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A Tumor Inhibitor in *Lampteromyces japonica.** (27889)

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In the fall of 1958, the senior author working in the laboratory of Dr. Ayao Yamamoto, Institute for Infectious Diseases, University of Tokyo, cooperated with Drs. Yamamoto, Ogata, Komatsu and Nakazama in studying the effect of saline extracts of *Lampteromyces japonica* (L.J.) on Ehrlich mouse tumors and Sarcoma 180. This work included *in vivo* and *in vitro* studies with findings of some anti-tumor activity. Lucas *et al.*(1,2) have demonstrated tumor inhibition by extracts of the fungus genus Calvatia and Holobasidiomycetes.

It seemed desirable to extend these preliminary observations and to determine the effect of fractions of L.J. on the Ehrlich mouse tumor and on human tumor cells growing in tissue culture.

Materials and methods. The tumor-inhibiting original extract was prepared from dried *Lampteromyces japonica,* the only species of this basidiomycete. The original samples were collected in Japan in 1959.

Ten g of dried material, ground in a 20-mesh Wiley Mill, were extracted with 100 ml of saline (0.85% NaCl) for 24 hours at 5°C with continuous shaking. The saline extract was then spun at 500 *g* for 20 minutes. The residue was reextracted with 100 ml of saline. Part of the pooled supernatants was dialyzed at 5°C against running tap water for 48 hours and then against distilled water for 3 hours. This saline extract was designated L.J., original extract. To the remainder of the pooled supernatant at a pH of 5.5-6.0, was added cold (5°C) ethanol to a final concentration of 30%. This was kept at 5°C overnight and then centrifuged at 5000 *g* for 30 minutes in an anglehead centrifuge. The 30% alcohol precipitate was dialyzed and lyophilized. This was designated as fraction No. 2. To the 30% ethanol supernatant, cold ethanol was added to a final concentration of 70%, then kept overnight at 5°C. This material was then centrifuged at 5000 *g* for 30 minutes and the supernatant was decanted, dialyzed and lyophilized, as was the precipitate. The latter was labeled fraction No. 3, and the lyophilized supernate fraction No. 4.

In vitro *inactivation of Ehrlich tumor cells.* Seven-day Ehrlich ascites tumor cells were washed free of red blood cells with 0.1% gelatin saline and made up to a 4% suspension by volume. Equal volumes of 1% and 0.1% initial concentrations of the L.J. fractions in saline were added to aliquots of the tumor cell suspension. After the mixtures had been incubated at 37°C for 30 minutes, 5 white mice (male, 18-20 g random bred Swiss) per test group were injected subcutaneously in the inguinal region, with each animal receiving 0.2 ml of the tumor cell suspension. This inoculum represents about 1.5 million cells, which gave 90-100% takes in controls. These mice were observed twice weekly for 3 weeks, and the development of tumors recorded.

In vivo *animal bioassay inactivation.* Mice

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were inoculated intraperitoneally with 2 million tumor cells suspended in 0.2 ml, then injected intraperitoneally one time only with 1.0 mg of the original extract or 0.5 mg of L.J. fractions one day or 2 days after the inoculation.

A second group of mice was inoculated subcutaneously with 2 million tumor cells, then treated intraperitoneally with 0.5 mg of L.J. fractions 2 days thereafter and every day for a total of 7 injections.

A third group of mice was inoculated subcutaneously with 2 million Ehrlich cells and then injected in the same area with 0.5 mg of L.J. fractions 2 days thereafter and every other day until 4 injections had been made.

Hemagglutination and hemolysis tests. Doubling dilutions of a 1% preparation of L.J. fractions were made in saline, and an equal quantity (0.5 ml) of a 0.5% suspension of washed mouse or human erythrocytes added. After incubating at 37°C, the tubes were stored at 5°C overnight. Hemagglutination and hemolysis titers were read and recorded as the highest dilution showing agglutination or hemolysis.

Anti-mammalian tumor tests in tissue culture. The freeze-dried L.J. fractions were made up as 1% solutions in Eagle's tissue culture medium containing 20% human serum, and 50 μg per ml of penicillin G and of streptomycin. A series of dilutions of the L.J. preparations were prepared in the tissue culture medium. An equal volume of each diluted L.J. fraction was added to a tissue culture cell suspension containing about 50,000 cells per ml. The human tumor cells, H. Ep No. 2, were collected from 5- to 7-days-old monolayer cultures grown on the surface of glass bottles. One ml of the mixture of diluted L.J. fraction and cell suspension was placed in vinyl plastic panel cups (Lat. No. 88, Fabri-Kal Corp., Kalamazoo, Mich.) of 1.2 ml capacity. The cups were then covered with 3-inch Scotch tape. The L.J.-tissue culture systems were incubated at 37°C and examined daily for 7 days. Acid accumulation and cytopathogenic changes in the cells were noted. The latter were indicated by increased granularity, sphericity, clumping of cells, and failure to adhere to the bottom of the cups.

The L.J. fractions were also tested for anti-growth activity against HeLa and Chang liver cells.

Results. As a first step, the original saline extract prepared from the dried fungus received from Japan was tested for its anti-Ehrlich tumor cell effect *in vitro*. Ehrlich tumor cells were inactivated by a 0.1% final concentration of the crude extract. Fractions No. 2 and 4 but not fraction No. 3 were also active against Ehrlich cells. Fraction No. 4 was active against the tumor cells in a concentration of 0.05% or less *in vitro* at 37° in 30 minutes.

In previous work with seed extracts(3) it has been found in this laboratory that such materials might be effective *in vitro* but not *in vivo*. Serum was found to inactivate the seed extracts. Obviously it was desirable to test the L.J. fractions *in vivo*. The first results were disappointing since 0.5 to 3.0 mg of the fractions or crude extract injected intraperitoneally into mice at the same time the tumor was planted subcutaneously had no effect on development of the tumor. However, when the tumor cells were given intraperitoneally and the L.J. fractions were administered by the same route 1 and 2 days later, both the original saline extract and fraction No. 4 completely protected 20 and 35 mice, respectively, inoculated with the tumor. Ninety-five to one hundred per cent of the control mice developed tumors in these tests. The marked difference in results obtained in the tests in which the agents were injected in a different site than the tumor might be related to the limited permeability of blood vessels to the passage of the L.J. materials into the tumor injected area. That there is limited capillary permeability to the passage of macromolecular substances in absence of inflammation in tumor bearing tissue has been stated by several investigators (4,5).

When mice were inoculated subcutaneously with the tumor and the L.J. fractions were injected 2 days later into the same area, No. 4 fraction afforded complete protection to 4 mice so treated. No tumors developed

TABLE I. Hemagglutinating, Hemolytic and Toxic Effects of L.J. Fractions Compared to Dilutions Affecting Mammalian Cells *in vitro*.

	Orig. extract	Fraction of L.J. No. 2	No. 3	No. 4
Hemagglutination titer				
Mouse R.B.C.	<1:200 (3)	<1:200 (3)	<1:200 (3)	<1:200 (7)
Human R.B.C.	<1:200 (3)	<1:200 (3)	<1:200 (3)	<1:200 (8)
Hemolytic titer				
Mouse R.B.C.	1:12,800 (3)	1:25,600 (3)	<1:200 (3)	<1:200 (7)
Human R.B.C.	1:25,600 (3)	1:51,200 (3)	<1:200 (3)	<1:200 (8)
Toxic dose for mouse				690 mg/kg body wt (1)
Anti-H. Ep. No. 2 titer in tissue culture	1:100,000 to 1:10,000 (3)	1:10,000 to 1:1,000 (3)	1:1,000 (3)	1:800,000 to 1:125,000 (10)
Anti-HeLa titer in tissue culture	1:50,000 (1)	1:1,000 (2)	1:1,000 (2)	1:250,000 to 1:50,000 (7)
Anti-Chang liver cell in tissue culture	1:50,000 (1)	1:10,000 to 1:1,000 (2)	1:5,000 to 1:1,000 (2)	1:250,000 (7)
Anti-Ehrlich titer *in vitro* tested by mouse inoculation	1:200 (2)	1:200 (2)	1:200 (2)	1:2,000 (3)

Number in parentheses represents No. of different tests each with 3 or more replications per test.

in 2 of 4 mice receiving the original extract, in 2 of 5 receiving fraction No. 3, whereas 5 of 5 mice given fraction No. 2 developed tumors.

Attempts to inhibit the growth of established subcutaneous tumors by injecting fractions of L.J. intravenously 7 days after tumor implantation were not successful. These results parallel those of Nungester and Fisher (6) with anti-tumor serum. Again, the failure of macromolecular substances to pass the blood-tissue barriers in the absence of inflammation is suggested.

To gain some insight into the effect of the L.J. fractions on mammalian cells, 3 cell lines, 2 neoplastic in origin and 1 from normal tissue, were exposed to varying doses of the L.J. fractions. These tissue cultures contained 20% human serum, a concentration which inactivated the anti-tumor activity of seed extracts as determined in previous studies. The data in the Table summarize the effects of L.J. fractions on growth of certain mammalian cells *in vitro*. It will be noted that fraction No. 4, in dilutions as great as 1:125,000 and to 1:800,000 on occasion, inhibited the growth of H. Ep. No. 2 cells in tissue culture.

The adverse effects on man of ingestion of this fungus are not great. One effect is said to be diarrhea. The toxicity of fraction No. 4 for mice injected intraperitoneally was about 690 mg/kilo body weight, yet the effective dose given at time of tumor implantation and in the same site was 50 mg/kilo body weight or less.

Possible effects of the saline extract and the 3 fractions on erythrocytes of mice and man were examined. Agglutination was minimal and was seen only in dilutions less than 1:200. However, hemolysis was noted with the original saline extract in high dilution, 1:12,800 for mouse RBC and 1:25,600 for human erythrocytes. The hemolytic factor was not present in the No. 3 or No. 4 fractions. These data are given in Table I along with dilutions of these fractions effective *in vitro* on human tumor cells H. Ep.

No. 2 and the mouse Ehrlich cells.

Discussion. In presenting these results, we wish to call attention to anti-mammalian cellular activity of extracts of the *Lamptero-myces japonica* (L.J.) fungus. One partially purified fraction appears to be active against human cancer cells in tissue culture in microgram quantities and is not inactivated by serum. It acts on the Ehrlich tumor cell *in vivo* in mice in subtoxic doses providing it comes in contact with the cells. Whether or not further purification and characterization of the active principle in fraction No. 4 of the L.J. extract will lead to a more active fraction remains to be seen. The senior author has made some progress in isolating and characterizing this substance which appears to contain little if any nitrogen. Whether or not such material injected intravenously will have an effect on an established tumor of animals or man is questionable unless procedures are developed for selectively increasing venule or capillary permeability to macromolecular substances in the tumor bearing area. This problem is being investigated.

The chemical differences in the surfaces of cancer cells and normal cells appear to be real but small, as indicated by current immunological studies. Furthermore, there is no reason to believe that all tumor cells have a common chemical surface component different from normal cells. Therefore, in searching for specific chemotherapeutic anticancer agents, one might well consider macromolecular substances which, by nature of their size, are most likely to have a characteristic surface configuration and thus have a specific action.

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Radiomanganese Studies in Pyridoxine Deficient Rats. (27890)

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Our earlier publication(1) and that of Hartsook and Hershberger(2) have shown that deficiency of pyridoxine in rats results in a disturbance of cellular equilibrium of sodium and potassium contents in tissues. Using isotopic technics, Hsu(3) has recently reported that pyridoxine deficiency decreases I^{131} uptake by the thyroid glands in rats. Further observations(4) in rabbits have indicated that administration of deoxypyridoxine, an antagonist of pyridoxine decreased the concentration of serum magnesium, and reduced uptake of Mg^{28} tagged chloride in kidneys, lung and bone. All the above findings demonstrated that pyridoxine plays an important role in maintaining the electrolyte balance. The present report indicates that deficiency of pyridoxine also disturbs the normal tissue distribution and rate of excretion of another indispensable trace metal, manganese, in rats.

Materials and methods. Pyridoxine deficiency was produced in young adult rats of both sexes of the McCollum strain by feeding a high protein diet* deficient in pyridox-

* Basal diet: Vitamin free casein 27, sucrose 59, corn oil 10, Salt mixture 4 and vitamin supplements: See Hsu and Chow, *Arch. Biochem. and Biophys.,* 1957, v72, 322.