CHEMOTHERAPY IN EXPERIMENTAL DIPHTHERIA INFECTION

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A study on the chemotherapeutic agent against C. diphtheriae was carried out already by Behring (1, 2) side by side with his study in the serotherapy. Boer (3), Eeiler (4-6), Burkard and Dorn (7), Langer (8), Neufeld, Schieman and Baumgarten (9), Reinhardt (10), Ritter and Schenkel (11), Brunner and Gonzenbach (12), Browning and Gulbransen (13), Goldschmidt (14), Braun and Goldschmidt (15), Shibanuma and Hata (16,17) followed after him in the study of the same field.

In the early stages they investigated about inorganic substances but later chiefly about organic substances and especially about acridine derivatives. The strong bacteriostatic action of acridine compounds in vitro attracted their interest and they have succeeded also in saving animals from experimental diphtheria infection. But no clinician has intended as yet to treat the infection of human beings with acridine compounds, because of the uncertainty of effect. Injection of antitoxin serum is the most trustworthy treatment; the serum is capable not only to neutralize the toxin, but to inhibit the growth of bacilli and cures the disease in its earlier stage with promptitude. But on the other hand the serum is somewhat inconvenient to treat with and troublesome in its production. The apparition of such a compound, as not only capable of inhibiting the growth of bacilli, but also to destruct or to neutralize toxin in vivo, if possible, might be desirable. Of course we have had comprehensive studies on the detoxication of diphtheria toxin, while those works were to gain an effective anatoxin for the use in preventing the disease. Formol, ketene, vanilline, adrenaline, sodium salicylate, urea, quinine salt, glutathion, soap, ricinoleic sodium salt, colloidal manganese, colloidal iron, various anorganic salts, ascorbic acid, hormones, enzymes etc. were namely the detoxicating agents in this sense, but none of these seems to be practically useful as therapeuticum, because they have no strong detoxifying ability in vivo.

Meanwhile penicillin appeared and a ray of hope has been thrown for a new treatment of diphtheria, yet so far we do not know about its final success.

On this occasion a number of compounds were investigated by us (18-21), in the bacteriostatic and bactericidal action against C. diphtheriae and among 120 chemicals, inclusive of 15 phenothiazine, 5 phenoxazine, 13 quinoline, 4 acridine, 31 diphenylsulfone, 17 diphenylsulfide, 5 diphenyldisulfide, 15 diphenylether, 9 tetronic acid derivatives, citrinin, koch acid, usnic acid and 3 others, usnic acid was found to be the only substance, that can not only save animals from experimentant diphtheria infection (22), but also detoxify the toxin.

The usnic acid, used in these studies, was extracted from Usnea longissima. Fp. 203°C \([\alpha]_D^{13} = +491.8°\) (in chloroform).
The acid has recently been reported to have strong bacteriostatic effect on the growth of the tubercle bacillus and the *Staphylococcus aureus* (23-25), but no one has ever reported in details about its effectiveness in diphtheria infection. The experiment here reported was designed in order to observe the effect of usnic acid in experimental diphtheria infection and its action on the toxin.

**EXPERIMENTAL**

**A. The bacteriostatic and bactericidal effect of usnic acid in vitro**

Materials and method:

*C. diphteriae* were the strain Park Williams No. 8 (Toronto strain).

Bacterial suspension: Cultures were grown on slants of Loeffler medium at 37°C. Twenty four hours cultures were weighed and 2.0 mg of moist culture was suspended in 5.0 cc of sterilized saline solution.

The effect of usnic acid on the growth of bacilli; One per cent solution of the usnic sodium salt, prepared aseptically using a Seitz filter, was further diluted with sterilized bouillon ten times as much. Now in a set of tubes, each containing 2.0 cc of bouillon (pH 7.4) the first tube received 2.0 cc of the above solution of usnic acid, 2.0 cc of the well mixed solution transferred into the next tube, the same quantity from the second tube to the third and thus varying concentrations of the chemical were prepared. A control tube contained only the nutrient fluid, without any addition of the chemical. Next each solution was inoculated with one drop (ca 0.05 cc) of the bacterial suspension and all tubes were incubated 48 hours at 37°C.

Turbidity, corresponding the growth of bacilli, was noted after 24 and 48 hours' incubation.

To test the bactericidal action of usnic acid, one drop in each test tube was replaced in a fresh bouillon medium after 24 and 48 hours' incubation, and the growth of bacilli was observed.

Results:

Usnic acid showed its bacteriostatic action yet in addition of 1:256,000 and its bacteriostatic action in dilution of 1:128,000. The same results were obtained after the observation of 24 and 48 hours.

**B. The effect of usnic acid in experimental diphtheria infection**

Method:

After the cultures of the same strain of *C. diphteriae*, grown on slants of Loeffler medium at 37°C for 24 hours, were weighed, they were washed twice with sterilized saline solution to remove the adhered toxin and then the bacterial suspension was made so as to contain approximately 2.0 mg of moist cultures per ml of saline solution. Repeated experiments showed that 0.1 cc (0.2 mg of cultures) of such a suspension killed
the guinea pigs of about 250 g in 5-8 days after the inoculation of bacilli, 0.2 cc in 2-4 days and 0.3 cc in 2 days, when it was subcutaneously injected. Hence most of the inoculations were held in these experiments with 0.2 cc of the bacterial suspension.

Now 24 male guinea pigs, weighing about 250 g, were inoculated subcutaneously in the back with the above dose of bacilli. They were divided into 8 equal groups. The first series of 3 groups received subcutaneous injections of usnic acid in the region of inoculation 2 hours later after the infection of bacilli. The dose of the drug was: group A, 0.5 mg per 100 g weight of animals; group B, 1.0 mg; and group C, 2.0 mg.

The next group, D, received the injection of 2.0 mg of acid 24 hours later after the infection.

The last 3 groups were injected 2 hours later after the infection subcutaneously opposite the inoculated part of the back; group E, 0.5 mg of acid; group F, 1.0 mg; and group G, 2.0 mg. The remaining one group, H, served as controls.

All animals were weighed daily. At death they were autopsied. Two weeks after infection all survivors were killed with chloroform and autopsied. In all instances the subcutaneous tissue of the inoculated part was scratched aseptically and cultivated on slants of Loeffler medium. Among the visceral organs the suprarenal glands were weighed, their tissual pieces were stained with hematoxylin eosin solution and histologically investigated.

Addendum. The minimum lethal dose of usnic acid was 0.35 mg per 10 g weight of mice and 10.0 mg per 100 g weight of guinea pigs, when used as sodium salt solution subcutaneously, hence less toxic in guinea pigs than in mice.

The same experiment was carried out once more with 21 animals, divided in 7 groups.

Table 1.

<table>
<thead>
<tr>
<th>Treatment with usnic acid</th>
<th>No. of animals</th>
<th>Surv. of animals in days</th>
<th>Surv. percent</th>
<th>mg. of usnic acid per 100 g. weight of animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Injected in the region of inoculation</td>
<td>Two hours after inoculation of bacilli</td>
<td>3 0.5</td>
<td>3 1 1 1 1 1 1</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>3 1.0</td>
<td>3 3 3 3 3 3 3</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 2.0</td>
<td>3 3 3 3 3 3 3</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Twent four hours after inoculation</td>
<td>3 2.0</td>
<td>3 2 1 0 0 0 0</td>
<td>0 0</td>
</tr>
<tr>
<td>Injected subcutaneously opposite the infected part of the back</td>
<td>Two hours after inoculation of bacilli</td>
<td>3 0.5</td>
<td>3 3 0 0 0 0 0</td>
<td>0 0</td>
</tr>
<tr>
<td></td>
<td>3 1.0</td>
<td>3 3 3 3 3 3 3</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 2.0</td>
<td>3 3 3 3 3 3 3</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>
Table 2.

<table>
<thead>
<tr>
<th>Treatment with usnic acid</th>
<th>No. of animals</th>
<th>mg of usnic acid per 100g weight of animals</th>
<th>Survival of animals in days</th>
<th>percent survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>Injected in the region of inoculation</td>
<td>Two hours after inoculation of bacilli</td>
<td>3</td>
<td>0.5</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>1.0</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>2.0</td>
<td>3</td>
</tr>
<tr>
<td>Injected subcutaneously opposite the infected part of the back</td>
<td>Two hours after inoculation of bacilli</td>
<td>3</td>
<td>0.5</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>1.0</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>2.0</td>
<td>3</td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Results:

As shown in Tables 1 and 2, animals treated with over 1.0 mg of usnic acid per 100 g weight of animals, survived when the injection was held 2 hours later after inoculation of bacilli, while no animal was able to withstand the infection even with the dose of 2.0 mg of the same acid per 100 g, when the treatment had been begun 24 hours after inoculation. And when bacilli were inoculated subcutaneously particularly opposite the infected part of the back of animals, they could all survive by the injection of 1.0 mg per 100 weight of animals in one time and of 2.0 mg in the other time of experiment. Subcutaneous hemorrhage in the inoculated part, the increase of weight and manifest diphtheric changes of adrenals were not recognized and no diphtheria bacilli were cultivated from the inoculated part in all survived animals, which were killed after 2 weeks observation.

C. The detoxifying effectiveness of usnic acid

a) Subcutaneous inoculation of diphtheria toxin, previously kept in contact with usnic sodium salt solution.

Materials and method:

Solutions were prepared by mixing saline solution of usnic sodium salt and diphtheria toxin in the following combinations:

1) 1.0 cc of saline solution, containing 1.0 mg of usnic sodium salt 1.0 cc of a dilution of diphtheria toxin, containing 2 MLD, incubated one hour at 37°C (pH=7.4).

2) The solution in the same combination as above, incubated 24 hours at 37°C.

3) One cc of saline solution, containing 2.0 mg of usnic sodium salt+1.0 cc of a dilution of diphtheria toxin, containing 2 MLD, incubated one hour at 37°C (pH=7.4).

4) The solution in the same combination as above, incubated 24 hours at 37°C.
In experiment 1, eight guinea pigs, weighing from 250 g to 280 g, were divided in 3 groups, each 3 animals in the 1st and 2nd groups and 2 animals in the 3rd group. Each animal of the 1st group was inoculated subcutaneously with the 1) solution and of the 2nd group with the 3) solution. The 3rd group served as controls, receiving the injection of diphtheria toxin, containing twice the number of MLD, only.

In experiment 2, eight guinea pigs, weighing from 250 g to 300 g, were divided quite in the same manner as above. Each animal of the 1st group received the injection of the 2) solution and of the 2nd group the injection of the 4) solution. The 3rd group was injected with 2 MLD of the original diphtheria toxin.

Diphtheria toxin (crude toxin, No. 26) was delivered from the Hokuriku Serum Industry. MLD = 1/1,500 cc, pH = 8.0.

Results:

Animals, which were injected with the mixture of 2 MLD of toxin and 1.0 mg of usnic acid, incubated 24 hours, could survive and those, injected with the mixture of 2 MLD toxin and 2.0 mg of usnic acid, could also survive, even when the mixture was reacted only for one hour.

There is now little doubt that usnic acid has not only the germicidal power against diphtheria bacilli but also the detoxifying ability of diphtheria toxin.

Table 3. Toxin + usnic acid, incubated 1 hour at 37°C.

<table>
<thead>
<tr>
<th>Guinea pig number</th>
<th>Injected material</th>
<th>Weight of animals</th>
<th>Survived (S.) or died (D.)</th>
<th>Surviving</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Toxin (MLD)</td>
<td>Usnic acid mg</td>
<td>pH of the solution</td>
<td>Before injection</td>
</tr>
<tr>
<td>104</td>
<td>2 (1.0 cc)</td>
<td>+1.0 (1.0 cc)</td>
<td>7.4</td>
<td>250</td>
</tr>
<tr>
<td>105</td>
<td></td>
<td></td>
<td></td>
<td>257</td>
</tr>
<tr>
<td>106</td>
<td></td>
<td></td>
<td></td>
<td>270</td>
</tr>
<tr>
<td>107</td>
<td></td>
<td>+2.0 (1.0 cc)</td>
<td></td>
<td>250</td>
</tr>
<tr>
<td>108</td>
<td></td>
<td></td>
<td></td>
<td>270</td>
</tr>
<tr>
<td>109</td>
<td></td>
<td>sterilized saline</td>
<td></td>
<td>250</td>
</tr>
<tr>
<td>110</td>
<td></td>
<td>solution (1.0 cc)</td>
<td></td>
<td>250</td>
</tr>
<tr>
<td>111</td>
<td></td>
<td></td>
<td></td>
<td>280</td>
</tr>
</tbody>
</table>

b) The intradermal test of detoxication with usnic acid

Method:

Mixed solutions of diphtheria toxin and usnic acid were prepared quite in the same combinations as above and 0.1 cc of them was injected each in a guinea pig, weighing
500 g, whose hairs were removed from an area of the back, intracutaneously. Two control animals received 0.1 cc of a dilution of toxin. Reactions were read as erythema 48 hours and as necrosis 96 hours later.

Results:

While control reaction showed the erythema of 20 mm in diameter 48 hours later and necrosis of 13 mm in diameter 96 hours later, any skin sensitivity of inflammation was missed, when the animals were injected with the mixture of 2 MLD of toxin and 1.0 or 2.0 mg of usnic acid, incubated 24 hours at 37°C. The reaction was also very feeble when the mixture of 2 MLD of toxin and 2.0 mg of usnic acid, incubated only one hour, was injected to an animal.

The results, obtained by the intradermal test, coincide therefore very well with that, obtained by the subcutaneous test.

Table 5. Detoxicating action of diphtheria toxin with usnic acid, tested by intracutan reaction in guinea pigs.
c) Subcutaneous injection of diphtheria toxin and usnic acid separately

Method:
In experiment 1, 1.0 cc of a dilution of diphtheria toxin, containing 1 MLD, was injected each in 3 guinea pigs, weighing from 250 g to 260 g, subcutaneously and simultaneously 1.0 cc of usnic sodium salt solution, containing 2.0 mg of the acid, injected also subcutaneously but in the different part of injection of toxin.

In experiment 2 the same dose of diphtheria toxin was injected in 3 animals, weighing about 250 g, subcutaneously and 1.0 cc of usnic sodium salt solution, containing 2.0 mg of the acid, three times, that is, directly after the inoculation of toxin, after 24 and 48 hours subcutaneously in the different part of the injection of toxin.

Results:
As results all animals died. The detoxication of toxin with usnic acid in vivo was not demonstrable, owing to the rapid fixation of the toxin in the tissue, even with use of much dosage of the acid in comparison with the amount of toxin injected.

D. Antigenic value of diphtheria toxin, detoxified with usnic acid

Method:
Two groups of young guinea pigs, 250-300 g in weight, which had previously given subcutaneous injections of neutral mixtures of toxin and usnic acid, the total number of MLD being 2 or 30, three times in an interval of one week, and a period of 2 weeks having elapsed since the last injection, received the subcutaneous injections of 2 MLD of the original diphtheria toxin or 0.6 mg of diphtheria bacilli.

Results:
All animals died within 2 or 3 days after the subcutaneous injections of the toxin or of bacilli. The detoxication of diphtheria toxin by means of usnic sodium salt deprives the toxin of its antigenicity.

<table>
<thead>
<tr>
<th>Number of</th>
<th>Fore-treatment</th>
<th>After-treatment</th>
<th>Fate of animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>animals</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1.0 cc of toxin (2MLD) — 1.0 cc of usnic acid (2.0 mg), incubated 24 hours at 37°C, injected.</td>
<td>Toxin (2MLD) injected.</td>
<td>All died within 2—3 days.</td>
</tr>
<tr>
<td>2</td>
<td>2.0 cc of NaCl sol. injected.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1.0 cc of toxin (2MLD) — 1.0 cc of usnic acid (2.0 mg), incubated 24 hours at 37°C, injected.</td>
<td>Bacilli (6.0 mg) inoculated.</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>2.0 cc of NaCl sol. injected.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1.0 cc of toxin (30MLD) — 1.0 cc of usnic acid (15.0 mg), incubated 24 hours at 37°C, injected.</td>
<td>Toxin (2 MLD) injected.</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>2.0 cc of NaCl sol. injected.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1.0 cc of toxin (30 MLD) — 1.0 cc of usnic acid (15.0 mg), incubated 24 hours at 37°C, injected.</td>
<td>Bacilli (6.0 mg) inoculated.</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>2.0 cc of NaCl sol. injected.</td>
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<td></td>
</tr>
</tbody>
</table>
The effect of usnic acid on the flocculation test of diphtheria toxin

Material and method:

100.0 cc of diphtheria crude toxin was equally divided in three portions. In the first portion usnic acid was dissolved as its sodium salt in 0.1% and in the second portion, in 0.01%. pH of both solutions became 8.4, hence the pH of the third portion was also adjusted to 8.4, adding a little quantity of 2 N. NaOH solution. All solutions were laid with toluene on them and after standing 24 hours or 7 days in an incubator at 37°C, Lf and Kf in the flocculation test were measured in a water bath at 50°C.

Diphtheria serum was delivered from the Hokuriku Serum Industry. It possessed 400 AU per cc.

Results:

Compared with values (Lf =26 and Kf =38') of the original diphtheria toxin after 24 hours in the flocculation test, the values (Lf =16 and Kf =65') of the toxin, added with usnic sodium salt solution in 0.1%, showed marked decrease of Lf and delay of Kf. Lf and Kf of the toxin, added with usnic acid in 0.01%, were about the same with that of the original toxin. These relations did not markedly change even when the toxin and usnic sodium salt solution were incubated for 7 days.

Table 7. The effect of usnic acid on the flocculation of toxin.

<table>
<thead>
<tr>
<th>Kinds of toxin, to be tested.</th>
<th>pH</th>
<th>Temp.</th>
<th>After 24 hours</th>
<th>After 7 days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lf</td>
<td>Kf</td>
</tr>
<tr>
<td>Usnic acid added to toxin in 0.01%</td>
<td>8.4</td>
<td>37°C</td>
<td>24</td>
<td>38'</td>
</tr>
<tr>
<td>Usnic acid added to toxin in 0.1%</td>
<td></td>
<td></td>
<td>16</td>
<td>65'</td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td></td>
<td>26</td>
<td>38'</td>
</tr>
</tbody>
</table>

Summary

1. Usnic acid has a relatively strong bactericidal action against *C. diphtheriae* in vitro.

2. When animals, infected with *C. diphtheriae*, are injected with a proper dose of usnic sodium salt solution, they are healed perfectly.

3. Usnic acid detoxicates the diphtheria toxin. When its sodium salt solution is placed with toxin for 1 hour at 37°C, 2 mg of acid is able to neutralize 2 MLD of toxin, and when the solution is placed for 24 hours at 37°C, 1 mg of acid to detoxicate the same quantity of toxin.

The fact was proved by the method of subcutaneous and intracutaneous injections in guinea pigs.

4. When usnic acid was added to toxin in 0.1% solution and incubated 24 hours or 7 days at 37°C, marked decrease of Lf and delay of Kf were confirmed.
5. Toxin, detoxicated with usnic acid, is deprived of its antigenic property.

We should like to express our sincere gratitude to Prof. T. Tani for his kindness to give us the strain of *C. diphtheriae* and Dr. N. Nishida and Mr. K. Wada for providing us with diphtheria toxin and antitoxin.

**REFERENCES**


21) Sasaki, T.: Report 5. The effect of usnic acid, citrinin, kojic acid and tetronic acideriva-


