The Japanese Medical Journal

Published Bimonthly by

The National Institute of Health of Japan

Volume 3

AUGUST, 1950

Number 4

BIOLOGICAL STUDIES ON THE ANTIBIOTICS PRODUCED BY STREPTOMYCES GRISEOLUTEUS I. BACTERIOSTATIC EFFECTS OF GRISEOLUTEIN

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(Received for publication, May 28, 1950)

Since the discovery of aureomycin¹⁾, yellow antibiotic substances produced by streptomycetes have been successively obtained. Aureothricin found by Umezawa and others,²⁾ luteomycin by Hata and others,³⁾ xanthomycin by Peterson and others,⁴⁾ and terramycin,⁵⁾ are all yellow antibiotics produced by streptomycetes. Griseolutein, which was found by Umezawa and the writer and others,⁶⁾ is also a yellow crystalline antibiotic. As shown in the previous report⁶⁾ the griseolutein-producing strain is a new species named as *Streptomyces griseoluteus*. From the fermented broth of this strain griseolutein is extracted with ethylacetate at pH 2-3 and after vacuum concentration and alumina chromatographic purification crystallized with ethylacetate. It melts at 193°C and it contains C 59.51%, H 4.36% and N 8.02%. Qualitative tests of sulfur and halogen are negative. It is different from aureothricin and luteomycin in the negative test of sulfur, from aureomycin in the negative test of chlorine, from xanthomycin in its slight toxicity and from terramycin in the melting point.

S. griseoluteus produces not only griseolutein but also the other antibiotics, depending on the cultural conditions. When it is cultivated in the yeast-glycerol medium, griseolutein and the other antibiotics are produced, and in the case of the cornsteepliquor-glycerol medium, the other antibiotic which can not be extracted with ethylacetate is produced.

In the present paper the bacteriostatic effects in vitro in various conditions and the toxicity are described.

EXPERIMENTAL

1. Experimental materials.

Griseolutein which was used for the experiment was obtained from the fermented broth and purified to yellow crystalline powder, according to the method in the previous report (6).

S. aureus, *E. coli* and many other bacteria which were used are those which are preserved in our laboratory.

The nutrient agar, if it is otherwise specified, contained 0.5% meat extract, 0.5% peptone, 0.3% NaCl and 2.0% agar at pH 7.0. The nutrient broth contained 0.5% meat extract, 0.5% peptone, 0.3% NaCl at pH 7.0.

2. Bacteriostatic effects against various kinds of bacteria.

Griseolutein was diluted with the nutrient agar, dissolved and cooled to 48°C, to various dilutions, and after solidification 24 hours' broth culture of various bacteria was streaked and after 20 hours the growth was examined.

Table 1. The bacteriostatic effects of griseolutein tested by agar streak method.

Bacteria	Maximum dilution of griseolutein necessary for the complete growth- inhibition
<i>S. aureus</i> 209-P	1 : 1,280,000
<i>S. aureus</i> (Terajima)	1 : 5,120,000
<i>B. anthracis</i>	1 : 2,560,000
<i>B. subtilis</i>	1 : 10,240,000
<i>B. natto</i>	1 : 2,560,000
<i>E. coli</i> communis	1 : 160,000
<i>E. typhi</i>	1 : 320,000
<i>S. paratyphi</i> A	1 : 320,000
<i>S. paratyphi</i> B	1 : 320,000
<i>S. typhi</i> murium	1 : 40,000
<i>S. dysenteriae</i>	1 : 640,000
<i>S. paradysenteriae</i> (Komagone B III)	1 : 1,280,000
<i>S. sonne</i>	1 : 320,000
<i>B. melitensis</i>	1 : 2,560,000
<i>P. vulgaris</i> (Warshaw)	1 : 10,240,000
<i>P. vulgaris</i> OX19	1 : 5,120,000
<i>P. aeruginosa</i>	1 : 80,000

The results are shown in Table 1. Griseolutein inhibits the growth of not only gram-positive bacteria but also gram-negative bacteria. It inhibits *S. aureus* at the dilution of 1 in 1,280,000 and very strongly spore-forming bacteria. The growth of *B. anthracis* and *B. natto* was completely inhibited at 1 in 2,560,000, and that of *B. subtilis* at 10,240,000. Gram-negative bacteria are generally more resistant than the positive bacteria, however *P. vulgaris* and *B. melitensis* are very sensitive, the former is inhibited at the dilution

of 1 in 10,240,000 and the latter at 5,120,000.

The bacteriostatic effect on *H. influenzae*, *H. pertussis*, *D. pneumoniae* and *S. hemolyticus* was tested on the blood nutrient agar, but the blood inhibited the effect of griseolutein and the clear results could not be obtained. The growth of *M. phlei* was tested on glycerol (1%) nutrient agar and inhibited at the dilution of 1: 160,000. The effect of griseolutein on the acid-fast bacteria seems to be weak.

3. The influence of pH on the bacteriostatic effect.

pH of the nutrient broth was adjusted and sterilized. After sterilization pH was measured and inoculated with one loop of 24 hours' broth culture of *S. aureus* 209-P. As shown in Table 2, the bacteriostatic effect is more strong at acid side than at alkaline side. The effect at pH 6.6-7.4 is 4 times stronger than at pH 7.8 and 8 times than at pH 8.0.

Table 2.

The influence of pH on the bacteriostatic effect of griseolutein against *S. aureus* 209-P.

pH	The maximum dilution of griseolutein necessary for the complete inhibition
6.6	1 : 1,280,000
7.0	1 : 1,280,000
7.4	1 : 1,280,000
7.8	1 : 320,000
8.0	1 : 160,000

4. Influence of cystein, cystin and serum.

The nutrient broth was sterilized for 20 minutes at 120°C and added with cystein or cystin and again sterilized for 20 minutes at 100°C. In the case of testing the influence of serum, the sterile nutrient broth was added with sterile serum to 10%. Griseolutein was diluted with these media and the 24 hours' broth culture of *S. aureus* 209-P was inoculated. After 24 hours' incubation the results were examined.

As shown in Table 3, cystin did not indicate any influence on the bacteriostatic

Table 3.

The influence of cystin, cystein and serum on the bacteriostatic effect of griseolutein.

The substance Added		The dilution of griseolutein ($\times 10^4$)									
	Amount of substance	2	4	8	16	32	64	128	256	512	∞
Cystin	0.5 mcg/cc	—	—	—	—	—	—	±	+	+	+
	20 "	—	—	—	—	—	—	±	+	+	+
	100 "	—	—	—	—	—	±	+	+	+	+
Cystein	0.5 "	—	—	—	—	—	±	±	+	+	+
	20 "	—	—	—	—	±	±	±	+	+	+
	100 "	±	±	±*	±	±	±	±	±	+	+
Serum	10%	—	—	—	—	—	+	+	+	+	+

effect of griseolutein, however, cystein inhibited the latter noticeably. When cystein was added at 100 mcg/cc, even in the dilution of 1 in 20,000 griseolutein did not completely inhibit the growth, though the growth in the effective range of griseolutein such as the dilution in 20,000–10,240,000 was very scarce. The serum decreased very slightly the bacteriostatic effect.

Since the bacteriostatic effect was influenced by cystein and slightly by serum, therefore whether the nutrient broth itself influences or not was tested by using synthetic medium and *E. coli*. *E. coli* which was used for the experiment grew in the synthetic medium containing KH_2PO_4 0.1%, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.07%, NaCl 0.1%, $(\text{NH}_4)_2\text{HPO}_4$ 0.4%, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.003% or glucose 1.0%. Griseolutein was diluted with this medium and the nutrient broth, and 24 hours' broth culture of *E. coli* was inoculated. In both cases *E. coli* was inhibited at the dilution of 1 in 40,000 and any influence of the material in the nutrient broth on the bacteriostatic effect could not be confirmed.

5. The effect of penicillinase on griseolutein.

Since griseolutein was extracted with ethylacetate at pH 3.0 and transferred to the water-layer at pH 7.0 and was thermolabile and resembled penicillin, the effect of penicillinase was examined.

In the nutrient broth containing penicillinase at 1 mg/cc which 1 mg destroyed 15,000 units of penicillin G, griseolutein was diluted and inoculated with *S. aureus* and incubated for 20 hours at 37°C. The growth-inhibiting effect of griseolutein in the broth was not changed with or without penicillinase. Griseolutein was not destroyed by penicillinase.

6. Toxicities of griseolutein.

Griseolutein was insoluble in water, therefore its fine powder was suspended in sterile saline and subcutaneously injected to mice (body weight 12 g). When 5, 10 or 20 mg was injected, all mice survived after the end of observation days (14 days). The mice injected with 10 mg or 20 mg were killed after 7 days, and each organ was histologically examined by Dr. Egashira at the Pathological Division of our Institute. In all organs except kidney, almost none of pathologic changes was observed. Slight swelling of some glomeruli and slight diffuse degeneration of tubular epithel was observed in the kidney.

DISCUSSION

The bacteriostatic effects of griseolutein cover almost all bacteria, however the activity is very various according to the species. Though gram-positive bacteria are rather more sensitive than negative bacteria, among the latter there are very sensitive species such as *P. vulgaris* and *B. melitensis*. The antibacterial spectrum resembles that of aureomycin, but differs in its strong effect against *P. vulgaris*. The comparison of the effect with that of aureomycin or terramycin will be reported in the other paper.

In chemical studies on griseolutein Maeda has found the existence of one carboxyl radical, and therefore griseolutein seems to be an acid antibiotic is usually more effective at acid side than at alkaline side. The experimental results of testing the

influence of pH on the effect of griseolutein indicates that griseolutein is an acid substance. However, it should be here noticed that griseolutein is more stable at acid reaction than at neutral or alkaline reaction. It was completely destroyed after heating at 100°C for 20 minutes when pH of the medium was 7.0 and about 90% remained after heating at pH 2.0. Therefore, the weak bacteriostatic effect at pH 7.8 and 8.0 may be due to the decomposition during the experiment.

Its stability at pH 2.0 and the no influence of penicillinase indicate that griseolutein molecule does not contain the specific configuration of penicillin. When the medium contained cystein, the bacteriostatic effect was much decreased. Griseolutein may combine the SH-group of cystein. The experiment indicates that in the medium with cystein *S. aureus* grew even in the existence of griseolutein at 50 mcg/cc, but the growth is very slight. It seems to indicate that griseolutein-SH compound may slightly dissociate and inhibit the growth, though it is not complete. The influence of serum was very slight, but in the case when griseolutein was dissolved in blood (55%) agar, its bacteriostatic effect was heavily inhibited. This influence of blood may also be due to the effect of SH-group-containing substances.

Among characters of griseolutein, its very slight toxicity is noticeable. Its minimal lethal dosis of subcutaneous injection to mice is larger than 2,000 mg per kilogram of the body weight and therefore its toxicity seems to be the same grade as aureomycin or chloramphenicol. The bacteriostatic effect and its low toxicity suggest its possible clinical use. However, the yield of griseolutein is at present very low, therefore the animal experiment is impossible at present and will be reported in future.

SUMMARY

1. The antibacterial spectrum of griseolutein was described. Gram-positive bacteria, especially spore-forming bacteria and *P. vulgaris* and *B. melitensis* were very sensitive.

2. On the acid side the bacteriostatic effect of griseolutein was more strong than at alkaline side.

3. The bacteriostatic effect was not inhibited by cystin, but slightly by serum and strongly by cystein.

4. The minimal lethal dosis of subcutaneous injection to mice was larger than 2,000 mg per kilogram of the body weight.

Here the writer wishes to express his sincere appreciation to Dr. Umezawa, Director of our Division for his kind direction and advices. He also wishes to thank Dr. Hayano, and Dr. Egashira at the Pathological Division for his kind advices and assistances.

REFERENCES

- 1) Duggar, B. M.: Aureomycin: A product of the continuing search for new antibiotics. Ann. New York Acad. Sci. **51**, 177, 1948.
- 2) Umezawa, H., Tazaki, T. and Maeda, K.: Isolation of a new antibiotic substance, aureothricin from a strain of streptomyces Jap. Med. J., **1**, 512, 1948: J. Penicillin, **2**, Suppl. A, 105, 1949.

- 3) Hata, T., Higuchi, T., Sano, Y. and Sawachika, K.: Isolation of a new antibiotic substance "Luteomycin" J. Antibiotics, **3**, 313, 1950.
 - 4) Thorn, C. B. and Peterson, W. H.: Xanthomycin A and B, new antibiotics produced by a streptomyces. J. Biol. Chem., **176**, 413, 1948.
 - 5) Finaly, A. C., Habby, G. L., Pan, S. Y., Regna, P. P., Routien, J. B., Seeley, D. B., Shull, G. M., Sabin, B. M., Solomons, J. A., Vinson, J. W. and Kane, J. H.: Terramycin, a new antibiotic. Science, **111**, 85, 1950.
 - 6) Umezawa, H., Hayano, S., Ogata, Y. and Okami, Y.: On a new antibiotic, griseolutein produced by streptomyces. Jap. Med. J., **3**, 111, 1950, J. Antibiotics, **3**, 1950.
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