgram of gramicidin was added to tubes containing 1 per cent suspensions of washed sheep erythrocytes, hemolysis was complete in twenty-four hours. This amount of gramicidin produced the same amount of hemolysis as did 1 microgram of the crude substance, tyrothricin. This indicates that gramicidin is more active than tyrothricin in causing hemolysis. Tyrocidine, on the other hand, is but slightly hemolytic, as indicated by the fact that hemolysis was observed in similar preparations at the end of twenty-four hours only when 40 micrograms of tyrocidine per cubic centimeter were added to the preparation.

[2] We are indebted to Drs. F. R. Heilman and Luther Thompson for bacterial strains used in this study.

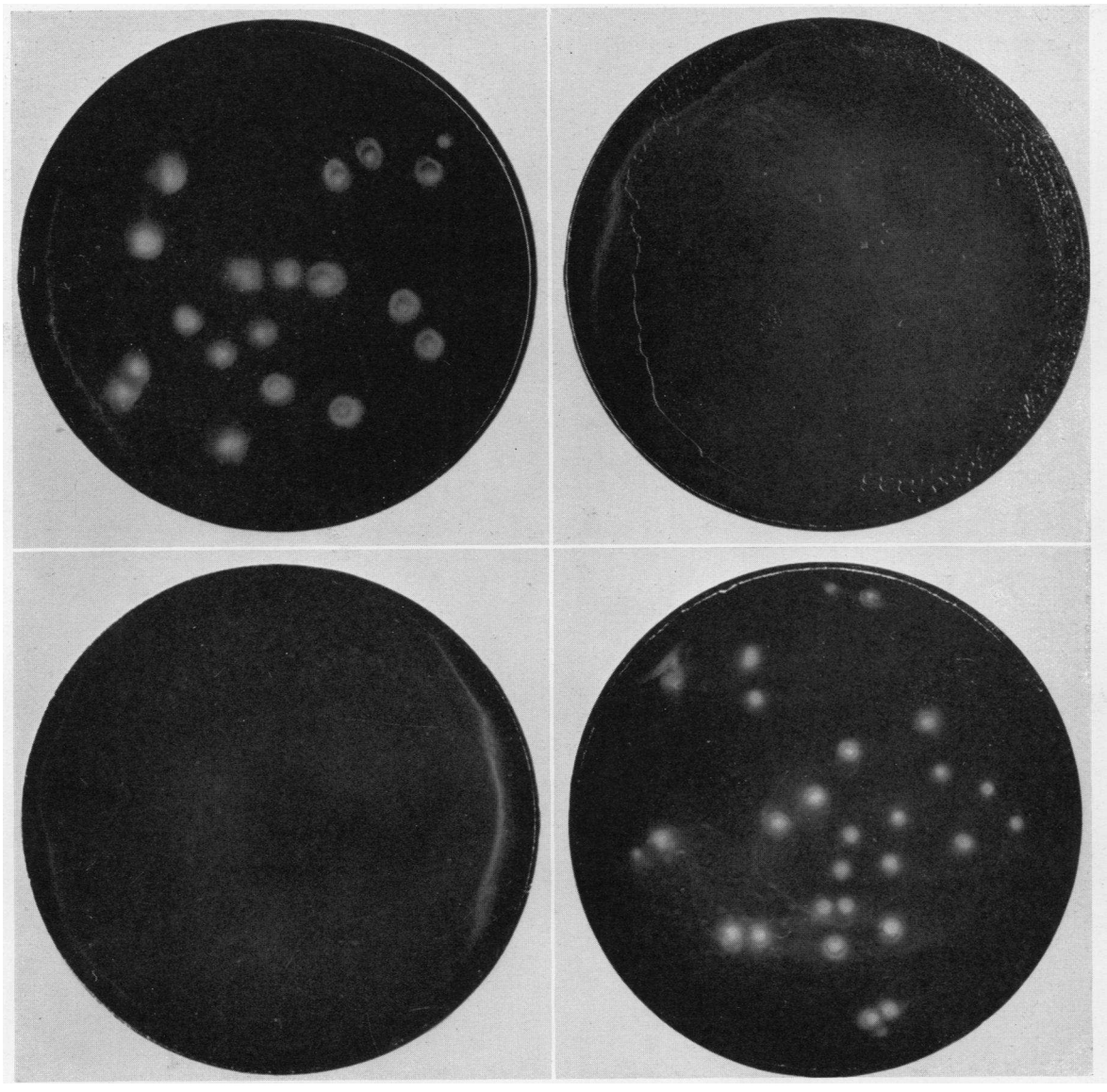

FIG. 1. TISSUE CULTURE CLOTS CONTAINING DIPLOCOCCUS PNEUMONIAE TYPE I AFTER FORTY-EIGHT HOURS' INCUBATION AT 37° C. (× 3.5)

Left upper: Control. *Right upper:* Clot treated with 2.5 micrograms tyrothricin per cubic centimeter media. Complete inhibition. *Left lower:* Clot treated with 1 microgram gramicidin per cubic centimeter. Complete inhibition. *Right lower:* Clot treated with 40 micrograms tyrocidine per cubic centimeter. No inhibition.

The hemolysis observed here in addition was only very slight. It seems possible that even this amount of hemolysis may be associated with the presence of minute amounts of gramicidin in the tyrocidine.

This hemolytic effect of the crude substance and its fractions was again studied in the tissue culture preparation containing 5 per cent of rabbit erythrocytes; 0.5 microgram per cubic centimeter of both tyrothricin and gramicidin resulted in complete hemolysis at the end of twenty-four hours of incubation (Figure 3), whereas no hemolysis was observed in similar preparations in which the clot contained 30 micrograms of tyrocidine per cubic centimeter. In one of these experiments dilutions of the bactericidal substance were made in serum

so as to avoid the introduction of electrolytes into the tissue culture medium. Under these conditions hemolysis was as great as before, indicating that the addition of electrolyte did not materially influence the reaction. Experiences with tyrocidine in our laboratories would indicate that further purification of crude tyrocidine causes progressive loss of the hemolytic activity, whereas the hemolytic activity of the gramicidin seems rather constant.

When suspensions of tyrothricin in Tyrode's solution at pH 8.0 are heated to 90° C. for ten minutes, there is a tenfold loss of hemolytic ac-

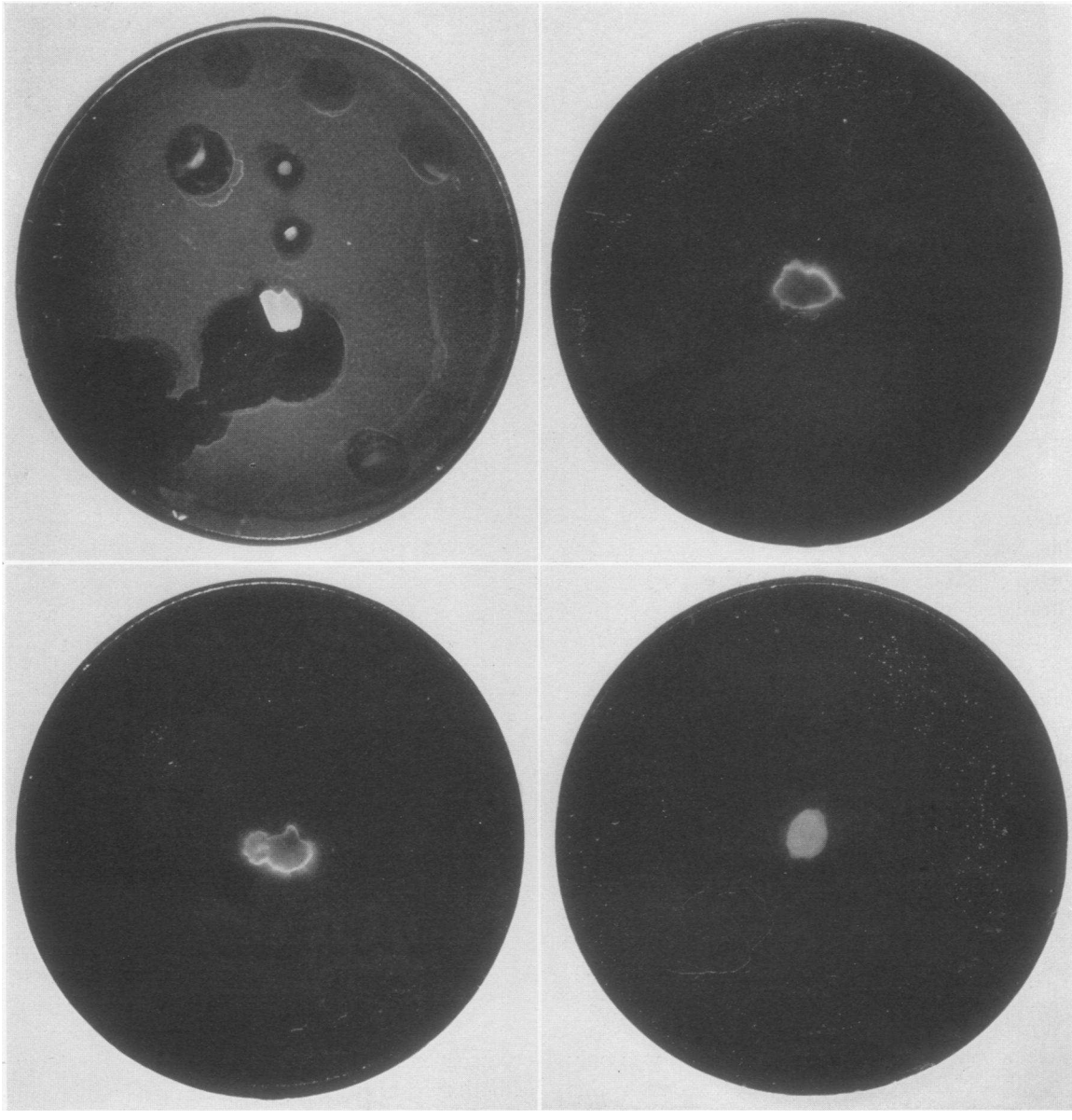

FIG. 2. TISSUE CULTURE CLOTS CONTAINING HEMOLYTIC STREPTOCOCCUS AND LYMPH NODE EXPLANTS AFTER FORTY-EIGHT HOURS' INCUBATION AT 37° C. (× 3.5)

Left upper: Control. Note liquefaction of plasma around colonies. *Right upper:* Clot treated with tyrothricin 10 micrograms per cubic centimeter. Complete inhibition. *Left lower:* Clot treated with 5 micrograms gramicidin per cubic centimeter. Complete inhibition. *Right lower:* Clot treated with 100 micrograms tyrocidine per cubic centimeter. Complete inhibition.

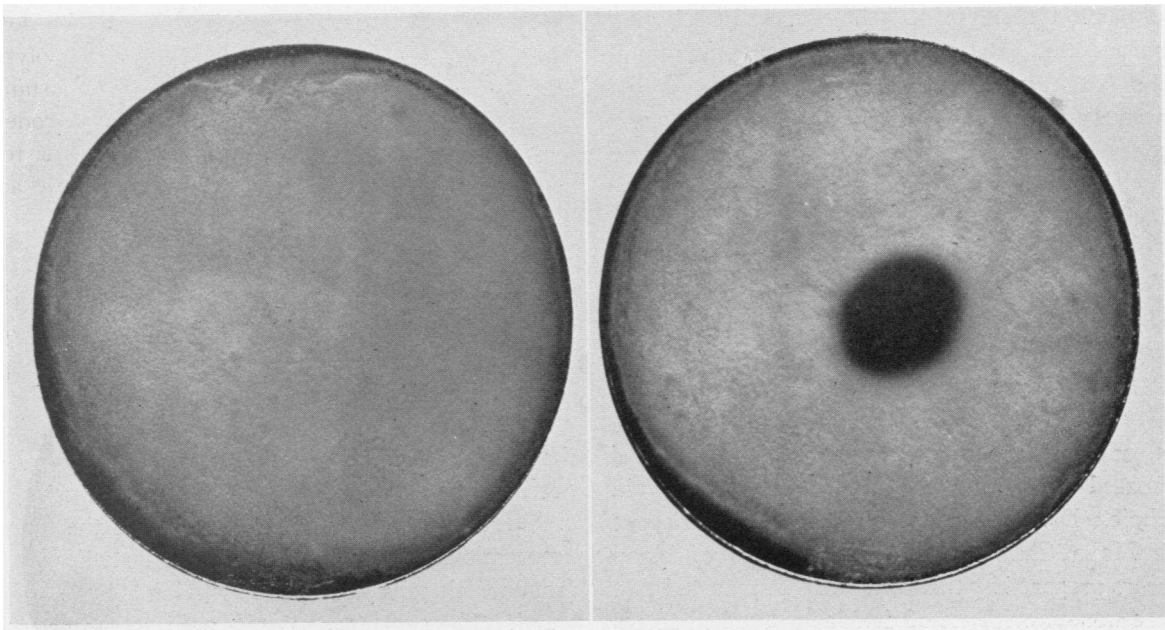

FIG. 3. TISSUE CULTURE CLOTS CONTAINING 5 PER CENT RABBIT ERYTHROCYTES INCUBATED EIGHTEEN HOURS AT 37° C. (× 3.5)

Left: Control. *Right:* Similar preparation to which was added 0.25 microgram gramicidin on the center of the clot. Hemolysis complete in eighteen hours

tivity in tests made on washed sheep erythrocytes. This loss of hemolytic activity does not result when tyrothricin dissolved in 95 per cent alcohol is heated to the same extent.

Suspensions of purified gramicidin were made in Tyrode's solution, the resulting suspensions having a pH of 7.8 to 8.0. These suspensions were divided into similar groups: one group was not heated; the second group was heated in a water bath at 90° C. for ten minutes; the third group was heated at 70° C. for thirty minutes; the fourth group was autoclaved at 15 pounds pressure and at 250° C. for thirty minutes. A sample of each dilution was tested in the tissue culture clot preparation containing 5 per cent of rabbit's erythrocytes, as previously described. The results of such a test are shown in Table II.

TABLE II

Hemolysis of rabbit's erythrocytes in the tissue culture clot at the end of incubation at 37° C. for twenty-four hours

	Grade of hemolysis*		
	1 μg. per cc.	10 μg. per cc.	100 μg. per cc.
Unheated gramicidin	4	4	
Gramicidin heated to 70° C. for thirty minutes	1	3	3
Gramicidin heated to 90° C. for ten minutes	0	2	3
Gramicidin autoclaved 15 lb. for thirty minutes		Very slight trace	±

* Graded on basis of 1 to 4.

With the loss of hemolytic activity there is also a loss of bactericidal activity for bacteria growing in tissue culture. Dubos has reported that heat removes the *in vivo* activity of the bactericidal substance but does not reduce its activity *in vitro*. In this respect the behavior of gramicidin in tissue culture resembles its action *in vivo*. Pneumococci, the growth of which is regularly inhibited in tissue culture by 1 microgram of unheated gramicidin per cubic centimeter, are not decreased in number by 10 micrograms of gramicidin which has been heated in Tyrode's solution at 90° C. for ten minutes. Whether or not the bactericidal property and the hemolytic property may be separated by other means will depend on the outcome of further investigation.

Studies have been made on the effect of grami-

cidin and tyrocidine on the leukocytes of whole human blood. Relatively large amounts of gramicidin and tyrocidine (50 micrograms and 100 micrograms per cubic centimeter of blood) have been added to freshly drawn heparinized human blood. Control samples to which nothing was added and samples containing suitable amounts of 95 per cent alcohol were studied at the same time. All tubes were incubated in a water bath at 37° C. for the duration of the experiment. Smears of each sample were made before the addition of the bactericidal substances and at fifteen minutes, thirty minutes, and one, one and a half, two, four, six, and eight hours thereafter. The films thus prepared were stained by the May-Grünwald technic. Dr. Watkins, one of the hematologists at the Mayo Clinic, examined the series of blood smears prepared by this method. He reported that there was no evidence of damage to the leukocytes by either gramicidin or tyrocidine. Preparations containing gramicidin showed marked lysis of erythrocytes after the first hour of incubation. Representative stained blood films are shown in Figure 4.

Clinical studies

An attempt has been made to apply in a clinical way the information obtained from the experimental studies available with regard to gramicidin. No attempt has been made to use the substance where it might come in contact with the blood stream or to administer gramicidin by mouth. It was felt, however, that the substance is quite safe for local use in the treatment of conditions in which a gram-positive organism has been found on culture. The crude substance, tyrothricin, has been used entirely because we have found experimentally that its bactericidal effect is essentially the same as that of gramicidin, although it may not be as active. The tyrothricin used has been obtained from Sharp and Dohme and is the same preparation used in the experimental studies and from which gramicidin and tyrocidine were prepared. The substance may be prepared for clinical use in one of two ways. The suspension used contains 200 micrograms of tyrothricin per cubic centimeter. It may be prepared by adding 200 mgm. of tyrothricin to 1 liter of a 1.5 per cent solution of aerosol OT (ester of a sulfonated bi-

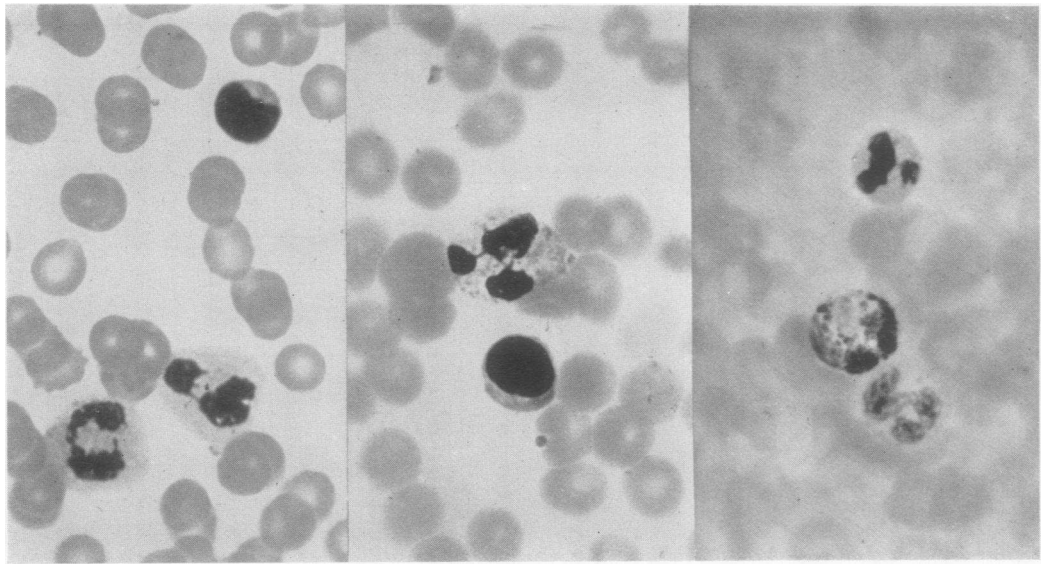

FIG. 4. WHOLE HUMAN BLOOD TREATED WITH TYROCIDINE AND GRAMICIDIN 100 MICROGRAMS PER CUBIC CENTIMETER

Smears shown made after four hours' water bath incubation at 37° C. (× 1000). *Left:* Photomicrograph of control blood smear. *Center:* Tyrocidine treated blood. Very minimal change in erythrocytes. Granulocytes appear normal. *Right:* Gramicidin treated blood. Marked agglutination of erythrocytes but no evidence of toxicity of granulocytes.

carboxylic acid [*di-octyl*]) in triple distilled water. The recent report by Petroff and Schain (7), in which it was stated that aerosol OT is also hemolytic, has caused us to abandon the preparation of tyrothricin in this substance for clinical use. Recently, however, we have prepared for clinical use a mixture of gramicidin in alcohol and glycerin: 200 mgm. of gramicidin are dissolved in 5 grams of alcohol and the solution is made up to 20 cc. with glycerin (U.S.P.). When 20 cc. of such a mixture are added to 1 liter of triple distilled water, the resulting solution contains 200 micrograms of tyrothricin per cubic centimeter and this is substantially isotonic with blood.

At the present time we have used the preparation in twelve cases of various types of infection. The type of infection, the gram-positive organism isolated, the amount of the preparation used locally and a summary of the results are shown in Table III. Two of the cases are of sufficient interest to warrant brief reports.

Report of cases

Case 1. A white man, twenty-nine years of age, came to the clinic January 2, 1941, because of an ulcer on the left leg. There was a history of ancient thrombophlebitis but the ulcer on the leg had appeared following an injury sustained seven months before he came to the clinic. He had had a great deal of local treatment although the ulceration was becoming larger and there was definite evidence of secondary infection. General physical examination, laboratory studies, and roentgenologic examination of the left leg did not reveal any abnormality. The patient was hospitalized on January 6, and bacterial cultures of the ulcer showed the presence of hemolytic streptococcus. A 1.5 per cent solution of aerosol containing 100 micrograms of tyrothricin per cubic centimeter was applied locally to the ulcer. By the third day there was remarkable improvement in the appearance of the ulcer. There was no evidence of any damage to the tissues. There was nothing about the patient which would suggest toxic effects; therefore, medication was continued for fifteen days. On the fifteenth day the condition of the ulcer was so good that skin grafting was carried out. Several days before, however, cultures taken from the ulcer failed to reveal any evidence of hemolytic streptococci. The pinch grafts took perfectly. Two weeks later the patient was dismissed from the hospital. When he was seen one month later the grafts had entirely healed and, except for a sponge dressing which was applied for one week, no treatment was necessary. The result was considered excellent by the consultants in the dermatology department who saw him.

Case 2. A man, aged thirty-four years, came to the clinic February 13, 1941, because of maxillary sinusitis on the left side. He had been ill for approximately seven weeks and there had been a great deal of purulent drainage from the left side. Roentgen therapy and suction had been applied to the left maxillary sinus but a great deal of purulent discharge as well as pain and tenderness had continued. The pain and purulent discharge had persisted for ten days after the treatment and he had been advised to undergo surgical treatment. At the time operation on the left maxillary sinus was considered, a very acute infection developed in the right maxillary sinus which had been entirely normal until that time. From this side a culture was obtained which showed the presence of hemolytic streptococcus. An attempt was made to treat this sinus with tyrothricin. The patient was admitted to the hospital and 20 cc. of the preparation of tyrothricin (200 micrograms per cubic centimeter) were introduced into the right maxillary sinus by means of the displacement technic; 20 cc. of this preparation were administered in this manner every three hours for two days. At the end of twenty-four hours the pain was markedly relieved and the patient was afebrile. The drainage decreased accordingly, and the patient's recovery was striking and uneventful. Surgical treatment on the left side was then carried out three days after the treatment of the right sinus with tyrothricin. A persistent sinusitis did not develop on the right side and no further treatment was necessary. Cultures from the nose revealed only gram-negative bacilli, and streptococci were no longer recovered from nasal cultures. A total of 320 cc. of the preparation was used.

COMMENT

The cases reported indicate that the results were somewhat irregular. This is due in part to the fact that in some instances inadequate amounts of tyrothricin were used in the beginning. On the other hand, there is some difference in the response of the conditions treated with relation to the pathogenic organism isolated. Infections caused by hemolytic streptococci seem to respond most readily, and the staphylococcic infections appear to be the most stubborn when similar amounts of tyrothricin are used. This is especially interesting in view of the fact that Staphyloccus aureus experimentally is much more resistant than most of the strains of streptococci studied by us in the tissue culture. It would appear that the most striking clinical results were obtained in the treatment of definite ulceration. This is especially true of the infected stasis ulcers. The few conditions in which infection occurred in a cavity into which adequate amounts of the substance could be placed also responded satisfactorily. Dermatitis, on the other hand, with the possible exception of

TABLE III

Results of local use of tyrothricin

Case	Diagnosis	Organisms	Solution of tyrothricin*		Response	Additional treatment
			Concentration	Total amount administered		
			μg. per cc.	*cc.*		
1	Ulcer of leg	Hemolytic streptococcus	100†	3,000	Ulcer became clean; culture became negative in one week	Pinch grafts on fifteenth day; all grafts took
2	Acute maxillary sinusitis	Hemolytic streptococcus	200	320	Striking clinical improvement; cultures became negative	None
3	Postoperative empyema	Staphylococcus aureus and hemolytic streptococcus	10	150	None; patient would not continue treatment	Sulfanilamide applied locally
4	Dermatitis of hands and feet	Hemolytic streptococcus	200	1,000	Good initial response; purulent drainage increased subsequently; tyrothricin discontinued	Sulfathiazole ointment; dressings of 0.5 per cent solution of aluminum subacetate
5	Bilateral otitis media	Non-hemolytic streptococcus	10	8	None; treatment inadequate	Abscess of right ear was drained
6	Extensive hidrosadenitis suppurativa of axilla; ulcer of leg	Hemolytic streptococcus	200	12,000	Very striking; drainage decreased, cultures became negative and lesions healed	Surgical excision of undermined portions of skin
7	Eczematoid dermatitis of hands and feet	Hemolytic streptococcus and gram-positive bacillus	200	3,000	Excellent, cultures became negative	Roentgen therapy
8	Eczematoid dermatitis of hands and feet	Hemolytic streptococcus and Staphylococcus aureus	200	500	Better on right side, but not striking	Left hand and foot treated with sulfathiazole ointment but not with tyrothricin
9	Stasis ulcer	Hemolytic streptococcus and Staphylococcus aureus	10	600	Ulcer became clean rapidly but never healed completely. Staphylococcus aureus persisted. Treatment probably inadequate	Solution of potassium permanganate used locally
10	Eczematoid dermatitis	Hemolytic streptococcus	200	1,000 (?)	Culture became negative but additional treatment was necessary	Wet dressings of aluminum subacetate; colloidal baths
11	Stasis ulcer	Staphylococcus aureus	200	500	Poor	Skin grafts
12	Very persistent cystitis	Streptococcus faecalis and Staphylococcus aureus	200 to 400	1,500	Excellent; first negative cultures in one year	Additional treatment none

* In triple distilled water unless stated otherwise.

† In 1.5 per cent solution of aerosol OT.

the dermatitis in Case 7 (Table III), did not respond well to this form of treatment, although in many instances the pathogenic organisms frequently disappeared. Additional therapy, however, almost always must be employed.

SUMMARY

The bactericidal effect of tyrothricin, tyrocidine, and gramicidin has been studied by using the tissue culture technic. Gramicidin is more effective than tyrocidine against most of the gram-positive bacteria studied. The tissue culture method permits not only the study of the bactericidal effect of these substances but at the same time an opportunity is afforded to observe the possible effects of the substance on bacteria growing in the presence of tissue fragments, serum and tissue extract. These conditions approach the circumstances which obtain *in vivo*.

Tyrothricin has a powerful hemolytic action on erythrocytes *in vitro*. The hemolytic effect of tyrothricin is due to the presence of gramicidin. When tyrothricin or gramicidin is heated in an aqueous suspension there is loss of hemolytic and bactericidal activity. Tyrocidine does not appear to be very hemolytic.

Neither gramicidin nor tyrocidine appears to produce any marked toxic effect upon the leukocytic elements of the human blood in amounts up to 100 micrograms per cubic centimeter over a period of eight hours.

Tyrothricin has been used locally in twelve cases of various types of infections in which gram-positive bacteria were present. Marked beneficial effect was noted in most cases in which the substance was used. No demonstrable damaging effects have been noted on the tissues. On the other hand, the healing of wounds has appeared to be considerably benefited in some instances. No evidence of toxicity has been observed following the use of the substance in the manner described. The hemolytic effect of gramicidin is great enough in the presence of constituents of the blood to render inadvisable the clinical use of this substance in any way except locally or perhaps to irrigate infected cavities which do not communicate with the blood stream.

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