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The Human Tumor-Egg Host System II. Discovery and Properties of a New Antitumor Agent, Hadacidin.* (27356)

C. O. GITTERMAN, E. L. DULANEY, E. A. KACZKA, D. HENDLIN, AND H. B. WOODRUFF
(Introduced by Karl Folkers)
Merck Sharp & Dohme Research Laboratories, Div. of Merck & Co., Inc., Rahway, N. J.

Dagg *et al.*(1) and Harris(2,3) have described the use of transplantable human tumors growing in embryonated eggs for chemotherapy studies. We have used the human tumor-egg host system to screen for new antitumor agents. Employing this system, we were successful in discovering a new antitumor agent, N-formyl hydroxyaminoacetic acid, designated hadacidin. Its isolation, structure determination, and synthesis are described(4).

Methods. The procedures of transplantation of tumors and testing of agents are based upon those described by Harris(3). Tumor implants were placed on the chorioallantoic membranes of 9-day embryonated eggs. The eggs were incubated 3 to 4 days, and those showing positive "takes" were selected for therapy. Test agents were injected into the yolk sac. Seven days after injection, the eggs were harvested and tumors and embryos from treated and control groups were weighed.

Results were expressed as follows. Ten eggs were sacrificed at time of injection to determine mean weight of the tumor. The value obtained was subtracted from the mean weight of treated and control tumors at time of harvest. Thus, the actual increase in weight of the tumors during the treatment period was determined. The per cent growth retardation ($100 - T/C \times 100$) compares the increase in weight of treated tumors with the increase in weight of control tumors. The per cent growth retardation for embryos was determined in a similar manner.

Primary screening was against the human adenocarcinoma, HAD #1(5). Additional tests were made against the human epidermoid carcinoma, HEP #3(6) and the human bronchogenic carcinoma, A-42(7).†

Microorganisms isolated from various habitats were grown in 250 ml Erlenmeyer flasks containing 50 ml of medium of the following composition: glucose, 4%; corn steep liquor, 1%; Edamine‡ (enzymatic digest of casein), 2%; distilled water to volume. The pH was adjusted to 6.8 with NaOH prior to sterilization. The flasks were incubated at 28°C on a rotary shaker moving at 220 rpm.

After 6 days' incubation, the fermentation broths were clarified by admixing with Super-Cel§ and filtering through a Buchner funnel. The clarified filtrates were sterilized by passage through Selas 03 filters‖ prior to testing. When necessary, dilutions were made with Earle's Balanced Salt Solution (ES) which was also used as diluent for water-soluble solids. One ml of filtrate or appropriate dilution was injected per egg. The control

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† All tumors carried in our laboratory were obtained initially through the generosity of Dr. John Harris, Sloan-Kettering Institute, New York.

‡ Sheffield Chemical Co., Inc., Norwich, N. Y.

§ Johns-Manville, New York.

‖ Selas Filter Corp. of America, Dresher, Pa.

TABLE I. Inhibition of HAD #1 Tumor in the Embryonated Egg by Filtered Broths* of *P. frequentans* Westling.

Broth filtrate	Tumor growth retardation, %			
	Test 1	Test 2	Test 3	Avg
1	64	69	63	65
2	53	67	67	62
3	41	61	59	56

* Each egg treated with 1 ml broth filtrate; 6-7 eggs were used per treatment.

series of eggs were injected with 1 ml ES.

For screening of fermentation broths, 6 eggs were used for each treatment group and 14 eggs for the control group. A sequential screening plan was used to aid in selection of broths for further study.

Results. A. *Fermentation studies.* Among the first 100 broth cultures evaluated against HAD #1, the broth produced by a culture identified as *Penicillium frequentans* Westling appeared to be of special interest. Sequential testing of the first 4 replicate growths of *P. frequentans* demonstrated that 3 were active, and that the 3 active broths inhibited the tumor to about 60% of the untreated controls (Table I). These results demonstrated not only the reproducibility of the fermentation but also that of the HAD #1-embryonated egg system.

Prior to initiation of chemical fractionation of broths from *P. frequentans,* optimal harvest time was established by assaying broths of various ages. The results of the time study are presented in Table II. Maximum antitumor activity was reached after 144 hours' incubation, after which there appeared to be a slow decrease in broth potency.

TABLE II. Inhibition of HAD #1 Tumor in Embryonated Egg by Filtered Broth* of *P. frequentans* Westling Sampled at Different Time Intervals of Fermentation Cycle.

Sample time, hr	Deaths	Growth retardation, %†	
		Embryo	Tumor
72	1/12	0	0
96	2/12	0	15
120	1/11	2	38
144	0/17	11	74
168	5/11	7	30
192	1/11	8	46

* Each egg treated with 1 ml broth filtrate.
† Results are derived from 2 different experiments except for the 144 hr sample where results are derived from 3 experiments.

B. *Guidance of the isolation of hadacidin.* Isolation of the antitumor activity in *P. frequentans* broth was undertaken. The results in Table III demonstrate that the bioassay can be used to guide isolation studies.

The antitumor activity of filtered broth proved stable to lyophilization. Separation of broth into 2 fractions by solvent extraction resulted in concentration of the activity in the methanol soluble fraction. No activity was detected in the residue. The crystalline product isolated from the methanol extract proved highly active. The product was designated hadacidin. Tumors from eggs treated with 3 mg of hadacidin were inhibited 63%.

TABLE III. Utilization of HAD #1-Embryonated Egg System to Guide Isolation Studies on Broth of *P. frequentans* Westling.

Preparation	Dose, mg/egg	Deaths	Growth retardation, %	
			Embryo	Tumor
Filtered broth	20*	0/17	11	74
Lyophilized filtered broth	20*	2/19	12	68
	10*	3/19	3	26
Methanol soluble	10	0/6	7	52
	5†	1/11	15	47
" insoluble	10†	1/11	0	−5
Hadacidin (free acid)	3*	4/18	8	63

* Results derived from 3 different experiments.
† Results derived from 2 different experiments.

C. *Antitumor spectrum of hadacidin.* Hadacidin was tested against 2 other transplantable human tumors growing in the embryonated egg, HEP #3 and A-42. Side by side experiments comparing the sensitivity of HAD #1, HEP #3, and A-42 are shown in Table IV.

All 3 human tumor lines growing in the embryonated egg were inhibited by hadacidin. In 3 of 4 determinations, A-42 was more sensitive than HAD #1. HEP #3 was the least sensitive of the tumors studied. A different strain of HEP #3 was inhibited 60% by a hadacidin-containing fermentation broth and thus was as sensitive as HAD #1. The

TABLE IV. Activity of Hadacidin (Sodium Salt) against HAD #1, HEP #3, and A-42 in Embryonated Egg.

Exp.	Hadacidin dose, mg/egg	Tumor	Deaths	Growth retardation, % Embryo	Tumor
1	10.0	HAD #1	0/6	30	73
		HEP #3	2/6	34	54
		A-42	1/6	18	92
	5.0	HAD #1	3/6	13	45
		HEP #3	1/6	17	29
		A-42	2/6	18	80*
2	7.5	HAD #1	0/6	2	80
		HEP #3	0/6	17	31
		A-42	0/6	11	57
	5.0	HAD #1	1/6	−8	52
		HEP #3	0/6	16	41
		A-42	1/6	3	85

* % value based on only 2 treated tumors because 2 other survivors appeared to be "no takes."

sensitive strain of HEP #3 differed from the resistant strain. It grew at a more rapid rate; in 7 days the tumors increased 10-fold in mass. Eleven days were required for a similar weight increase with the more resistant strain of tumors. Experiments with the sensitive strain were terminated 7 days after implantation of tumors because of a high embryo mortality commencing with the 8th day. The high mortality rate was not observed with the more resistant, slower growing strain of HEP #3, and experiments were not terminated until 10 to 11 days after implantation.

D. *Dose level and tumor inhibition.* A dose response curve was determined for the sodium salt of hadacidin against HAD #1. The effect of dose on embryo weight gain and mortality also was noted. The results of one experiment are summarized in Table V. The

TABLE V. Response of HAD #1 Tumor and of Chick Embryo to Different Levels of Hadacidin. Na salt injected into yolk sac of 13-day embryo 4 days after tumor implantation.

Dose hadacidin, mg/egg	Embryo mortality, dead/total	Weight gain Embryo, g	Tumor, mg
0 (ES 1 ml)	3/16	17.5	1682
2.7	0/12	18.0	948
4.1	3/12	17.0	966
6.3	3/12	15.0	463
9.75	5/12	11.5	379
15.0	8/12	15.0	111

statistical analysis of all experiments completed to date yield the following values.

	mg/egg	95% confidence limits
Tumor inhibitory dose 50% (ID_{50})	3.3	2.5- 4.1
Embryo inhibitory dose 15% (EID_{15})	5.3	3.3- 8.9
Embryo lethal dose 50% (LD_{50})	13.2	10.8-19.1

It is apparent from the above figures that the ID_{50} is about one-fourth the LD_{50} *i.e.*, the level of hadacidin which causes a 50% inhibition of tumor growth is one-fourth the hadacidin concentration necessary to kill 50% of the embryos.

E. *Activity of salts and derivatives of hadacidin.* Results obtained on testing the sodium, potassium, and ammonium salts and the methyl ester of hadacidin are summarized in Table VI. Also shown for comparison are results obtained with known antitumor agents, triethylene melamine, azaserine, and 6-mercaptopurine.

All of the hadacidin derivatives were active against HAD #1 in the embryonated egg. The alkylating agent, triethylene melamine is about 100-200 times as active as hadacidin on a weight basis. On the other hand, azaserine and 6-mercaptopurine are active in the same range as hadacidin.

Discussion. The human tumor-egg host system has been used previously to evaluate substances for antitumor activity(1,2). We have employed such a system in our laboratory to test fermentation broths for tumor-inhibiting substances. In addition, we have used the tumor-egg host system as an assay to guide fermentation and chemical studies which led to isolation of the active agent in broths of *Penicillium frequentans*. To our knowledge this is the first instance in which the human tumor-embryonated egg method was successfully employed to guide the isolation of an antitumor agent from fermentation broth.

Although the original observation of the antitumor activity was with the HAD #1 tumor, hadacidin is active also against HEP #3 in the egg.

TABLE VI. Growth Retardation of HAD #1 in Embryonated Egg by Hadacidin and Other Antitumor Compounds.

Compound	Dose, mg/egg	Embryo mortality	Growth retardation, % Embryo	Tumor
Hadacidin—Monosodium salt	6.0	3/24*	3.5 (0– 7)†	61 (56– 65)†
	7.5	4/21*	20 (18–22)†	68 (62– 73)†
Monopotassium salt	6.0	0/6	14	59
Monoammonium salt	5.0	1/6	27	52
Methyl ester	7.5	0/6	14	57
Triethylene melamine	.025	2/48‡	10 (0–23)†	74 (48–100)†
Azaserine	2.0	5/9	41	75
	1.0	2/9	20	62
6-Mercaptopurine	8.0	3/9	6	63
	4.0	0/9	3	27

* Results derived from 2 different experiments.
† No. in parentheses refer to range of values observed.
‡ Results derived from 8 different experiments; 6 eggs/treatment.

Our results with HAD #1 and HEP #3 have been confirmed by Harris and Yap-Guevera who found hadacidin effective also against A-42 and HS #1(8). We have confirmed their observations with A-42. In our hands the strain of HEP #3 tumor now in use proved the least sensitive of the tumors studied. However, an earlier employed strain of HEP #3 was as sensitive to hadacidin as HAD #1.

Summary. A new crystalline antitumor agent, hadacidin, has been discovered in fermentation broths of *Penicillium frequentans* Westling. The human tumor-embryonated egg system was used to guide fermentation and isolation studies. Against HAD #1 the tumor ID_{50} of hadacidin is about 3 mg per egg. Hadacidin is active also against HEP #3 and A-42 in the embryonated egg. On a weight basis, hadacidin has about the same range of activity as azaserine and 6-mercaptopurine but has only one per cent the activity of the alkylating agent, triethylene melamine.

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Observations on the Properties and Prevalence of Coe Virus.* (27357)

ANDREW S. ABRAHAM (Introduced by F. S. Cheever)
Department of Microbiology, University of Pittsburgh School of Medicine, Pittsburgh, Pa.

During viral isolation studies on material obtained from the Bangkok (Thailand) cholera outbreak in 1958, an agent was isolated (1) which bore some resemblance to the Coe virus. In comparing the 2 agents, which

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