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In vivo Activity of Colloidal Hamycin, an Antifungal Antibiotic. (29525)

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Hamycin is a polyene antifungal antibiotic obtained from *Streptomyces pimprina*. The antibiotic has been studied intensively in India, where it is produced, but has received scant attention in the U.S.A. Hamycin given orally or intraperitoneally to mice has been able to eradicate infection due to *Cryptococcus neoformans*(1). The drug is active *in vitro* against *Candida albicans* and has been used successfully to treat thrush in man(2).

Hamycin is insoluble in water. Experience with another water-insoluble polyene, amphotericin B, has indicated that a colloidal dispersion of the drug was easier to dispense, was more active *in vivo*, and probably persisted longer in the circulation than the crystalline form(3). For this reason, a colloidal dispersion of hamycin was prepared, bioassayed, and its chemotherapeutic activity assessed in mice infected with *Cryptococcus neoformans* and *Histoplasma capsulatum*. Acute toxicity of the preparation was assessed in mice and rabbits.

Materials and methods. Unnumbered preparations of hamycin were received 1/22/63 and 10/29/63. They will be referred to as numbers 1 and 2 respectively. The yellow powders were dried *in vacuo* at 75-100 μ Hg for 6 hours and stored at 4°C. Solutions were prepared as 2 mg/ml in dimethyl sulfoxide (DMS). For bioassay, fresh DMS solutions were diluted in phosphate buffered saline pH 7.4 (PBS) to concentrations of 0.04-0.32 μg/ml. To determine absorption coefficients, DMS solutions were diluted 300-fold in a solvent containing 3 volumes ethanol and 2 volumes distilled water. Absorption maxima as measured in a Beckman DU spectrophotometer were 364, 384 and 408 mμ. Absorption coefficients were expressed as $E^{1\%}_{1\,cm}$ at $\lambda = 384$ mμ.

A colloidal dispersion of hamycin #1 was prepared by a method similar to that described for amphotericin B(4). Hamycin 2.42 g and sodium desoxycholate 3.1 g were added to 375 ml distilled water and stirred in an ice bath until the suspension reached 7°C. Addition of 8.2 ml N sodium hydroxide resulted in a pH rise to 12.6. After several minutes of stirring, the solution became nearly clear. It was then neutralized with N hydrocloric acid. Centrifugation at 5°C removed 20 mg sediment. The optically clear yellow supernate was divided into portions, frozen in alcohol-dry ice, and stored at —42°C. A portion was lyophilized, revealing a dry weight of 15.21 mg per ml solution.

On 2 occasions, colloidal hamycin solutions were thawed, divided into 3 ml samples, and quickly returned to —42°C storage. One sample on both occasions was not refrozen but bioassayed. A similar operation was per-

formed with the lyophilized powder, which was reconstituted in distilled water to 15.21 mg/ml, divided, bioassayed, and returned to —42°C.

Colloidal hamycin formed clear, stabile solutions in either distilled water or 5% dextrose in water (D5W). Solutions in 60% ethanol gave absorption maxima identical to the original material. Cultures of thawed solutions for bacteria and fungi were negative.

Bioassay. A selected strain of *Paecilomyces varioti* was grown on Sabouraud agar at room temperature for 7-14 days. The spores were harvested in saline, pipetted forcibly to break spore chains, and filtered through a sterile cotton plug. Ten milliliters of this suspension, containing approximately 2×10^7 spores/ml by haemocytometer count, were mixed with 150 ml horse meat infusion agar cooled to 40°C. The mixture was poured into flat-bottomed Petri dishes, 30 ml/dish. Dishes were cooled on a level surface. Wells 8 mm diameter were cut in the hardened agar at points equi-distant from the center of the Petri dish and at least 2.0 cm apart. The bottoms of the wells were sealed with molten agar. After holding the plates at room temperature for 6 hours, 0.1 ml of hamycin solutions were placed in the wells and incubated at 30°C for 48 hours. All solutions were run in sextuplicate and all zones of inhibition read 3 times independently.

Preparation of infectious inocula. The isolate of *Cryptococcus neoformans* (#3749) had been maintained for 5 years by biweekly transfers on Sabouraud's agar at 37°C. A 48-hour growth was harvested in saline, counted in a hemocytometer, and diluted to give an inoculum of 4×10^6 cells in 0.5 ml. The isolate of *Histoplasma capsulatum* (#6656) had been carried for one year by biweekly transfers on blood-cysteine-glucose agar at 37°C. A 48-hour growth was harvested in saline, counted in a hemocytometer, and diluted to give an inoculum of 2.75×10^6 cells in 0.5 ml. The inocula were injected into a lateral tail vein of mice.

Animals. Female general purpose 18-21 g NIH albino mice and 2.0-2.5 kg New Zealand rabbits were employed. The mice in any one experiment did not vary more than 2 g in weight and were randomized by weight within the experiment.

Hamycin administration. To determine acute toxicity in mice, colloidal hamycin was given intraperitoneally or into a lateral tail vein as a single injection of 0.5 ml. Logarithmically spaced doses were given to groups of 10 mice. Using the mortality at the end of 72 hours, the LD_{50} was computed by the method of Irwin and Cheeseman. The standard error was computed by the Spearman-Karber method. Rabbits were given colloidal hamycin into a marginal ear vein.

For oral therapy, hamycin was prepared daily from material stored in vials at —42°C. The hamycin was diluted in distilled water and dispensed in calibrated drinking bottles as the only source of water. The stability of hamycin in distilled water at room temperature for 24 hours was measured on 2 occasions. The losses of activity on bioassay were 25% and 18%; the fall in the absorption coefficient on the latter occasion was 21%. Solutions for parenteral therapy were prepared in D5W every 2 weeks and stored at 4°C.

Ten control mice in each experiment were given the same quantity of vehicle which treated mice received. The vehicle was D5W for parenteral therapy and aqueous sodium desoxycholate for oral therapy. The desoxycholate concentration in each experiment was equivalent to that being given in the highest dose of colloidal hamycin.

Treatment programs were begun 72 hours after infection and continued over a 4 week interval. Ten mice were given each parenteral dose. Twenty mice were given each oral dose. Oral therapy was given 7 days per week, intraperitoneal 5 days per week and intravenous 3 days per week. Mice were housed in groups of 10 and given dry food *ad lib.* Deaths and oral drug intake were recorded daily. Seven days after the last treatment, surviving animals were killed and cultured. From the Cryptococcus-infected mice, a half of each brain was taken. From the Histoplasma-infected mice, the spleen

TABLE I. Parenteral Therapy of Mice Infected with *Cryptococcus neoformans.*

Hamycin		Cumulative mortality, % by week post infection					Survivors	
Route	mg/mouse/wk	1	2	3	4	5	Hydrocephalus	Pos. brain culture
Intravenous	0	0	0	40	100	100	—	—
	.030	0	0	0	0	0	0/10	10/10
	.045	10	10	10	10	10	0/9	9/9
Intraperitoneal	0	0	10	90	100	100	—	—
	.015	0	0	70	100	100	—	—
	.030	0	0	0	10	10	6/9	9/9
	.060	10	10	10	10	10	0/9	0/9

TABLE II. Oral Therapy of Mice Infected with *Cryptococcus neoformans.*

	Hamycin						Cumulative mortality, % by week post infection					Survivors	
Exp	Drinking water conc, μg/ml	Wk	Avg consumption, mg/mouse/wk 1	2	3	4	1	2	3	4	5	Hydro-cephalus	Pos. brain culture
1	0		—	—	—	—	0	0	90	100	100	—	—
	8.6		.30	.17	.04	0	0	0	65	100	100	—	—
	43		1.3	1.3	1.3	1.3	0	0	5	20	20	3/16	16/16
2	0		—	—	—	—	0	0	10	100	100	—	—
	86		1.9	2.2	2.4	2.4	0	0	0	0	0	0/20	12/20
	129		2.9	3.2	3.5	3.5	0	0	0	0	0	0/20	6/20

and one-fourth of the liver were taken and minced with scissors. Tissues were smeared over the surface of Sabouraud agar slants and incubated at 30°C for one month.

Results. Assay. Absorption coefficients for hamycins #1 and #2 were 840 and 1060 respectively. Compared with hamycin #2, hamycin #1 was 81% active on bioassay. Because of its higher optical and biological activity, hamycin #2 was used as reference in stating the activity of the colloidal preparations. Bioassay of the colloidal solution revealed 23% activity on a dry weight basis. Four bioassays of the lyophilized, reconstituted colloidal hamycin over a 2 months' interval gave an average activity of 20% and a range of 16-24%. There was no apparent decrease in activity during storage at —42°C. The absorption coefficients for both colloidal preparations indicated 25-26% hamycin. The discrepancy between optical and biological activity was attributed to alterations of the hamycin molecule not affecting the chromophoric linkage. The presence of DMS in concentrations up to 0.016% in the standard solutions and sodium desoxycholate in concentrations up to 0.01% in the colloidal solutions could not be shown to influence the assay comparisons. Both colloidal preparations contained approximately 52% sodium desoxycholate and 8% sodium chloride on a dry weight basis, leaving the remaining 17-20% attributable to degraded hamycin and other impurities.

Toxicity. The toxicity of colloidal hamycin, expressed in milligrams of hamycin activity, was determined in the mouse:

Inj route	LD_{50} mg/kg	LD_{50} μg/mouse	$LD_{50} \pm 1.96$ S.E., μg/mouse
Intrav	1.0	19	18–21
Intraper	1.7	33	30–38

One rabbit anaesthetized with pentobarbital 20 mg/kg and 4 unanaesthetized rabbits were given colloidal hamycin intravenously while an electrocardiogram was recorded using a midsternal V lead. Two rabbits were given each minute an injection of one-second duration, each dose containing 0.05 mg/kg as 3.0 mg/ml. Two rabbits were given each minute an injection of 30 seconds duration, each dose diluted in D5W as 0.06 mg/ml and containing 0.05 mg/kg for one rabbit and 0.06 mg/kg for the other. In all 4 rabbits, 1-2 doses caused marked sinus bradycardia, while 2-4 doses caused increased T wave amplitude,

TABLE III. Intraperitoneal Therapy of Mice Infected with *Histoplasma capsulatum*.

Hamycin, mg/mouse/wk	Cumulative mortality, % by week post infection		Survivors
	1	2-5	Pos. culture
0	0	10	9/9
.0074	0	0	6/10
.015	0	0	7/10
.030	0	0	2/10
.060	20	20	1/8

lengthening of the QRS interval, ST segment changes, runs of ventricular tachycardia, and death by ventricular fibrillation or arrest. A fifth rabbit was given 0.29 mg/kg as 0.012 mg/ml over an 11-minute interval, resulting only in an increase in T wave amplitude.

Chemotherapy. The results are summarized in Tables I-III. Colloidal hamycin by the intraperitoneal route was able to eradicate Cryptococcus from the brains of infected mice at a dosage level which caused an early death of one mouse. The oral route was less effective but caused no apparent toxicity. Mice receiving the 2 highest doses by mouth never appeared ill, and increased their weight by 142% during the 5 weeks. At autopsy, Cryptococcus was seen on squash smears of brain from all surviving mice treated orally. Many of the cells had bizarre morphology. Therapy by the intravenous route protected the Cryptococcus-infected mice from death but did not eradicate the infection even at a toxic dosage.

Some but not all of the mice infected with Histoplasma were freed from infection by intraperitoneal therapy.

Discussion. The method previously devised for preparing colloidal amphotericin B(4) was found applicable to hamycin. The 2 colloidal dispersions lost 30%-40% biologic activity during preparation. If provision could have been made to minimize exposure of the hamycin to light, oxygen, and high alkalinity during preparation, no doubt less loss of active material would have resulted. The preparations nevertheless retained significant chemotherapeutic activity. Colloidal hamycin by both oral and intraperitoneal routes eradicated Cryptococcus from the brains of the majority of mice, an effect which has not been observed by the authors in similar experiments with amphotericin B. Success has been achieved by others with crystalline hamycin given either orally or intraperitoneally(1). The ability to eradicate histoplasmosis from the mouse and to prevent death from cryptococcosis in the mouse is manifest by both hamycin and amphotericin B(5). These limited experiments suggest that, of the 2 drugs, colloidal hamycin has a lesser difference between effective and toxic parenteral dosage. If parenteral hamycin therapy in man were limited to intravenous colloidal drug, as has been the case for amphotericin B, this toxicity may be an important limiting factor. Impurities might account for some of the toxicity noted. The cardiotoxicity found was similar to that reported for crystalline hamycin(6) and, with higher doses, for amphotericin B(7).

The definite chemotherapeutic effect of hamycin administered orally, which has been reported previously for crystalline hamycin and noted here for the colloidal preparation, suggests that this may be the preferred route of administration. Patients in India tolerated 400 mg doses of crystalline hamycin by mouth(1). It should be remembered that both crystalline(5) and colloidal(8) amphotericin B were effective by the oral route in treating mice with systemic mycoses. Use of these preparations in treating patients with such infections was disappointing(9,10).

Summary. A colloidal dispersion of hamycin was prepared, bioassayed, and given parenterally to mice infected with *Cryptococcus neoformans* or *Histoplasma capsulatum*. Either infection was sterilized in many mice when doses of hamycin approached toxic levels. Colloidal hamycin given orally controlled cryptococcosis in mice and caused no obvious toxicity. The rabbit was more sensitive than the mouse to the lethal effects of colloidal hamycin injected intravenously. The cause of death in the rabbit was cardiotoxicity.

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Succinic Dehydrogenase and RNA in the Supraoptic Nucleus and Hypothalamic Anterior Area in Dehydrated Rats.* (29526)

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The purpose of this investigation was to develop a quantitative method for determining the degree of activity of neurohumoral cells in the hypothalamus of rats. Succinic dehydrogenase activity (SDH) and ribonucleic acid (RNA) content were chosen as possible indicators as it seemed feasible to combine known analytical methods for these substances into a procedure that would give quantitative results for both from the same tissue. The supraoptic nucleus (SON) was selected for study since dehydration is known to increase the ribonucleic acid (RNA) content(1) of the nucleus and an increase in SDH has also been reported in these neurons (2). However, conflicting reports exist concerning SDH in the SON as some investigators have found little or no activity there(3). The anterior area of the hypothalamus (AA) was included in the present study for comparative purposes.

Materials and methods. Eighty female rats (Charles River Breeding Laboratories) weighing approximately 250 g were divided into 8 groups of 10 each. Half of the groups were deprived of water for 7 days while the others served as normal controls. The animals were killed in groups of 10 by decapitation, the hypothalamus removed, immediately frozen and sectioned in a cryostat at 32 μ thickness. The sections were affixed to 75 mm $\times$ 25 mm glass slides by warming to room temperature for 30 minutes. The slides were then placed in Coplin jars and incubated for 15 minutes in a succinate-tetrazole mixture at room temperature. This mixture was prepared according to the method of Nachlas *et al*(4) with the exception that 2-(p-iodophenyl)-3-nitrophenyltetrazolium chloride (INT) was substituted for the Nitro-BT of the original. The slides were then dried overnight in a vacuum desiccator at 9°C. Under a dissecting microscope the SON and the AA were removed by scraping. The material from each rat in a group was pooled and analyzed for formazan, RNA and DNA.

The analysis procedure was that of Scott *et al*(5) for RNA and DNA. The procedure was modified to the extent that the ethanol washing of step 4 and the ethanol-ether mixture of step 5 were saved as the formazan was extracted in these washings. The formazan was measured in these solutions in a Beckman DU spectrophotometer with a Lowry-Bessey attachment at 500 mμ.

Results and discussion. Table I summarizes

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