The Effect of Various Fungi on Mouse Tumors with Special Reference to Sarcoma 37*

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Some years ago Protti (7) described the lytic action of several species of Saccharomyces on transplanted carcinoma by a process which he called "cytophotolysis"; and Castelli and Gaggini (1) repeated these experiments, not only with yeasts, but also with "*Oidium*" *albicans*, which lysed tumor cells. These experiments and those of Lewisohn *et al.* (3) were reviewed in an earlier paper (2) in which we confirmed the lytic action of several species of Candida on Sarcoma 37 ascites and Ehrlich ascites tumors *in vitro*. Some inhibition of mitosis in Sarcoma 37 ascites cells *in vivo*, with reduction of ascites swelling, was observed following intraperitoneal injection of nonpathogenic species of Candida. However, with a single exception, there was no prolongation of survival time. Cells of the pathogenic species, *Candida albicans*, were quickly phagocytized by leukocytes, and the effect on the ascites tumor *in vivo* was negligible. Examination of the influence of Candida species, as well as some other yeastlike organisms and molds on solid tumors (5), showed that many of these organisms caused tumor regression. The result of treating solid Sarcoma 37 with these organisms is the subject of the present paper.

MATERIALS AND METHODS

Solid tumors were obtained by injecting Sarcoma 37 ascites fluid subcutaneously into Swiss mice (0.5 cc/mouse). Fluid was withdrawn aseptically from donor mice, pooled, and diluted 1:4 with sterile saline. Each sample was injected into a number of mice which were divided into two groups, one of which served as control. Repeated samplings were made in the same way until the desired number of animals had been inoculated. These tumors show a lower percentage of spontaneous regression (6 per cent) and are more uniform in size than tumors derived from trocar implant. Furthermore, we have found them to be more satisfactory for microscopic studies, since there is no residual necrotic fragment of the original implant, as is the case with trocar-introduced tumors. The tumors were allowed to grow for 1 week, or until they attained a diameter of approximately 1 cm.

The following organisms, obtained from the American Type Culture Collection, Washington, D.C., were tested for oncolytic effect: Candida species, *Cryptococcus glabratus*, *Geotrichum lactis*, *Histoplasma capsulatum*, *Nocardia intracellularis*, *Nocardia asteroides*, *Pichia fermentans*, *Rhodotorula rubra*, *Saccharomyces cerevisiae*, *Torulopsis candida*, and *Torulopsis utilis*. They were grown on modified Sabouraud's medium, or glucose-peptone medium, with the exception of *Histoplasma capsulatum*, which was cultured on blood-containing medium, in wax-sealed tubes, to promote the yeast phase of this organism used exclusively in these experiments.

The organisms to be tested were washed with sterile saline, centrifuged at 2,000 r.p.m. for 15 minutes, and prepared in a 1:100 volumetric dilution in sterile saline. Candida preparations contained approximately 4×10^6 viable cells/c.mm.

Since some of the organisms, e.g., *Candida albicans*, are known pathogens, it was necessary to kill them before injection. On the basis of our previous experiments (4), we knew that almost all Candida species may be pathogenic, but that *C. guilliermondi*., *C. parakruzei*, and *C. pseudotropicalis* can be inoculated in the living state with no lethal consequences. Other species, such as *C. albicans*, *C. tropicalis*, and *C. krusei*, have to be killed before inoculation into mice. This was accomplished either by heating (60° C. for 1 hour) or by chemical procedures (5 per cent phenol for 1 hour, followed by centrifugation and three washes in sterile saline).

Because of the great difficulty in breaking down the pellicle in which *Candida lipolytica* grows on solid medium, it was necessary to treat this organism in a Waring Blendor for several minutes to obtain a homogeneous suspension. The apparatus was packed in ice to avoid temperature rise.

Preparation of crude filtrate.—The most satisfactory preparations resulted from treatment of the organisms in a Waring Blendor. Seven gm. of dry yeast was mixed with 50 gm. of Superbrite glass beads and 15 ml. of buffer (Na_2HPO_4) at pH 7.2 and homogenized for 3 minutes. This preparation was centrifuged, and the resulting supernate was passed through an ultrafine Büchner funnel with fritted glass disc (porosity, 1–2μ) and tested for sterility by incubation on Sabouraud's agar.

Inoculation.—Different routes of inoculation were employed, but most of the experiments were made by intravenous administration. All control mice were given injections of sterile saline in the same volume and by the same route.

White cell counts were sampled on hosts from each group of tumor implants treated with *Candida guilliermondi* and *Rhodotorula rubra* and their corresponding controls. Blood was drawn from the tail vein prior to treatment and at daily intervals after each injection.

RESULTS

Almost all *Candida* species tested showed oncolytic activity with respect to Sarcoma 37. This activity was more pronounced following the injec-

* Supported in part by a grant from the Eli Lilly Co. and in part by an institutional grant from the American Cancer Society.

Received for publication December 24, 1956.

tion of living cultures of such organisms as *C. guilliermondi*, *C. pseudotropicalis*, and *C. parakruzei* than was that obtained with killed organisms (*C. albicans*, *C. tropicalis*, *C. kruzei*). *Candida guilliermondi* was perhaps slightly more active than other species of this genus. The percentage of regression following injection of this organism is shown in Table 1.

Similar oncolytic effects were obtained following the injection of *Cryptococcus glabratus* (approximately 90 per cent regression), *Rhodotorula rubra* (80 per cent regression), and several other organisms shown in Table 6. Only one of the Candidas, *C. lipolytica* (which is considered by some authorities to belong to another genus), appeared to have no effect on Sarcoma 37.

Cell division in this tumor was arrested within a few hours after injection of active organisms. In the case of *C. guilliermondi*, 60 per cent of all animals treated showed only small scabs at the tumor site 7 days after initial injection of the organism. One week later an additional 20 per cent of the tumors had totally regressed. In other words, 80 per cent of all tumors treated were permanently sloughed by 14 days postinjection. The mean survival time of untreated Sarcoma 37-implanted mice and of treated mice with nonregressed tumors was the same (23 days postimplantation).

The host mice showed no signs of toxicity following the injection of most of these organisms. Occasionally, following injection of *Candida parakruzei*, and more frequently following injection of *Cryptococcus glabratus*, some mice developed evidence of cerebral effects such as twisting of the head to one side and twirling movements when held by the tail.

Most of the injections were by the intravenous route in amounts of 0.2 cc. of the 1:100 dilution, 3 times weekly; but larger doses (0.5 cc. and 1.0 cc. in a single injection) of such organisms as *C. guilliermondi* were equally well tolerated. Table 2 shows the effect of varying dosages.

In this set of experiments, daily injections of 1.0 cc. caused regression of all tumors. However, it will be noted that 0.5 cc. was almost as effective, and 0.2 cc. not much less active, in tumor destruction.

To determine the effect of injection by other routes, two organisms were tested intravenously, intraperitoneally, and subcutaneously. The results are shown in Table 3. Intravenous injection was

TABLE 1

EFFECT OF LIVING *Candida guilliermondi* ON SARCOMA 37

(Combined data from ten samples of pooled donor ascites)

No. of mice	Treatment: Dosage	Treatment: No. inj.	Treatment: Total amt.	Treatment: Route	Tumor regression: Regressed	Tumor regression: Nonregressed	Host mice surviving at 2 mos.
100*	0.2 cc.	5 (every other day)	1.0 cc.	Intravenous	80	17	80
100	Saline-injected controls				6	94	6

* Three mice in this group died because of clumping of the organisms in the needle. Air was accidentally injected in the attempt to free the obstruction.

TABLE 2

TUMOR REGRESSION AS RELATED TO SIZE AND FREQUENCY OF DOSAGE

(*C. guilliermondi* living)

Dosage:	Controls	0.2 cc.	0.2 cc.	0.5 cc.	0.5 cc.	0.5 cc.	1.0 cc.	1.0 cc.	1.0 cc.
Frequency of injection	Non-injected	Every second day	Every day	Single injection	Every second day	Every day	Single injection	Every second day	Every day
Duration of injections in days	*	12	12		7	7		7	7
No. of tumors regressed	1/20	12/16	16/17	6/12	13/14	12/12	6/6	12/12	12/12

* The experiment was continued until all mice with nonregressed tumors had succumbed (approx. 4 weeks).

TABLE 3

TUMOR REGRESSION AS RELATED TO ROUTE OF ADMINISTRATION

Organism	Route of administration	No. of tumor-bearing mice injected	No. of tumors regressed
Candida guilliermondi	Intravenous	36	32
	Intraperitoneal	36	19
	Subcutaneous	36	11
Cryptococcus glabratus	Intravenous	32	29
	Intraperitoneal	32	19
	Subcutaneous	32	6

the most effective (80–90 per cent regressions). About 50 per cent of the tumors regressed following intraperitoneal injection; and only 20–30 per cent after subcutaneous administration.

Although killed organisms were not so effective as living ones, Table 4 shows that many tumors regressed following the intravenous injection of these preparations, accompanied by only slight toxicity. Heat-killed organisms were decidedly less effective than chemically killed ones.

TABLE 4

INFLUENCE OF INTRAVENOUSLY INJECTED KILLED ORGANISMS ON SOLID SARCOMA 37

	No. of tumors	No. regressed	Lethality
Chemically killed:			
C. albicans	34	27	1
C. tropicalis	38	24	
C. kruzei	28	15	1
C. guilliermondi	32	18	
Cryptococcus glabratus	36	29	
Heat-killed:			
C. albicans	36	17	3
C. tropicalis	32	12	2
C. kruzei	24	11	1
C. guilliermondi	32	16	
Cryptococcus glabratus	32	14	2

Crude filtrates, prepared as previously indicated, were incubated on Sabouraud's medium to test whether or not living cells persisted. Only those preparations from which no growth was obtained were employed in the experiments summarized in Table 5. They showed some tumor-inhibiting activity and caused the death of about 10 per cent of the treated mice.

TABLE 5

THE INFLUENCE OF INTRAVENOUS INJECTION OF CRUDE EXTRACTS OF ORGANISMS ON SOLID SARCOMA 37

Treatment of organism	Organism	No. of tumors	No. regressed	Lethality
Lyophylized	*C. guilliermondi*	37	13	3
	Cryptococcus glabratus	31	11	4
Homogenized	*C. guilliermondi*	24	7	2
	Cryptococcus glabratus	30	11	4

A comparison of the effects of the various other organisms tested is shown in Table 6.

TABLE 6

COMPARISON OF EFFECTS OF INTRAVENOUSLY INJECTED LIVING FUNGI OTHER THAN CANDIDA SPECIES ON SOLID SARCOMA 37

Name of organism	No. of tumors	No. regressed	Lethality
Cryptococcus glabratus	102	95	
Cryptococcus neoformans	18	0	
Geotrichum lactis	12	1	1
Histoplasma capsulatum	18	2	4
Nocardia intracellularis	14	2	3
Nocardia asteroides	12	2	4
Pichia fermentans	18	4	1
Rhodotorula rubra	100	80	
Saccharomyces cerevisiae	40	25	2
Torulopsis candida	16	9	
Torulopsis utilis	18	6	4
Controls	101	6	

Although the original observations of Protti (7) concerned effects of *Saccharomyces cerevisiae*, and most of the yeast extract studies (4) also were made with this organism, our experiments show that it appears to be less active than the Candidas.

The injection of glucose-peptone medium in which organisms had been grown produced no tumor regression.

Pre-injection.—Some experiments were performed to determine whether pre-injection of organisms would prevent growth of tumor transplants. The results were negative.

Microscopic observations.—Tumor tissues and corresponding normal tissues from host mice were prepared in paraffin section. Samples were taken at hourly intervals postinjection up to 12 hours and thereafter each day until the tumor had been completely resorbed. Sections were stained with the McManus-Hotchkiss technic (Kligman modification) for demonstration of fungi in tissue and were counterstained with Harris' hematoxylin.

At 1 hour postinjection with *C. guilliermondi*, the greatest concentration of organisms was found in the lung capillaries. Occasionally, free yeast cells appeared in the spleen and also in the liver. They were also located in the peripheral stroma of the tumor but were not in contact with the tumor cells, and there was no evidence of cellular damage. One hour later (2 hours postinjection), tumors showed slight gross hemorrhage. However, the yeast cells were found only in the peripheral vascular system, although tumor cells showed slight coagulation and vacuolization, and some were still dividing. Yeast cells were found also in the liver and in the lungs, where they were already being phagocytized by polymorphonuclear leukocytes. No marked differences were observed at 3 hours; but, at 4 hours postinjection, tumor necrosis had become extensive, and cellular distortions were noticeable. Most

of the fungus cells were aggregated in the small capillaries of the tumor and were not in contact with the degenerating tumor cells.

At 5 hours Candida cells were scattered throughout the tumor, and the tumor cells were distorted and dissociated (Fig. 1). They could also be found in the lungs, the liver, the heart, the spleen, and a few in the pancreatic tissues. A survey of 1,000 cells (ten fields of 100 cells each) per tumor revealed no cells in any stage of mitosis.

Yeast forms were found throughout the tumor at 6 hours, but they were no longer free. They had been phagocytized either by histiocytes or by tumor cells, which showed large vacuoles. The organisms had disappeared from the lung but were found in large numbers in the liver capillaries and also in the interstitial spaces. Many had been phagocytized by Kupffer cells. Some of these organisms in the liver appeared to be disintegrating.

At 24 hours after the initial injection, broad bands of necrotic tissue appeared in the tumor, and most of the cells were pyknotic (Fig. 3). Although the areas of necrosis in tumor tissue became ever greater in the period from 2 to 5 days, only a few fungus cells or debris persisted, and some tumors showed none at all. Disintegration of tumor cells was almost complete at 72 hours (Fig. 4). However, large numbers of yeast cells were still present in the liver at this period, with a heavy concentration of McManus-positive debris, especially in the vascular areas. On the 7th day, the greatest concentration of organisms appeared in the spleen, where they were being phagocytized by macrophages and were undergoing destruction. On the 8th day, nothing remained of the tumor except fibrous tissue and capsule, and the organisms were being destroyed in both liver and spleen.

Viability of organisms in host tissue.—In order to test viability of *C. guilliermondi* and *Rhodotorula rubra* in treated mice, we cultured blood withdrawn from the tail vein prior to treatment and thereafter every 2d day. The organisms could be recovered on Sabouraud's agar up to 3 weeks post-injection.

White blood cell counts.—Table 7 shows changes in the peripheral blood following injection of *Rhodotorula rubra* and *Candida guilliermondi* into tumor-bearing mice. Though the total white cell count increased slightly in both sets of controls during the experimental period, as well as in Rhodotorula-treated mice, there was a decrease in number of white cells following injection with *C. guilliermondi*. Differential counts over a 5-day period showed an increase in the percentage of lymphocytes following treatment with both organisms and a transitory rise in percentage of monocytes in mice treated with *Rhodotorula rubra*.

TABLE 7

WHITE BLOOD CELL COUNT IN SWISS MICE IMPLANTED WITH SARCOMA 37 FOLLOWING INTRAVENOUS INJECTION OF *R. rubra* AND *C. guilliermondi* AT 2-DAY INTERVALS

Treatment	Total W.B.C. count			Differential W.B.C. count								
	Pre-inject.	24 hr. after 1st inject.	24 hr. after 2d inject.	Pre-inject.			24 hr. after 1st inject.			24 hr. after 2d inject.		
				Lymph.	Neutr. (per cent)	Mono.	Lymph.	Neutr. (per cent)	Mono.	Lymph.	Neutr. (per cent)	Mono.
R. rubra (0.5 cc/dose)	25,937	30,187	29,500	26.0	61.0	12.0	38.0	22.0	39.0	49.0	36.0	13.0
Untreated controls	23,410	23,810	29,281	27.0	55.0	14.0	26.0	58.0	15.0	37.0	51.0	10.0
C. guill. (0.5 cc/dose)	24,190	19,925	12,967	20.8	65.2	14.0	49.5	40.0	10.5	45.5	48.0	6.5
Untreated controls	20,070	26,250	29,281	25.2	64.2	10.6	38.5	53.5	8.0	35.5	56.0	8.2

Response of tumors other than Sarcoma 37.—Spontaneous mammary tumors of Swiss mice occasionally diminished in size following injection of the host with *C. guilliermondi*. Mammary tumors of the C3H strain showed no regression but remained static for several weeks under similar treatment. However, this does not appear to be the result of a selective action of the organisms on sarcomas, since Krebs 2 solid tumors, implanted subcutaneously in Swiss mice, were sloughed in approximately the same percentage as was Sarcoma 37, thus confirming our earlier *in vitro* observation (3) with respect to the oncolytic action of Candida cultures against carcinoma cells. These experiments are being repeated on a larger scale, as well as with other organisms, since cultures that were most effective against carcinoma cells *in vitro* were not always those that caused the greatest damage to Sarcoma 37.

A transplantable rhabdomyosarcoma carried in C3H mice was not affected by injection of the host with *C. guilliermondi*, though in several cases survival time was prolonged.

DISCUSSION

These studies have shown that there is a large group of organisms, mostly yeasts and yeastlike fungi, capable of exerting a destructive action against Sarcoma 37. All Candida species tested, with the exception of *C. lipolytica*, possess this capacity. It is interesting to note that killed cultures of the yeast phase of *Histoplasma capsulatum* gave negative results. Histoplasmosis is a disease of the reticulo-endothelial system, and we anticipated that the injection of dead Histoplasma cells might stimulate the defense mechanisms of the host to inhibit tumor growth; but this was not the case.

The mechanism of oncolytic action of these organisms is not clear. However, when nonpathogenic species are employed, there are no toxic, inflammatory, or hemorrhagic reactions on the part of the host. Our *in vitro* studies (3) showed that there is a direct destructive action of the organisms quite apart from any possible response of the reticulo-endothelial system. However, intraperitoneal as well as subcutaneous injections were effective, though to a lesser extent than intravenous injections, despite the fact that organisms have not been observed in tumor tissue and cannot be recovered from blood following intraperitoneal and subcutaneous administration. One must therefore assume the existence of some other mechanism, though the experiments of Pillemer *et al.* (6) with zymosan, a polysaccharide component of the yeast cell wall, suggest the possibility of some immune activity. Some preliminary studies of a similar polysaccharide isolated by Pillemer's formula from *C. guilliermondi* indicate oncolytic activity with respect to Sarcoma 37. Protein and carbohydrate fractions prepared by Dr. T. H. Lavine of this Institute and his associates, tested *in vitro*, have not proved as effective as living organisms.

SUMMARY

Injection of mice bearing transplantable Sarcoma 37 with living and killed Candida species caused sloughing of the tumors in varying degrees (over 80 per cent with *Candida guilliermondi*). The intravenous route of administration proved the most effective. Heat- and chemically killed cultures, as well as crude filtrates, were active, though to a lesser degree. Several other organisms, e.g., *Cryptococcus glabratus*, *Rhodotorula rubra*, *Torulopsis candida*, *Torulopsis utilis*, and *Pichia fermentans*, were also effective. Some organisms were without effect on these tumors: *Cryptococcus neoformans*, *Histoplasma capsulatum*, *Geotrichum album*, *Nocardia intracellularis*. Response of the spontaneous tumors thus far tested has been very limited.

Following intravenous injection, there was widespread distribution of the organisms in normal tissues, as well as in tumors, but no evidence of destruction of cells other than those of the tumor was noted. No toxic symptoms developed in mice injected with nonpathogenic species in tumor-necrotizing doses.

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Figs. 1–4.—Paraffin sections of Sarcoma 37 from mice given injections intravenously of *Candida guilliermondi*. Bouin fixation, McManus stain.

Fig. 1.—Five hours postinjection. Yeastlike forms of the organism scattered through the tumor tissue, which is becoming vacuolated. ×1,800.

Fig. 2.—Untreated tumor 10 days postimplantation (control for Fig. 4). ×1,800.

Fig. 3.—Six hours postinjection. Note pyknosis of tumor cells. ×900.

Fig. 4.—72 hours postinjection. No viable cells can be detected microscopically. ×900.